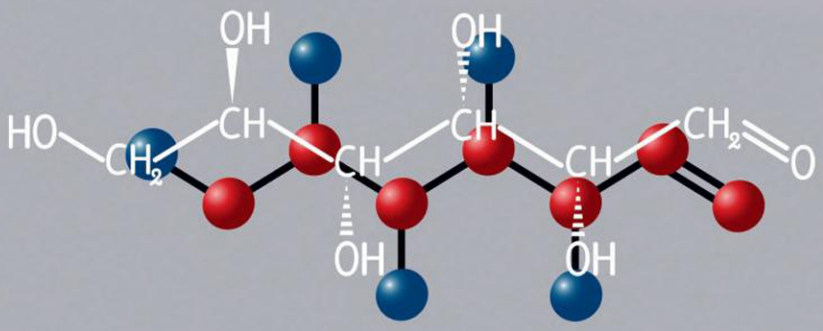
Molecular Nutrition Series

Molecular Nutrition
Carbohydrates



Edited by
Vinood B. Patel

MOLECULAR NUTRITION: CARBOHYDRATES

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Series Preface

In this series on Molecular Nutrition, the editors of each book aim to disseminate important material pertaining to molecular nutrition in its broadest sense. The coverage ranges from molecular aspects to whole organs, and the impact of nutrition or malnutrition on individuals and whole communities. It includes concepts, policy, preclinical studies, and clinical investigations relating to molecular nutrition. The subject areas include molecular mechanisms, polymorphisms, SNPs, genome-wide analysis, genotypes, gene expression, genetic modifications, and many other aspects. Information given in the Molecular Nutrition series relates to national, international, and global issues.

A major feature of the series that sets it apart from other texts is the initiative to bridge the transintellectual divide so that it is suitable for novices and experts alike. It embraces traditional and nontraditional formats of nutritional sciences in different ways. Each book in the series has both overviews and detailed and focused chapters. Molecular Nutrition is designed for nutritionists, dieticians, educationalists, health experts, epidemiologists, and health-related professionals such as chemists. It is also suitable for students, graduates, postgraduates, researchers, lecturers, teachers, and professors. Contributors are national or international experts, many of whom are from world-renowned institutions or universities. It is intended to be an authoritative text covering nutrition at the molecular level.

V. R. Preedy
Series Editor

Preface

In this volume in the *Molecular Nutrition Series*, the authors focus on *Carbohydrates*. Carbohydrates can be classified into three forms: monosaccharides, disaccharides, and polysaccharides, with storage of sugars as either glycogen, starch, or cellulose. Derived from these complex sugars is the simple sugar glucose, which can also be obtained from sucrose; other simple/monosaccharide sugars include fructose. While carbohydrates are an important contribution to the diet, the role of simple sugars is an area of much debate in relation to diabetes and liver diseases. Sugars are known to act via the gut-brain axis promoting food intake. A high-sugar diet increases the risk of developing type 2 diabetes, with subsequent increased risk for cardiovascular disease; whereas independently fatty liver disease arises from a high-carbohydrate diet leading to nonalcoholic fatty liver disease, a leading cause of liver cirrhosis. Furthermore, a diet high in fructose can also lead to fat deposition in the liver and ischemic damage in a variety of organs.

Sugars also play a significant role in the development and progression of cancer, due to the increase in glycolysis and the TCA cycle. Contributing to cancer development is an upregulation of carbohydrate response element-binding protein which further promotes lipogenesis and tumor growth. This has also led to research investigating the inhibition of GLUT1 glucose transporters as a mechanism to inhibit glucose uptake and tumor growth. Despite the negative effects of glucose in certain conditions, glucose is essential for CD4 and CD8 T-cell functions, regulating a normal immune response.

The book *Molecular Nutrition: Carbohydrates* contains three sections. *Part 1* covers the general aspects of carbohydrate metabolism, carbohydrates in the diet and insulin resistance, dietary sugars, lipoproteins, glucose transporters, fats, and glycoproteins. In *Part 2* the topics covered are fructose and insulin signaling, carbohydrate-responsive element-binding protein, metabolic syndrome, glucose metabolism in T cells, effects of D-galactose, sugars and sweet taste receptors, peroxisome proliferator-activated receptors, and the beneficial effects of glucosamine. *Part 3* includes genetic machinery and its function, and provides coverage of nutrigenomics, glucose and DNA methylation, GALT gene, OLR1 and IL17A genes, and glucose metabolism.

The Editor

PART 1

General and introductory aspects

CHAPTER

1

Glucose homeostasis and the gastrointestinal tract

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OUTLINE

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SUMMARY POINTS

- Dietary carbohydrate (Fig. 2) is largely ingested as polysaccharides in the form of starch (amylopectin and amylose) with smaller contributions of disaccharides (sucrose, maltose, and lactose). All carbohydrates must be broken down into monosaccharide to allow absorption. Glucose is the major monosaccharide produced by the digestion of dietary carbohydrate.
- The rate of carbohydrate delivery into the small intestine (i.e., gastric emptying rate) is a key determinant of blood glucose levels after a meal (postprandial glycemia). It influences the absorption rate of glucose and the secretion of gut peptides qualitatively and quantitatively.
- The release of gut hormones depends on nutrient sensing. A major factor in nutrient sensing is the absorption of nutrients.
- Gut hormones regulate appetite, control food intake, adjust gastrointestinal motility (including gastric emptying), regulate the secretion of digestive juices, and stimulate insulin secretion.
- The gut peptides, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are responsible for the so-called incretin effect—the augmentation of glucose-stimulated insulin secretion after oral ingestion of nutrients.
- Manipulation of gastrointestinal function by pharmacological, nutritional, and in particular surgical interventions has the capacity to change postprandial glucose handling and overall glucose homeostasis.

Key facts

- Gastric emptying rate (GER) is the rate at which nutrients are dispensed into the small intestine from the stomach. The normal range spans 1–4 kcal/min.
- Insulin is released together with C-peptide in equal (molar) amounts. Both peptides are produced from the same precursor peptide. While insulin is extracted by the liver (30%–50%), this is not the case for C-peptide. Therefore C-peptide has been used as a surrogate measure to better estimate the total amount of secreted insulin.

Introduction

Homeostasis is a term used to describe the maintenance of a stable inner environment by complex interactions between multiple organs (Bernard, 1974) ultimately serving to maintain the functions of the brain. Glucose is the preferred fuel of the brain, and a steady supply is critical. Glucose is a monosaccharide, a fundamental carbohydrate unit, and the key energy currency in the body. Glucose homeostasis is a term used to denote the maintenance of stable

glucose concentrations in the blood stream within a narrow concentration range (euglycemia) while avoiding high glucose levels (hyperglycemia) and low glucose levels (hypoglycemia). During fasting, glucose is released from the liver to ensure a steady supply of glucose either by glycogenolysis (the release of glucose stored as glycogen in the liver) or by gluconeogenesis (the generation of glucose from other energy substrates).

The fasted state sets in after the complete digestion and absorption and storage of nutrients from preceding meals. Depending on the amount of food eaten during a given meal and the macronutrient composition of the meal, the fasted state may be reached after a few hours (dilute nutrient solutions) up to several hours (large, solid fatty meals). In the modern world, where the food supply is plentiful, it is not unusual for humans to enter the fasted state only during sleep because snacks between regular meals provide a more or less continuous supply of nutrients to the gastrointestinal tract.

Food intake breaks the fast. To dampen changes in glucose concentrations when food is ingested, a range of behavioral and gastrointestinal checks have evolved. Some are recruited concurrently, while others are engaged consecutively. Entry of nutrients into the circulation is steered by a system of reflexes and regulatory hormones; this includes various characteristic patterns of motility in the gastrointestinal tract, as well as the release of digestive juices, absorption of nutrients, and changes in the intestinal blood flow.

Determinants of the amplitude and duration of glucose excursions after a meal include the glucose concentration before the meal, the physical characteristics of the ingested foods, the nutrient composition of the meal, the rate of small intestinal nutrient delivery, intestinal glucose transport, insulin and glucagon secretion, hepatic glucose handling, and the insulin sensitivity of peripheral tissues.

The phases of a meal

A meal may be perceived as a two-step process made up of a preabsorptive and an absorptive phase (Katschinski, 2000). In more detail, a meal can be divided into phases defined by the progress of nutrients from one sensory domain or anatomical compartment of the alimentary tract (Fig. 1, Table 1) to the next—the cephalic phase, the gastric phase, and the intestinal phase.

TABLE 1 Functions of the individual segments of the alimentary tract.

Segment of alimentary tract	Function
Oral cavity	Mechanical and enzymatic breakdown of foods, tasting, tasting, cephalic responses
Esophagus	Conductive (brings masticated foods from the oral cavity to the stomach)
Stomach	Storage of foods, mechanical and enzymatic breakdown of foods. Gastric acid production, hormone responses
Small intestine	Enzymatic breakdown of nutrients, absorption (water electrolytes, and nutrients) hormone responses
Large intestine	Absorption of water, electrolytes and some nutrients, hormone responses, storage of feces. Houses colonic bacteria

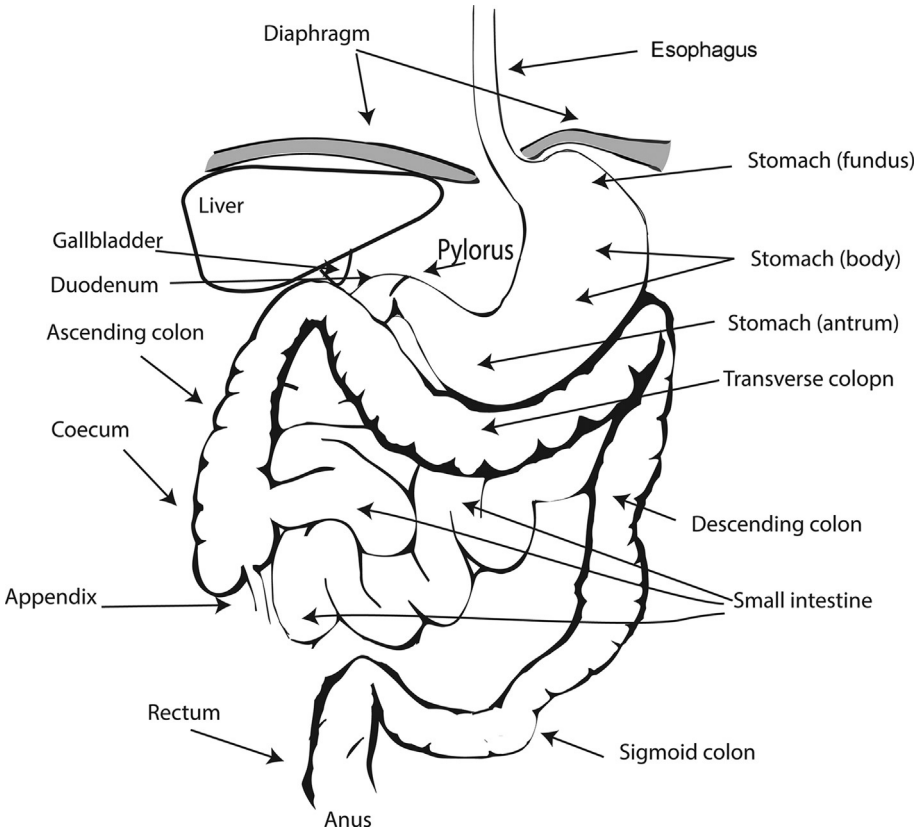

FIG. 1 Overview of the gastrointestinal tract.

The preabsorptive meal phase encompasses the cephalic and gastric phases (Gidãœck et al., 1987). The cephalic phase is triggered by meal expectations (accompanied by hunger sensations), later by the sight and smell of food, followed by the taste and chewing sensations when food enters the oral cavity. Neural signals triggered by these sensations are transmitted to and relayed in the central nervous system. The central integration of sensory impulses generates nerve signals that are transmitted via the vagal nerves, a major pathway of brain-gut communication, to prepare gastrointestinal target organs (Berthoud, 2008) for the incoming nutrients. Cephalic phase responses have been investigated by so-called sham feeding studies where participants experience a broad sensory exposure to foods before premature termination of ingestion by spitting out foods (Konturek et al., 1981; Veedfald et al., 2016).

Swallowing moves food into the esophagus. The gastric phase sets in when food enters the stomach. Stretching of the gastric wall activates vagal sensory nerves giving rise vago-vagal reflex activity (Schöön et al., 1980). When foods have been processed in the stomach, ground

into smaller particles, and mixed thoroughly with gastric acid, the resulting fluid, chyme, is squirted into the duodenum.

This initiates the intestinal phase. Rapidly, free enzymes carried in pancreatic juices and membrane-bound enzymes on intestinal epithelial cells break down nutrients into absorbable molecules. Shortly thereafter, nutrients begin to appear in blood leaving the small intestine demonstrating that the absorptive phase has begun.

Should meal ingestion be prolonged, as is often the case, the just described processes will overlap, as incoming foods elicit cephalic activation while previously ingested foods are stretching the stomach wall or being absorbed by intestinal cells. Neurohormonal responses triggered by nutrients in one gastrointestinal compartment may in such cases modulate handling of nutrients in segments both up- and downstream.

Dietary carbohydrate

In most diets, carbohydrate is principally ingested in the form of polysaccharides—starch and fiber. Most ingested carbohydrate comes from grains and other types of plant materials with only a small portion stemming from animals (glycogen). The intake of disaccharides, such as lactose, sucrose, and maltose, is quite variable. Free monosaccharides make up only a minute fraction of a normal western diet.

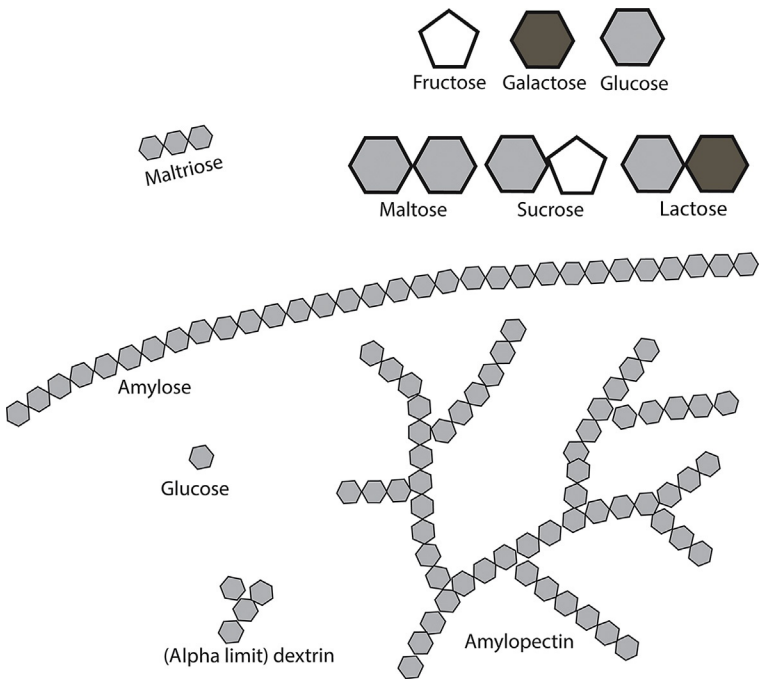

FIG. 2 Dietary carbohydrates. Schematic presentation of the most common dietary carbohydrates.

The end goal of carbohydrate digestion is the conversion of polysaccharides and disaccharides (Fig. 2) into monosaccharides—the only absorbable molecular forms of carbohydrates.

Glucose is the major monosaccharide component in our diet. Starch and fibers are made up of glucose. The disaccharides are either made up of fwo glucose molecules (i.e. maltose, glucose-glucose) while glucose makes up only half of the disaccharides lactose (glucose-galactose) and sucrose (glucose-fructose). The average carbohydrate component of a western diet for an adult over 24 h amounts to about 200-g glucose, 50-g fructose, and 10-g galactose (Southgate, 1995).

Carbohydrate handling in the alimentary tract: A physiological walkthrough

The oral cavity: Mechanical breakdown and enzymatic digestion

In the oral cavity, foods are masticated into smaller bites suitable for swallowing. During the masticatory process, a profuse release of saliva, triggered via cephalic activation (Richardson and Feldman, 1986) helps to coat foods so that they may travel smoothly through the esophagus. Depending on the size of the ingested food morsels and the degree of mastication, the surfaces available for enzymatic activity may vary greatly. Dissolved in saliva are various enzymes including alpha amylase (ptyalin), which contribute to the digestion of polysaccharides. Amylase cleaves starch into smaller fragments (Southgate, 1995) such as maltose, maltotriose, and dextrins (Fig. 2).

The stomach: Storage, digestion, and emptying

Storage

After initial mechanical and enzymatic processing in the oral cavity, swallowed foods are passed within a few seconds via the esophagus, a conduit without digestive or absorptive functions, to the stomach. Prior to the arrival of foods in the stomach, gastric wall tension is reduced due to vagal signals. This makes it possible for food to enter the stomach without rises in the luminal pressure (*receptive relaxation*). As more foods are ingested, and as the amount of gastric acid produced increases, the volume of accumulated gastric contents increases. However, due to a vago-vagal reflex, the tension of the gastric wall is further reduced preventing the luminal pressure from rising (accommodation reflex). These mechanisms allow unimpeded and continued meal ingestion as long as foods have not yet been sufficiently degraded to be allowed into the small intestine.

Digestion

In the stomach, the enzymatic digestion of carbohydrates slows down, but mechanical processing of solid foods continues. Food particles with diameters exceeding 2 mm are not allowed to pass beyond the pylorus (gr. meaning “the gatekeeper”) a muscular tract restricting the entry of gastric contents into the intestine (Fig. 1). The delay between the ingestion of solid foods and the initiation of emptying, which spans around 20–40 min, is termed the lag phase. During this period of time, foods are ground into 1–2-mm particles.

This is accomplished by the coordinated contractions of the thick muscular layer of the antral stomach wall, repeatedly propelling foods towards the closed pylorus.

Gastric emptying

Ground food materials mixed with gastric juices and ingested fluids is termed chyme. This nutrient-enriched fluid is passed on to the small intestine in squirts (Hausken et al., 1992).

Liquids, even when they are ingested together with solids, are emptied without much delay—in an exponential pattern if the solution is nutrient dilute and, more slowly, in a linear pattern, if the solution is nutrient concentrated (Horowitz et al., 1993). The rate at which nutrients exit the stomach depends not only on food particle size and consistency/viscosity but also on the nutrient composition of the ingested foods (Horowitz et al., 1994) and the osmolarity of the chyme (Hunt, 1960), which increases dramatically once the nutrient molecules reach the free and membrane-bound enzymes of the small intestine (Ladas et al., 1983).

When glucose solutions of the same volume but containing increasing amounts of glucose are ingested, gastric emptying will be completed earlier for dilute glucose solution than for higher concentration solutions (Bagger et al., 2011). In healthy individuals the rate of gastric emptying (expressed as kcal/min) varies between individuals within the range of 1–4 kcal/min (Brener et al., 1983). The delivery of 25-g (∼100 kcal) glucose solution to the small intestine therefore takes between 25 (4 kcal/min) and 100 min (1 kcal/min), while a 100-g (∼400 kcal) glucose solution will be emptied within 100 min (4 kcal/min) and 400 min (1 kcal/min). That gastric emptying is a key determinant of postprandial glucose excursions influencing both the duration of intestinal glucose and peak glucose levels after oral glucose exposure (Horowitz et al., 1993), has been elegantly demonstrated by graded instillation of glucose via duodenal catheters (Ma et al., 2011).

The rate of gastric emptying is determined by a finely tuned interplay between the small intestine, the autonomic nervous system, and the stomach (Table 2). Nutrients present in the small intestine stimulate the secretion of a whole range of gut hormones, many of which influence the rate of gastric emptying—including but not limited to GLP-1 (Schirra et al., 2000), CCK (Rehfeld, 2004), Peptide YY (Witte et al., 2009) and, perhaps, neurotensin and pancreatic polypeptide (Schmidt et al., 2005; Thor et al., 1983). To allow further fine-tuning of gastric emptying, hormones such as ghrelin and motilin may accelerate gastric emptying (Ohno et al., 2010). The time of day has also been suggested as a determinant of gastric emptying because the motor activity of the small intestine fluctuates during fasting (Thompson et al., 1982).

It follows that the rate of gastric emptying is not constant over the course of a mixed meal because the nutrient composition of foods remaining in the stomach will not be the same throughout a meal, the consistency of foods will change, and nutrients already passed into the small intestine will have stimulated the release of various gut peptides. The concentration of absorbed nutrients in the circulation may also modulate gastric emptying. Thus hyperglycemia slows gastric emptying (Schvarcz et al., 1997), whereas low blood glucose (hypoglycemia, ∼2.5 mmol/L) markedly accelerates gastric emptying (Schvarcz et al., 1995), both involving hypothalamic and vagal mechanisms (Hjelland et al., 2005).

TABLE 2 Gut hormones.

Peptides	Distribution in GI tract	Function
Gastrointestinal tract		
Ghrelin	Fundus and body of the stomach	Stimulates gastric emptying, stimulates food intake. Inhibits insulin secretion
Motilin	Stomach and small intestine	Regulates intestinal motility, migrating motor complex activity
Gastrin	Stomach	Stimulates gastric acid secretion
Cholecystokinin (CCK)	Upper small intestine	Stimulates gallbladder emptying, inhibits gastric emptying and gastric acid secretion, stimulates pancreatic enzymes release. Lowers appetite
Glucose-dependent insulinotropic polypeptide (GIP)	Small intestine	Stimulates insulin secretion, stimulates glucagon secretion during hypoglycemia
Glucagon-like peptide 1 (GLP-1)	Small and large intestine	Slows gastric emptying, inhibits gastric and pancreatic secretions. Lowers appetite and reduces food intake. Stimulates insulin secretion, inhibits glucagon secretion
GLP-2	Small and large intestine	Stimulates growth of intestine, intestinal blood flow
Neurotensin	Small intestine	Slows gastric emptying, inhibits acid secretion
Peptide YY (PYY)	Small and large intestine	Lowers appetite, reduces food intake, slows gastric emptying
Pancreas		
Insulin	Pancreatic islets (beta cells) Released together with equal amounts of C-peptide (1:1). Secretion is stimulated by glucose, amino acids, gut hormones (Baggio and Drucker, 2007; Rehfeld, 2011)	Lowers blood glucose by increasing glucose uptake in liver, muscle, and fat cells. Inhibits gluconeogenesis and glycogenolysis. Stimulates protein synthesis
Glucagon	Pancreatic islets (alpha cells) Released in response to hypoglycemia, elevated amino acids. Secretion is suppressed by hyperglycemia (Bagger et al., 2014) and GLP-1 (Lund et al., 2011)	Stimulates gluconeogenesis and glycogenolysis during fasting

The small intestine: Digestion and absorption

The small intestine is not a simple hollow tube. The mucosal surface area is augmented by circular and semicircular infoldings of the gut wall (plicae circulares). These folds

are most developed in the proximal parts of the intestine where the exposure to nutrients is greatest. The small intestinal surface is further augmented by the presence of finger-like mucosal infoldings (villi). Villi are taller and more closely set in the proximal small intestine than in the distal small intestine. These anatomical structures allow many more intestinal cells (enterocytes) to inhabit a given length of small intestine than would have otherwise been possible. Enterocytes are the smallest digestive and absorptive units in the small intestine.

Digestion

Upon entering the small intestine from the stomach, carbohydrate moieties not already broken down into monosaccharides will undergo enzymatic degradation by (1) pancreatic enzymes and (2) enzymes present in the luminal membranes ("brush border") of the enterocytes. The term brush border stems from the microscopical appearance of the luminal membranes of enterocytes (Fig. 2). The brushlike appearance is due to the presence of long thin membrane projections, which serve to markedly increase the luminal surface area of the enterocyte. The larger membrane area allows more enzymes and nutrient transporters to be presented on the surface of the cell.

Pancreatic enzymes are carried in the pancreatic juice, an enzyme-enriched sodium bicarbonate solution, that is released in response to neural activation (Katschinski et al., 1992) and hormones released upon nutrient and chemical stimulation of duodenal gut hormone producing cells (Isenberg et al., 1977).

Like the salivary amylase enzyme, pancreatic amylase breaks down alpha 1,4 bonds between glucose molecules in branched and unbranched glucose chains of starch molecules generating maltose (two glucose molecules), maltotriose (three glucose molecules), and oligosaccharides such as dextrins (glucose molecules connected by alpha 1,4 and alpha 1,6 bonds) (Fig. 2).

The membrane-bound enzyme alpha glucosidase hydrolyzes oligosaccharides, maltose, and maltotriose into glucose. Other examples of membrane-bound enzymes are isomaltase (α-1,6 glucosidase), maltase, sucrase, and lactase, which, respectively, cleave dextrins, maltose, sucrose, and lactose into monosaccharides (Fig. 2). Worldwide, many adults are intolerant to lactose due to a gradual loss of lactase activity after weaning (Southgate, 1995).

For the enzymes in the brush border of the intestinal cells (enterocytes) in the mucosa to function optimally, it is essential that the mucosa is continuously bathed with new chyme. This is accomplished by the segmental movements (annular nonpropulsive contractions) of the small intestinal wall. After the completion of the final digestion steps, monosaccharides engage transporters in the luminal membranes of the enterocytes.

Absorption

The gastrointestinal segments upstream from the small intestine do not contribute to the absorption of monosaccharides. Therefore, the rate at which carbohydrates are allowed to enter the small intestine is a major determinant of glucose absorption and the blood glucose after a meal (postprandial glycemia). Dampening small intestinal motility will also delay the uptake of glucose and reduce the peak glucose levels (Chaikomin et al., 2006).

Enterocytes are equipped with transport proteins that help to import monosaccharides from the intestinal lumen. Sodium-glucose-linked transporter 1 (SGLT-1) transports 1 glucose or 1 galactose molecule together with two sodium (Na^+) ions. This transport process is secondary active because the sodium gradient (low intracellular sodium concentration vs high extracellular sodium concentration) is established by the sodium potassium ATPase of the enterocyte. Coupling glucose and galactose transport to sodium therefore allows the absorption of these two monosaccharides even when the luminal concentration of glucose/galactose is low. From within the enterocytes glucose and galactose are exported via the GLUT2 transporter—relying on a concentration gradient to drive transport (facilitated transport). Thus GLUT2 transporters will take glucose and galactose from where the concentration is high to where it is lower. Therefore, should glucose concentration in the bloodstream be higher than inside the cell, the GLUT2 transporter will import glucose rather than export it.

Fructose is taken up from the intestinal lumen by specific transporters (GLUT5) present in the enterocyte membranes (Douard and Ferraris, 2008) and exported from enterocytes via GLUT2 transporters. Transport through both transporters is driven by a concentration gradient.

Malabsorption

Incompletely digested carbohydrates cannot be absorbed. This is the case for fiber and a variable fraction of nonfiber carbohydrates. These carbohydrates, in particular fiber, help to retain water in the intestinal lumen and add bulk to the stools. Fiber and incompletely digested nonfiber polysaccharides also serve as fodder for the bacteria colonizing the colon (Flint, 2012). It is estimated that between 10% and 20% of ingested carbohydrate passes through the small intestine into the colon. If larger amounts of malabsorbed carbohydrate reach the colon, this will give rise to excessive production of gasses and irritants resulting in flatulence and/or diarrhea. Disaccharides and monosaccharides are almost completely absorbed in the upper small intestine. The capacity for fructose absorption is highly variable, and for most individuals, an intake of more than ~35 g/day will result in malabsorbtion.

Postabsorptive disposition of glucose

After absorption, monosaccharides are brought from the small intestine to the liver via the portal vein. In the liver, galactose and fructose are converted into glucose.

A fraction of the glucose arriving to the liver after a meal may be transported across the hepatocyte membranes via GLUT2 and phosphorylated by glucokinase allowing it to enter the pathway to glycogen synthesis facilitated by glycogen synthase (an enzyme that glues glucose together into large storage molecules—glycogen) activated by insulin, which also inhibits the enzyme that breaks down glycogen. Glucose stimulates the secretion of insulin (see in the succeeding text) and inhibits the secretion of the counteracting hormone glucagon (which stimulates the breakdown of glycogen and inhibits the integration of glucose into glycogen). When the influx of glucose from the intestine ceases, the liver will start releasing glucose into the blood leaving the liver by hydrolyzing glycogen (glycogenolysis) and by generating glucose from other substrates (gluconeogenesis).

Signals from the gut

The roles of gut hormones

When nutrients reach the mucosal surfaces of the stomach and intestine, they will not only be absorbed by enterocytes but also be sensed by a diverse population of chemosensory hormone-producing (endocrine) cells that are interspersed between glandular cells (stomach) and digestive/absorptive cells (small intestine). When these endocrine cells are activated by one or several factors e.g. (hyperosmolarity) (Veedfald et al., 2018), nutrient composition (Herrmann et al., 1995; Lindgren et al., 2011), pH, and mechanical stimulation (e.g. distortion or stretching) (distortion/stretch) (Schöön et al., 1980), they discharge hormones (Dirksen et al., 2013; Hornnes et al., 1980) (Table 2).

By engaging a broad panel of gut hormones, it is possible to encode with more precision the responses to incoming nutrients on the gut—for example, according to their type, load, and spatial progress.

These hormones may act locally or on distant target organs. Local effects may be elicited by acting on enteric nerves under the mucosa or on neighboring absorptive cells, hormone producing cells, or smooth muscle cells. Effects on distant target organs may be communicated by hormones released into the circulation or by activation of, for example, vagal sensory nerve fibers (Dockray, 2014; Holst and Deacon, 2005).

These peptides help to orchestrate not only gastrointestinal functions but also ensures coordination with other organ systems (e.g., regarding blood supply during a meal). As previously mentioned a key role of gut hormones is to regulate the rate of nutrient entry into the small intestine (Rehfeld, 2004; Schirra et al., 2000; Schmidt et al., 2005; Thor et al., 1983; Witte et al., 2009) and digestive processes, but they are also involved in intestinal housekeeping (Ohno et al., 2010), cytoprotection and maintenance (Drucker et al., 1996), and importantly in the regulation of appetite and food intake (Degen et al., 2005; Steinert et al., 2014; Zander et al., 2002).

Inhibition of appetite and reduction of food intake elicited by gut hormones is an important element in nutrient balance and body weight regulation. Body weight and adiposity in turn regulate insulin sensitivity and thereby fasting and postprandial glucose concentrations.

A subset of gut peptides have turned out to be key factors in the regulation of insulin and glucagon release (Baggio and Drucker, 2007). This is important because the balance between insulin and glucagon signaling determines whether nutrients such as glucose and amino acids are cleared from the circulation into the liver, muscles, and adipose tissue for storage (the fed state, insulin dominance) or liberated from existing stores (the fasted state, glucagon dominance).

The incretin effect and the incretin hormones

The incretin effect

The incretin effect is a term used to denote the importance of intestinal factors for glucose handling. Oral glucose (Perley and Kipnis, 1967) or glucose administered into the small intestine (Marathe et al., 2014) provokes more insulin secretion than does intravenous glucose infused to obtain the same blood glucose concentration curve (isoglycemia) as that observed on the oral or intestinal glucose stimulation days, respectively.

The augmented insulin response to intestinal glucose absorption occurs because the secretion of insulin is stimulated not only by glucose circulating in blood but also by gut hormones released from endocrine cells nested in the intestinal lining between the digesting and absorbing enterocytes.

These hormones inform target cells in the brain, stomach, and small intestine that nutrient absorption is underway. Insulin and glucagon secreting cells in the pancreatic islets adjust their secretory pattern accordingly, increasing insulin secretion and adapting glucagon secretion to the meal in question. When gut hormone levels return to fasting levels, it suggests that the small intestine is no longer being exposed to nutrients—the stomach is empty.

If only small amounts of glucose (<25 g) are ingested (Bagger et al., 2011) or if the intestinal infusion rate of glucose is modest, the difference in insulin secretion between the oral/intestinal glucose day and the isoglycemic day need not be remarkable (Marathe et al., 2014). However, as the oral glucose load increases, the incretin effect becomes increasingly important (Bagger et al., 2011; Marathe et al., 2014). For relatively small glucose loads, it is notable that if the same amount of glucose is ingested and infused, the amount of insulin secreted may be about the same, but in that case, blood glucose levels during the infusions are considerably higher (Madsbad et al., 1983).

The incretin hormones

The two gut hormones currently considered to be physiologically important incretin hormones from the intestine are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) (Vilsbøll et al., 2003). Both are readily released from the small intestine minutes after the ingestion of an oral glucose or galactose drink (Herrmann et al., 1995). In comparison, oral fructose modestly stimulates the secretion of GLP-1, but does not stimulate the secretion of GIP (Kong et al., 1999).

The secretion of GLP-1 and GIP is also stimulated by lipid and protein (Herrmann et al., 1995; Lindgren et al., 2011).

The insulin-stimulating (insulinotropic) effects of GLP-1 and GIP are dependent on permissive glucose levels (Vilsbøll et al., 2003). Thus while GLP-1 and GIP stimulate insulin secretion at fasting glucose levels, the effect will rapidly disappear as glucose levels decrease. Meanwhile, the effect of both hormones is potentiated when glucose increases. Thus the incretins potentiate glucose-induced insulin secretion. Another way of describing their activity is to say that they enhance beta cell glucose sensitivity, which can be nicely demonstrated experimentally in humans (Kjems et al., 2003).

The activity of these hormones is rapidly terminated due to enzymatic degradation by the enzyme dipeptidyl peptidase 4 (Table 3). Meanwhile, in healthy individuals, augmenting the concentration of the active peptides does not impact the glycemic response to an OGTT (Rhee et al., 2014). However, during intraduodenal infusion of glucose, regulation was improved (Wu et al., 2014)—ostensibly because the variation imparted by gastric emptying was excluded.

Other peptides, CCK, gastrin, and secretion, have been found to have insulin-stimulating effects, but the physiological importance remains uncertain (Rehfeld, 2011).

Harnessing the full potential of the gut

One of the most illustrative examples demonstrating the potential of the gut to regulate postprandial blood glucose is the changes observed when relatively modest changes in the gastrointestinal architecture are instituted. In particular, changes causing (1) an acceleration of gastric emptying and (2) increasing the delivery of nutrients to distal parts of the small intestine have pronounced effects. A common denominator for both approaches is remarkable changes in gut hormone responses. Roux-en-Y gastric bypass (RYGB) surgery (Fig. 3)—one of the most efficacious treatments for obesity and type 2 diabetes—combines both elements.

Briefly, the top of the stomach is sectioned, and a small proximal gastric pouch (25–30 mL) receiving swallowed food is created. Next, the small intestine is sectioned 75–100 cm from the pylorus, and the distal part is joined to the small reservoir, while the proximal end is anastomosed to the small intestine another 100–200-cm distal to the reservoir. In this way, food is brought immediately into contact with the digestive and absorptive mucosa of the more distal small intestine. The storage capacity of the stomach is lost, and the majority of the stomach no longer receives food. Gastric acid and bile are still released, but flow through the duodenal (biliopancreatic) limb unmixed with food. These secretions make contact with nutrients in the distal small intestine at the point where the proximal part (the biliary-pancreatic limb) is joined to the small intestinal (now termed the common limb).

After RYGB surgery the postprandial release of many small intestinal peptides is increased (Jacobsen et al., 2012; Jørgensen et al., 2013). Interestingly, varying the length of the biliopancreatic limb (in effect, moving the point where digestive secretions meet ingested food) has been shown to further the hormonal responses (Patrício et al., 2018). Moreover, further evidence underscoring the importance of the route taken by nutrients has been contributed by studies where RYGB surgeries have been reverted (Borghede et al., 2018; Svane et al.,

TABLE 3 Drugs influencing glucose regulation by modulating gastrointestinal function

Drug	Main site(s) of action	Effects
Acarbose	Small intestine	Inhibits the breakdown of complex carbohydrates by blocking the activity of amylase and the brush border enzymes. Increases GLP-1 secretion and lowers GIP secretion when taken with a mixed meal (Enç et al., 2001)
GLP-1 receptor agonists	Brain, brainstem, pancreatic islets, stomach, small intestine	Lowers appetite, reduces food intake Stimulates insulin secretion, inhibits glucagon secretion
Metformin	Small intestine, liver	Increases glucose metabolism in the intestine, inhibits glucose production in the liver. Increases GLP-1 secretion
DPP-4 inhibitors	Intestine and blood	Block the enzyme dipeptidyl peptidase 4, which inactivates GLP-1 and GIP by removing two amino acids from the N-terminals of peptides. It also influences the activities of PYY peptides (Aaboe et al., 2010)
SGLT-1 inhibitors	Small intestine	Block the absorption of glucose from the small intestinal lumen (Zambrowicz et al., 2013a). Increases GLP-1 secretion while reducing GIP secretion

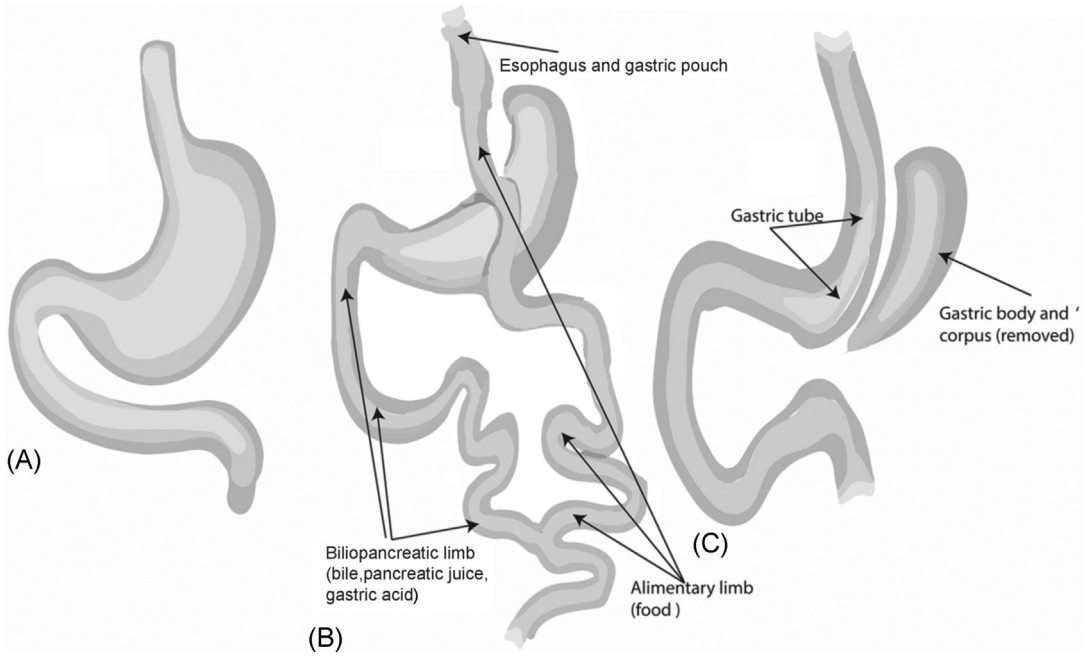

FIG. 3 Anatomy after bariatric surgery. (A) Normal gastrointestinal architecture, (B) Roux-en-Y gastric bypass, and (C) Vertical sleeve gastrectomy.

2017). The changes observed after gastric bypass surgery may not only be due to rerouting. Adaptive changes in the endocrine cell populations and absorptive capacity may further drive the qualitative and quantitative changes in gut hormone responses (Dirksen et al., 2013; Jacobsen et al., 2012).

Interestingly, gut peptide profiles are quite similar after RYGB surgery and truncal vagotomy with pyloroplasty (Plamboeck et al., 2013). A pyloroplasty is a procedure where gastric emptying rate is dramatically increased due to permanent augmentation of the pyloric conduit. This suggests that the key factor responsible for the changes in gut hormone responses is the rapid small intestinal exposure. In recent years a different type of surgery has received attention as an approach to surgically induced weight loss—the vertical sleeve gastrectomy operation. Rather than bypassing the stomach, the size of it is markedly reduced by resection, leaving only a thin conduit through which foods can be transferred from the esophagus to the small intestine.

The gut hormone meal responses after sleeve gastrectomy are less pronounced than after RYGB but still markedly higher than in nonoperated controls (Romero et al., 2012). The mechanism underlying the augmentation of gut hormone secretion is ostensibly accelerated gastric emptying (Melissas et al., 2013) undoubtedly due in part to the reduced ability to accommodate ingested foods. Interestingly, because most of the stomach body is removed, ghrelin secretion is markedly reduced.

Efforts to emulate the consequences of anatomical changes have prompted the search for drugs or novel nutrient regimes that might mimic the effects of these surgeries. Possibly,

inhibiting the proximal digestion of carbohydrates (Enç et al., 2001), delaying the absorption of nutrients (Zambrowicz et al., 2013b) so that they might stimulate gut hormone populations in more distal segments of the small intestine, accelerating gastric emptying, or increasing the consumption of slowly digested carbohydrates could offer advantages.

Glossary

Absorption Uptake of nutrients, electrolytes, and water by enterocytes from the intestinal lumen.
Digestion The mechanical and enzymatic breakdown of foods into absorbable molecules.
Enterocyte Digestive and absorptive cells of the small intestine.
Gluconeogenesis The generation of glucose in the liver (and kidney) from other substrates.
Glycogenolysis The breakdown of glycogen, a starch-like storage molecule in the liver.
Incretin effect The augmentation of glucose-stimulated insulin secretion by hormones released from the small intestine.
Lumen The space enclosed by the mucosal surface of the gastrointestinal wall.
Mucosa The innermost layer of the gastrointestinal wall which surrounds the lumen.
Pylorus Section of the stomach regulating the entry of nutrients into the small intestine.
The vagal nerves Nerves supplying most of the gastrointestinal tract with motor and sensory fibers. Exerts control on gastric emptying rate and gastric acid and pancreatic juice secretion.
Vago-vagal reflex A long reflex arch involving sensory vagal nerve fibers and vagal motor nerve fibers.

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CHAPTER

2

Glucose transporters and their cellular form, role and function

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OUTLINE

SUMMARY POINTS

Disturbance in the expression or function of several glucose transporters is observed in diseases like DM, cancer, IBD, obesity, and psychiatric disorders. However, complete physiological aspects are not known for most of these transporters. More studies are required for understanding their physiology and to utilize them as disease targets.

Key Facts

- Oral rehydration therapy, based on understanding of SGLT1-mediated glucose transport, has saved millions of lives till date.
- Glucose transporters present in proximal renal tubule have been of recent interest as a potential target for the treatment of diabetes mellitus. What would be the safety profile of SGLT1

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inhibitors, given a fact that genetic SGLT1 dysfunction causes osmotic diarrhea postsugar administration?
- GLUT1 deficiency syndrome is characterized by altered brain glucose homeostasis and impaired brain development; early life replenishment with GLUT1 may relieve the symptoms.
- PET imaging used for cancer diagnosis exploits the fact that specific GLUTs are overexpressed and overused in such tissues.
- How protease inhibitors, used in treatment of AIDS, cause insulin resistance and diabetes mellitus?

Introduction

Glucose is a vital source of energy to all species including humans. Every cell needs and utilizes glucose. However, due to its polar nature and relatively large size, it cannot cross the cell membrane by passive diffusion. It requires specific transporter proteins that carry glucose across the membrane. As various tissues differ in their glucose needs, there are different types of glucose transporters. This whole family of glucose transporters is divided in two main types, namely, sodium-glucose linked transporters (SGLTs) and facilitated diffusion glucose transporters (GLUTs).

Structure of SGLTs

SLC5A is the gene encoding SGLTs (Wright, 2001). There exists 50%–70% identity and 67%–84% similarity in the SGLT2–6 sequences, with respect to SGLT1. SGLT1 and SGLT2 are extensively studied and well characterized as compared with the newer members SGLT3-6. Fig. 1 depicts the phylogenetic tree of SGLTs in humans. Table 1 describes the substrates and tissue distribution of various SGLTs in human body.

Structure of SGLT1 and SGLT2 has been extensively studied. SGLT1 consists of 14 transmembrane helices (TM). N terminal (TM1) and C terminal (TM13) are both extracellular. Fig. 2 represents the schematic diagram of SGLT from *Vibrio parahaemolyticus.* TM 7–11 (white) are inverted repeats of TM 2–6 (green). Discontinuous helices, TM 2, and TM 7, are at core of the sugar binding region. TM 4 and TM 9 are highly tilted, and there are helical structures in intracellular (TM 3–TM 4) and extracellular (TM6–TM7, and TM 8–TM 9) loops.

Functional aspects of SGLTs

SGLTs transport sugar by secondary active transport mechanism. They use Na^+ electrochemical gradient as driving force to transport glucose against its chemical gradient. Na^+ gradient is maintained by Na^+–K^+ ATPase pump.

Intestine and renal proximal tubule are the prominent locations of SGLT1 expression. In intestine, SGLT1 is present on apical membrane of intestinal brush border (Gorboulev et al., 2012). Here, it is involved in the absorption of nutrients like glucose and galactose from intestinal lumen. The absorbed glucose enters blood via GLUT2 on basolateral membrane

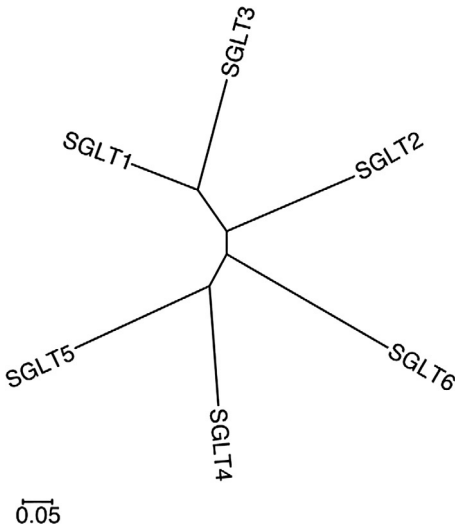

FIG. 1 The phylogenetic tree of human SGLTs. Multiple sequence alignment performed with ClustalW and the results were presented with MEGA6. *Reprinted from Deng, D., Yan, N., 2016. GLUT, SGLT, and SWEET: structural and mechanistic investigations of the glucose transporters. Protein Sci. 25, 546–558. Copyright 2016 by Cambridge University Press. Reprinted with permission.*

TABLE 1 SGLT genes, substrate specificity, and tissue expression in human body (Wright, 2013)

SLC name	Protein name	Substrates	Tissue and cellular expression
SLC5A9	SGLT1	Glucose and galactose (urea and water)	Small intestine, trachea, kidney, heart, brain, testes, prostrate
SLC5A10	SGLT2	Glucose	Kidney, brain, liver, heart muscle, thyroid, salivary glands
SLC5A9	SGLT3	Na^+ (H^+)	Small intestine (cholinergic neurons), skeletal muscle, kidney, uterus, testis
SLC5A9	SGLT4	Mannose, fructose, glucose	Kidney, small Intestine, brain, liver, heart, uterus, lung
SLC5A10	SGLT5	Mannose, fructose, glucose	Kidney cortex
SLC5A11	SGLT6	Myoinositol, chiroinositol	Thyroid, brain, heart, muscle, spleen, liver, lung

(Yang et al., 2010). SGLT1 is high-affinity (K_m 0.3 mM) transporter, which works at coupling stoichiometry of 2 Na:1 sugar. Stan Schultz in 1985 gave the most concise description of its cotransport kinetics (Schultz, 1986). Patch clamp studies indicated that SGLT1 also allows reverse transport, but the sugar selectivity and transport kinetics are different in both directions (Quick et al., 2003).

The most important application of SGLT1 cotransport mechanism lies probably in the discovery of oral rehydration therapy (Ruxin, 1994). This therapy as a treatment for secretory

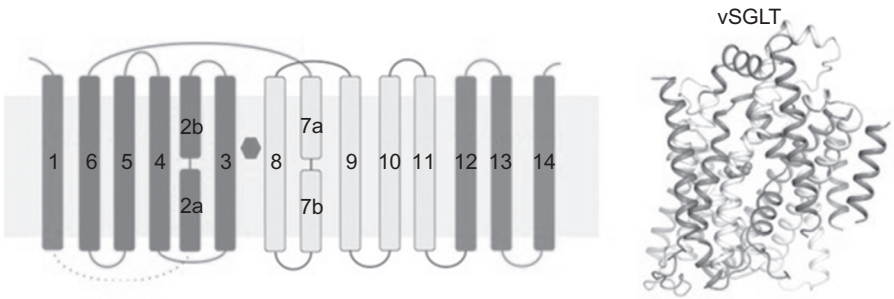

FIG. 2 Schematic and 3D structure of vSGLT. The transmembrane helices TM2-11 constitute the "5+5" inverted repeats of the LeuT-fold of vSGLT. The two repeats are colored *grey* and *white*. The additional transmembrane helices 1,12,13, and 14 are also colored gray. The substrate (galactose) is indicated by the black hexagon. Shown on the right is the structure of the inward-occluded vSGLT (PDB accession code 3DH4). *Reprinted from Deng, D., Yan, N., 2016. GLUT, SGLT, and SWEET: structural and mechanistic investigations of the glucose transporters. Protein Sci. 25, 546–558. Copyright 2016 by Cambridge University Press. Reprinted with permission.*

diarrhea has saved millions of lives till date. Secretory diarrhea especially that of cholera is fatal for children, elderly, and immunocompromised patients. Rotavirus is also known to inhibit SGLT1 resulting in loss of water and electrolytes from intestinal lumen (Halaihel et al., 2000). Rehydration of patient with such diarrhea can be a massive task as it requires administration of nearly 80 L of intravenous fluids over 5 days (Hirschhorn and Greenough, 1991). This task can be accomplished simply by using the efficient SGLT1 cotransport system, by coadministration of Na^+ and glucose. Oral rehydration solution (ORS) administration can replenish as good as 6 L of fluid per day in an adult, via absorption from the enormous surface area available in the intestinal brush border.

Mutations in SGLT1 gene result in abnormalities in glucose and galactose absorption (Lindquist and Meeuwisse, 1962). The presence of these hexoses (but not fructose) in food results in severe watery diarrhea, which is relieved on removing offending sugars from diet. This genetic disorder is typically detected in newborns. Affected subjects need to eliminate sugar and carbohydrate from diet to spend normal healthy life (Hediger et al., 1989). In the same lines, inhibition of SGLT1 as a therapeutic target was feared to have diarrhea as a side effect. However, no such adverse effect was observed in phase I clinical trials of sotagliflozin (a nonselective SGLT2/1 inhibitor) or GSK-1614235 (a selective SGLT1 inhibitor) (Zambrowicz et al., 2012; Dobbins et al., 2015).

Yu et al. (2017) demonstrated a protective role of SGLT1 giving 100% protection against LPS-induced apoptosis in Caco-2 cell model. SGLT1 expression was found to be inversely proportional to TNFα expression in these cells indicating role of SGLT1 in intestinal inflammatory conditions (Merigo et al., 2018). SGLT1 is also found to have a protective role in the epithelium of patients with inflammatory bowel disease through the inhibition of intestinal epithelial cell activation mediated by phosphorylation of Nf-κB.

SGLT1 is also found in enteroendocrine L cells and K cells (Vrhovac et al., 2015). Here, it is involved in the secretion of incretins, glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic peptide (GIP). Intestinal expression of SGLT1 is regulated by a number of factors. Moreover, increase in dietary glucose content increases SGLT activity and density (Dyer et al., 2007).

SGLT1 present in proximal renal tubule (S3 cells) has been of recent interest as a potential target for the treatment of diabetes mellitus. SGLT1 is present downstream to SGLT2 and is involved in reabsorption of glucose that escapes SGLT2 or exceeds its capacity. Selective SGLT1 inhibitors and mixed SGLT1–SGLT2 inhibitors are developed as blood glucose lowering agents. They are anticipated to serve dual advantage by decreasing intestinal absorption of glucose and reducing its reabsorption from renal tubule.

As per a recent study, SGLT1 is abundantly expressed in human and murine heart. Inhibition of SGLT1 by phlorizin averted the protective function of SGLT1 on ischemia reperfusion model in isolated murine heart. Further studies in the direction may reveal important role of SGLT1 in cardiac ischaemic diseases and its treatment (Kashiwagi et al., 2015). SGLT1 is responsible for nearly 80% of SGLT-mediated glucose uptake in the midbrain. It may also have a role in glucose homeostasis at ventromedial hypothalamus (Fan et al., 2015)

SGLT2 is prominently expressed in the early parts of proximal tubules (S2 cells) of the kidney. It is a low-affinity (K_m 2 mM) high-capacity system. It is responsible for the reabsorption of more than 90% of filtered glucose load in renal proximal tubule. It has recently received attention as a target for treatment of diabetes mellitus. Canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin are some of SGLT2 inhibitors, approved by USFDA for use in type 2 DM. SGLT2 is found to be abundantly expressed in pancreatic and prostate adenocarcinomas (Scafoglio et al., 2015). This indicates potential implications for SGLT2 inhibitors in the treatment of these cancers. Mutations in SGLT2 results in familial renal glucosuria, where there is loss of glucose in urine. However, in spite of glucosuria, patient does not suffer from hypoglycemia or any renal complications (Wright et al., 2011).

SGLT3 is a glucose sensor in enteric nervous system and skeletal muscles. It has high affinity for glucose and imino sugars. Similar to SGLT1, it also has two sodium binding sites. The F453 outer gate in SGLT3 closes after sugar binding, making this site inaccessible. This may be a possible reason for its inability to transport sugar (Sala-Rabanal et al., 2011). Studies on SGLT3 are limited due to unavailability of specific agonists, blockers, and antibodies. SGLT3 has low affinity for D-glucose (K_m 20 mM) and high affinity for imino sugars, such as 1-deoxynojirimycin (K_m 4 μM). Therefore miglitol and miglustat are potent ligands for SGLT3. SGLT3 found in the rat hepatic portal vein acts as glucose sensor reducing food intake. There is also an indirect evidence of SGLT3 involved in the secretion of GLP-1 in gut (Soták et al., 2017).

Little is known about glucose transporters SGLT4, SGLT5, and SGLT6. SGLT4 is expressed in small intestine and kidney, where it is possibly involved in mannose absorption and reabsorption (Tazawa et al., 2005). SGLT4 mRNA has also been found in the lung, liver, small intestines, brain, uterus, and heart. It exhibits higher affinity for mannose followed by other sugars, glucose, fructose, and galactose. SGLT5 was first cloned from human kidney cDNA. SGLT5 is also primarily a high-affinity mannose transporter, which is speculated to reabsorb mannose from renal filtrate. SGLT6 also known as SMIT2 (myoinositol/Na^+ symporter) transports inositol instead of glucose. It is widely expressed in various tissues like the skeletal muscle, brain, heart, liver, spleen, lungs, neurons, and placenta in human body. *SLC5A11* gene expressing SGLT6 is known to interact with immune-related genes like PDCD1, TNF-α, LTA, and C4 to alter the immune response (Tsai et al., 2008). It may be involved in the induction of apoptosis through TNF-α and PDCD1 pathway in systemic lupus erythematosus.

GLUTs: Facilitated diffusion glucose transporters (Fig. 3)

Structure of GLUTs

GLUTs are transporter proteins containing 12 transmembrane helices with intracellularly located amino and carboxyl terminals. They are encoded by the SLC2 genes. All 14 GLUT proteins show 28%–65% homology against GLUT 1. They are divided in three subclasses (classes I, II, and III) based on multiple sequence alignment studies. These proteins transport glucose (or other sugars) according to their concentration gradient across membrane (Navale and Paranjape, 2016) (Fig. 4).

Functional aspects of GLUTs

Class I facilitative glucose transporters

These include GLUT1 to GLUT4 and a newly added member GLUT14. GLUT 1 was the first of the GLUT family to be identified and cloned in 1985 (Mueckler et al., 1985). It is a ubiquitous glucose transporter present in all cells; however, it has important roles in RBCs and blood-brain barrier. It is found to play an important role in the activation of $CD4^+$ T cells. GLUT1 is abundantly present in the microvasculature of the brain, allowing glucose entry to the brain. Mutation in gene encoding GLUT1 (*SLC2A1*) results in debilitating neurodevelopmental disorder, GLUT1 deficiency syndrome. These patients suffer from hypoglycorrhachia, impaired brain development, microcephaly, seizures, spasticity, dystonia, and ataxia. A recent study suggested that several features of this syndrome can be reversed by early supplementation of GLUT1 (Tang et al., 2017). GLUT1 is also present on erythrocyte membrane and offer entry and exit of glucose, which is altered in patients with

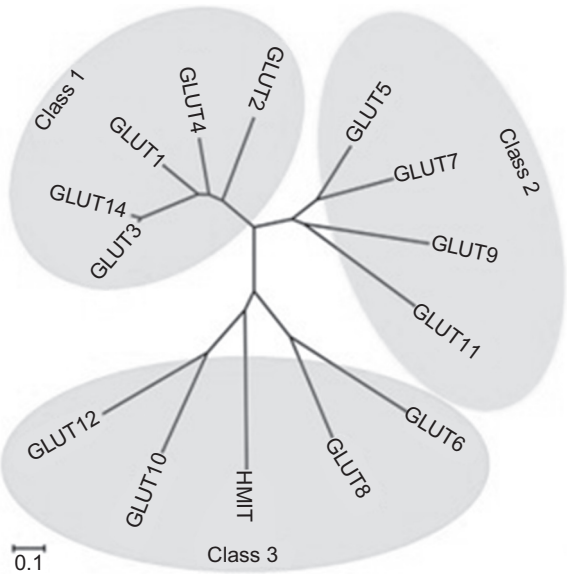

FIG. 3 Dendrogram of the glucose transporter (GLUT) family. The three classes of GLUT proteins are indicated as class 1, class 2 and class 3. The scale bar represents 0.1 substitutions per amino acid position. HMIT, H^+-coupled myo-inositol transporter. *Reprinted from Deng, D., Yan, N., 2016. GLUT, SGLT, and SWEET: structural and mechanistic investigations of the glucose transporters. Protein Sci. 25, 546–558. Copyright 2016 by Cambridge University Press. Reprinted with permission.*

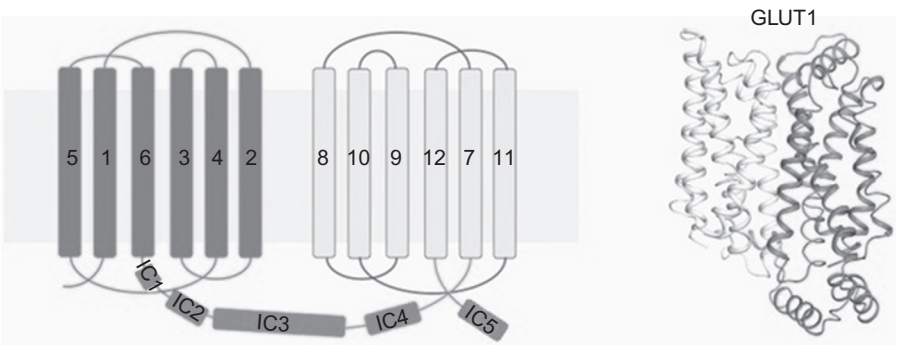

FIG. 4 Schematic and 3D structures of GLUTs. The 12 transmembrane helices were divided into an N domain and a C domain, which are colored grey and white, respectively. The intracellular helices domains (ICH) are colored grey and indicated with IC1 to IC5. Shown on the right is the structure of the human GLUT1 (PDB accession code 4PYP). *Reprinted from Deng, D., Yan, N., 2016. GLUT, SGLT, and SWEET: structural and mechanistic investigations of the glucose transporters. Protein Sci. 25, 546–558. Copyright 2016 by Cambridge University Press. Reprinted with permission.*

type 2 DM (Hu et al., 2000). GLUT1 has also been found to be abundantly expressed in malignant cells, providing the additional energy to cope up with the rampant tumor growth. A recent study proved its role in tumorigenesis and tumor progression in prostate cancer via mediating glycolysis (Xiao et al., 2018). As elevated expression of GLUT1 in tumor cell is associated with poor therapy effectiveness, it may also serve as a good prognostic biomarker for various cancers (Yu et al., 2017). Additionally, as cancer cells are dependent on GLUT1-mediated glucose entry for anaerobic metabolism, inhibition of GLUT1 by small molecule blockers can be an effective approach for controlling tumor growth (Ojelabi et al., 2016). One such GLUT1 inhibitor WZB117 has shown encouraging results in various preliminary studies, while more data on safety and efficacy of the molecule are awaited (Chen et al., 2017; Zhao et al., 2016). GLUT1 present in hepatocytes is associated with regulation of bidirectional transport of glucose under influence of thyroid hormone.

GLUT2 is expressed in the pancreatic beta cells, liver, kidney, intestine, and central nervous system (Thorens, 2015). Murine pancreatic beta cell has GLUT2 as the major glucose sensor involved in the regulation of insulin secretion. However, human beta cells have GLUT1, GLUT2, and GLUT3 as low affinity but high-capacity glucose sensors (McCulloch et al., 2011). GLUT2 has low affinity for glucose (K_m 17 mmol/L) and high affinity for glucosamine (K_m 0.8 mmol/L). Mannose, fructose, and galactose are other low-affinity substrates (Uldry et al., 2002). Due to low affinity and high capacity to glucose, it can achieve quick equilibrium between extracellular and intracellular glucose. Thus, glucose gets virtually unrestricted access to glucokinase, the rate limiting factor in glucos-stimulated insulin secretion. Diabetic rat and mice, which had impaired insulin secretion, exhibited reduced expression of GLUT2 on the beta cell membrane (Thorens et al., 1990). Thus, glucose sensors of beta cells may have important role in pathophysiology of T2DM. GLUT2 is ubiquitously present in the brain, neurons, endothelial cells, astrocytes, and tanycytes. Here, GLUT2-mediated glucose sensing is involved in the regulation of feeding behavior via melanocortin pathway. Apart from leptin dysregulation GLUT2 knockout mice also had defective thermoregulation (Thorens, 2015). Nervous glucose sensing via GLUT2 also plays a major role in the regulation of beta cell

proliferation via parasympathetic nervous system (Seyer et al., 2013). Opposite to this, GLUT2-expressing neurons of nucleus tractus solitarius are inhibited by glucose. They are activated in hypoglycemia, stimulating release of glucagon from pancreas. Mutations in human GLUT2 gene results in Fanconi-Bickel syndrome, a condition associated with liver enlargement, growth retardation, renal Fanconi syndrome, and neonatal DM (Santer et al., 1998). GLUT2 is present in the basolateral membrane of intestinal epithelial cells and proximal tubule of kidney allowing entry of glucose from the epithelial cell to the blood. GLUT3 referred to as neuronal glucose transporter is mainly distributed in the brain. It has high affinity and capacity for glucose, which ensures sufficient intake of glucose in neurones, where the glucose levels in the surrounding fluid (1–2 mM) is lower than that in serum (5–6 mM). As surrounding structures like endothelial cells of BBB and glial cells have GLUT1, neurons get preferential access to glucose due to the presence of GLUT3. Tissue samples from Alzheimer's disease patients have shown a significant decrease in GLUT3 expression in several brain regions (Simpson et al., 1994). GLUT3 is also important in the function of sperm cells and placental cone. The fact that GLUT1 and GLUT3 are overexpressed in cancerous cells and these cells show increased glucose uptake (the Warburg effect) is exploited in the diagnosis of cancer by PET imaging using isotope-labeled glucose derivative ^{18}F-FDG.

GLUT4 is insulin-responsive glucose transporter, found in the skeletal muscle, heart, adipose tissue, and brain. GLUT4 is present in vesicles in cytoplasm of the cells. Binding of insulin to insulin receptor causes translocation of GLUT4 to cell membrane. GLUT4 vesicles in the skeletal muscle are either transferrin positive or negative and are translocated by different stimuli. Muscle contraction causes recruitment of transferrin-positive GLUT4 vesicles, while insulin and exercise affect transferrin-negative vesicles. Thus, insulin and exercise are two independent stimuli causing GLUT4 recruitment to the membrane in muscle and adipose cells. Interestingly, GLUT4 is inhibited by protease inhibitors, thus causing development of insulin resistance and diabetes mellitus in treated patients. Recent advancements in imaging technique have provided opportunity to understand GLUT4 recycling; the detailed review is provided by Huang (Huang and Czech, 2007). In brief, GLUT4 is the major glucose transporter involved in insulin-mediated glucose uptake by most tissues of body.

A new facilitative glucose transporter GLUT14 was recently identified. *SLC2A14* gene is located on chromosome 12p13.3 (17.1M). Two isoforms of GLUT14 have been identified, the shorter form (GLUT14-S) and long form of GLUT14 (GLUT14-L). Both isoforms of GLUT14 are found in testis, at an mRNA level four times higher than that of GLUT3 (Wu and Freeze, 2002). Another study speculates role of GLUT14 in pathophysiology of IBD (Amir Shaghaghi et al., 2017).

Class II facilitative glucose transporters

GLUT5, GLUT 7, GLUT 9, and GLUT 11 constitute class II of facilitative glucose transporters. Out of these GLUT5 is an exclusive fructose transporter, while GLUT7 and GLUT11 can transport both glucose and fructose. GLUT9 is a urate transporter. GLUT5 is located in the small intestine, kidney, and testes where it plays important physiological and pathological roles. Its overexpression is linked to type 2 diabetes mellitus, obesity, and cancer. Inhibitors of this transporter are potential targets for these conditions, especially cancer (Douard and Ferraris, 2008). Overactivity of GLUT5 has been found in several tumors including breast cancer and pancreatic cancer. Fluorinated fructose-mediated novel PET imaging technique is under development for diagnosis of cancers overexpressing GLUT5. Two natural products

rubusoside and astragalin-6-glucoside are found to have GLUT5 inhibitory activity. These molecules though less potent have been helpful for the identification of ligand-binding differences between GLUT1 and GLUT5. Based on this, *N*-[4-(methylsulfonyl)-2-nitrophenyl]-1,3-benzodioxol-5-amine (MSNBA) was successfully recognized as a selective and potent GLUT5 inhibitor (Thompson et al., 2016).

GLUT7 is expressed in the apical membranes of the small intestinal epithelial cells. It has a high affinity (<0.5 mM) for glucose and fructose. However, distribution of GLUT7 along small intestine does not match with availability of these sugars, indicating that there may be yet another natural ligand for this transporter (Ebert et al., 2017).

GLUT9, the last member of this class, is a urate transporter. It is expressed predominantly in the liver and kidney. It also has a low affinity for deoxyglucose (Augustin et al., 2004). Immunohistochemistry has shown its presence in proximal tubules of kidney (Li et al., 2007). Polymorphisms in *GLUT9* gene affect glucose and uric acid metabolism. It is upregulated in DM, and its genetic variants are associated with hyperuricemia (Keembiyehetty et al., 2006).

GLUT11 is also expressed as two splice variants. The long form is a fructose transporter with 503 amino acid residues and is expressed in the liver, lung, trachea, and brain. The shorter form of GLUT11 has 593 amino acids and is found in the heart and skeletal muscle. It has low affinity for glucose and somewhat higher affinity for fructose (Doege et al., 2001). Its ability to transport fructose is consistent with its structural similarity with GLUT5. It is also proposed to be a mannose transporter for N-glycan synthesis (Ichikawa et al., 2014).

Class III facilitative transporters

There are five known facilitative transporters in this class, namely, GLUT6, GLUT8, GLUT10, GLUT12, and GLUT13 (HMIT). These transporters differ in location of glycosylation site. It is located on loop 9 in this class, in contrast to classes I and II transporters in which it is located on loop 1 (Asano et al., 1991). Another important feature of these transporters is the presence of targeting motifs that may be involved in regulating their functional characteristics. However, their characterization and significance is yet to be determined.

GLUT6 is located in the spleen, leucocytes, and the brain. It has low affinity (K_m 5 mM) for glucose. Insulin cannot induce membrane translocation of GLUT6. The dileucine (LL) motif located at the amino terminus of the transporter is demonstrated to play an important role in translocation and internalization of the transporter (Lisinski et al., 2001). Apart from glucose, other substrate specificity of GLUT6 is not known.

Like GLUT6, GLUT8 also has 12 transmembrane helices, with glycosylation site on exoplasmic loop between helices 9 and 10. The LL motif in amino terminal is found to serve as an internalization signal. It has a wider distribution as compared with GLUT6. Its mRNA has been found in tissues including the muscle, brown and white adipose tissue, placenta, brain, adrenal gland, spleen, heart, and liver. Apart from this, it shows highest expression in adult testis, which is altered by gonadotrophins. The presence of this transporter in hippocampus in STZ diabetic rats and the absence of other glucose transporters indicate its contribution in supporting glucose requirements of hippocampal neurons and their cognitive function (Reagan et al., 2001). Insulin, osmotic shock (sorbitol), or phorbol myristate acetate could not induce its translocation to plasma membrane. However, glucose challenge to rat hippocampal cells caused their redistribution to the endoplasmic reticulum. This may point to a unique role of this transporter as an intracellular glucose transporter. In liver, its location

is limited to single layer of hepatocytes surrounding central veins, indicating its involvement in linking hepatic response to systemic glucose concentration. Further, this localization of GLUT8 is altered in DM indicating its role in glucose homeostasis (Gorovits et al., 2003).

GLUT10 has highest sequence homology with GLUT8. It has two hydrophilic loops, one intracellular (between TM 6 and 7) and another exofacial (between TM 9 and 10). It is abundantly expressed in the liver and pancreas. Other tissues including the heart, skeletal muscle, placenta, kidney, lung, brain, salivary gland, thyroid, adrenal gland, ovary, prostate, and skin have also shown its presence. It has high affinity for glucose (K_m 0.3 mM), and this transport is blocked by galactose but not fructose. It's vicinity to a known T2DM susceptibility locus indicates its possible involvement in the pathophysiology of T2DM. However, gene polymorphisms in GLUT10 were not associated with T2DM in several populations (Augustin, 2010).

GLUT12 was first cloned from human breast cancer cell line MCF-7. An elevated expression of GLUT12 is found in in situ ductal cell carcinoma of breast. In normal tissues, it is present in the skeletal muscle, prostate, small intestine, and heart. The substrate selectivity has been defined in the order of D-glucose > 2-DOG > D-galactose > D-fructose > L-glucose (Pujol-Giménez et al., 2013). It is translocated to cell membrane under the influence of insulin. It acts as a secondary insulin sensitive glucose transporter in body and is abundantly coexpressed with GLUT4 in type 1 fibers of skeletal muscle. Based on its study in fetal development and mammary gland, it is proposed as insulin sensitive transporter before the appearance of GLUT4 in fetus (Macheda et al., 2002). Further, it is the only glucose transporter present at apical membrane of mammary gland epithelial cells and is responsible for glucose transport in milk. As per recent studies, GLUT12 along with GLUT1 is involved in the Warburg effect. The p53 transcription factor (tumor suppressor gene) interacts with GLUT1 and GLUT12 genes to reduce their expression in tumor cells. It has also been shown to be involved in mTOR signaling pathway, associated with diabetic kidney nephropathy (Wilson-O'Brien et al., 2007). GLUT12 mRNA expression was found to be increased in a canine model of chronic heart failure. Role of GLUT12 as a backup mechanism for insulin-dependent transport in conditions of insulin resistance and its role in intestinal glucose absorption need to be studied further.

GLUT13/HMIT, encoded by *SLC2A13* gene, is mainly expressed in the brain in areas like hypothalamus, hippocampus, cerebellum, and brainstem (Augustin, 2010). This H^+-coupled myoinositol symporter (K_m 100 μM) is located intracellularly and is inhibited by typical GLUT inhibitors like phloretin, phlorizin, and cytochalasin B. Depolarization and protein kinase C activation cause its translocation to plasma membrane. In the brain, myoinositol is involved in phosphatidylinositol formation, an important mediator in various neuronal signaling pathways. HMIT may be involved in intracellular myoinositol transport rather than that from extracellular compartment (Di Daniel et al., 2009). Apart from the brain, HMIT is found in the kidney, placenta, pancreas, heart, skeletal muscle, and lungs.

SWEET family of glucose transporters

SWEETs are newly added sugar transporter family for humans. SWEETs are well-studied sugar transporters in plant kingdom. However, their discovery as human glucose transporter is a recent development. SWEET1 is the first identified human SWEET transporter, encoded

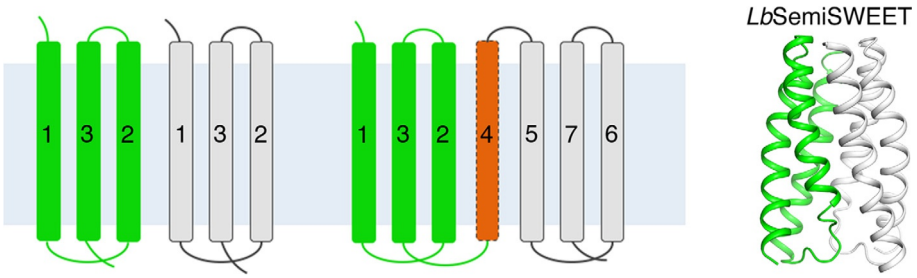

FIG. 5 Schematic and 3D structure of semi-SWEETs. The topology of semi-SWEETs and predicted topology of SWEET1 in human are shown on the left. Shown on the right is the structure of LbSemi-SWEET (PDB accession code 4QNC). *Reprinted from Deng, D., Yan, N., 2016. GLUT, SGLT, and SWEET: structural and mechanistic investigations of the glucose transporters. Protein Sci. 25, 546–558. Copyright 2016 by Cambridge University Press. Reprinted with permission.*

by gene *SLC50A1.* Several semi-SWEETs from microorganisms are studied. Fig. 5 shows structure of one of the bacterial semi-SWEET compared with human SWEET structure. It consists of dimer of two protomers, with 3 TM helices in each. These TM helices are located in pattern of 1, 3, 2. The two protomers in the diagram are indicated in white and green colors (Deng and Yan, 2016). Human SWEET has one additional TM helix, indicated in orange. They mediate efflux of glucose in humans and are ubiquitous in human body, with highest expression in the oviduct, epididymis, and intestine. Immunolocalization data from the Human Protein Atlas indicate their localization in absorptive intestinal epithelial cells (Chen et al., 2010). Further studies are required to discover SWEET physiology in humans.

Glossary

Warburg effect Warburg effect is the observation that most cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells.
Hypoglycorrhachia Low glucose concentration in the CSF.
Microcephaly Condition where the head (circumference) is smaller than normal.
Tanycytes Specialized astrocyte lining part of the third ventricle.

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CHAPTER

3

Molecular aspects and biochemical regulation of diabetes mellitus

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OUTLINE

SUMMARY POINTS

1. This chapter focuses on the molecular aspects and the biochemical regulation of diabetes mellitus (DM).
3. DM is one of the major diseases associated with high morbidity and mortality, with more than 422 million people affected globally.
3. Based on the etiology and clinical presentation, there are two main forms of the disease: T1DM and T2DM.
4. T1DM results from T cell-mediated autoimmune destruction of pancreatic beta cells, involving cellular and humoral

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immunity as well as genetic and environmental factors, leading to absolute insulin deficiency.

5. T2DM is caused by insulin resistance in peripheral tissues and/or pancreatic insulin secretory dysfunction.
6. Due to failure of physiological roles of insulin, there is derangement in not only carbohydrate metabolism but also protein and lipid metabolism.
7. Uncontrolled DM can lead to complications such as nephropathy, neuropathy, stroke, and retinopathy.

Key facts of human leucocyte antigen

- The human leucocyte antigen (HLA) also termed major histocompatibility complex (MHC) is located on chromosome 6p21 and contains more than 200 genes that are arranged into three subregions: class I, class II, and class III.
- The class I genes encode class I molecules that are found on all nucleated cells in the body and present processed antigen to receptors of cytotoxic ($CD8^+$) T lymphocytes.
- Class II genes code for class II molecules expressed on antigen-presenting cells such as dendritic cells, mononuclear phagocytes, and B cells that processed antigen for the recognition by helper ($CD4^+$) T cells.
- The class III genes also encode a range of molecules including complement components (C2 and C4), tumor necrosis factor (TNF), and heat shock protein (Hsp70).
- HLA is a heterodimeric molecule made up of α and β chains, and hence, alteration in the amino acid sequence at critical sites on either chain can significantly increase or decrease the binding capacity of the relevant autoantigens leading to disease susceptibility.
- The HLA complex polymorphic alleles account for about 30%–50% of the genetic susceptibility of developing T1DM.

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases in which there is a consistently high blood glucose level (hyperglycemia) as a result of impaired insulin secretion and/or insulin action according to American Diabetic Association (ADA) (ADA, 2005). Classical symptoms of DM include polyuria (excessive urine production), polydipsia (increased thirst), polyphagia (increased hunger), weight loss, fatigue, and skin and mucosal infections. The word *mellitus* (Latin, meaning "sweetened with honey") differentiates the disease from diabetes *insipidus*, which has a similar presentation but results from insufficient antidiuretic hormone production or impaired renal reabsorption of water (Berg et al., 2002).

The Cappadocian physician, Aretaeus, first used the term "diabetes" in the second century AD to describe a condition that was associated with excessive urine production. Shortly after,

Galen (a Roman physician) also described two cases in which the patients were presented with polyuria and polydipsia. The association of polyuria with a sweet-tasting substance in the urine, however, was first unveiled by two Indian clinicians, Susruta and Sharuka in the 5th or 6th century (Fernandez-Mejia, 2006).

Since these discoveries, a lot of researches have elucidated many facts about DM in terms of its pathophysiology, symptoms, treatment, and management. However, it has taken its place globally as one of the top three killer disorders along with cardiovascular disease and cancer. The World Health Organization (WHO) report on DM indicated that more than 422 million people are affected with the disease globally, increasing its economic burden on countries every other day. For instance, DM accounted for about 1.6 million deaths in 2016, making it the seventh leading cause of death in that year alone (WHO, 2018). This chapter focuses on the general overview of DM, molecular aspects, and biochemical regulation.

Types of diabetes mellitus

There are two main types of DM (Table 1); type 1 DM (T1DM) (old names: juvenile or insulin-dependent diabetes) and type 2 DM (T2DM) (old name: non-insulin-dependent diabetes), though other types also exist. T1DM results from absolute insulin deficiency due to autoimmune destruction of pancreatic beta cells (Noble et al., 1996; Arneson and Brickell, 2007). Patients with this type of diabetes therefore depend on exogenous insulin for survival, and this type forms about 10% of all diabetic cases (Fernandez-Mejia, 2006). This prevalence increases at a rate of 3% per year globally with the highest incidence occurring among young children—approximately 86,000 children develop T1DM each year. Research has also shown that the life expectancy of affected subjects is decreased by about 13 years (Livingstone et al., 2015). Again, Scandinavian countries are reported to have the highest incidence of the disease with Finland and Sweden recording incidence rates of about 60/100,000 per year and 43/100,000 per year, respectively. To the other extreme, China has a low incidence of 0.1/100,000 per year (Skyler et al., 2017). Although T1DM is usually predominant among children, roughly 5%–15% of adults diagnosed of T2DM are also presented with T1DM (Stenström et al., 2005).

In the T2DM, there is insulin secretory defect in the pancreatic beta cells and/or insulin resistance in peripheral tissues particularly in the muscle and adipose tissues (Russo et al., 2014). This type constitutes about 90% of all diabetic cases (Fernandez-Mejia, 2006). It is estimated that, in years to come, incidence and prevalence rates of T2DM are bound to increase, in view of the rapid epidemiological transition associated with changes in dietary patterns, reduced physical activity, and other sedentary lifestyles, especially among the urban populace.

Although both forms of the disease are characterized by hyperglycemia, the glucose in the blood is not efficiently made available to cells that utilize glucose as source of metabolic fuel. These cells are "starved" and therefore result to other alternatives for energy. In some cases, certain organs and tissues exposed to the high glycemic environment may be harmed, if not well managed, leading to disabling and life-threatening health complications (Reynolds and Helgeson, 2011; Hagger et al., 2016), such as cardiovascular diseases, renal failure, vision loss,

TABLE 1 Differences between type 1 and type 2 diabetes mellitus

	Type 1 diabetes	Type 2 diabetes
Pathogenesis	• T cell-mediated autoimmune etiology involved • Marked atrophy and severe destruction of pancreatic β-cells • Absolute or severe insulin deficiency	• Does not involve autoimmune components • Focal atrophy and mild/partial β-cell depletion • Relative insulin deficiency or insulin resistance in peripheral tissues
Clinical	• Rapid onset; common among young people (<20 years) • Normal or reduced blood insulin level (hypoinsulinemia) • Insulitis present during early stages of the disease • The presence of islet cell autoantibodies in the serum of patients • Ketoacidosis is a common feature	• Insidious onset; predominant among adults (usually >30 years) • Normal or increased blood insulin level (hyperinsulinemia) • Insulitis absent at the onset of the disease • Autoantibodies are not usually detected in the serum of these patients • Ketoacidosis is rare
Prevalence	• It accounts for about 10% of all diabetic cases	• It accounts for approximately 90% of worldwide diabetic cases
Genetics	• Human leucocyte antigen associated • Has relatively low concordance rate of about 50% among identical twins	• No human leucocyte antigen associated • Has relatively high concordance rate of approximately 60%–80% in twins
Other Disease Association	• Associated with usually autoimmune diseases, for example, celiac disease, thyroiditis, Addison's disease	• Associated with usually metabolic syndrome, for example, obesity
Treatment	• Depends absolutely on exogenous insulin for survival	• Depends on oral hypoglycemic drugs and lifestyle modification like diet and exercise

and limb amputation. Insulin therapy has mainly played a major role in controlling T1DM; however, it may also be used in T2DM especially if management becomes intricate with oral hypoglycemic drugs and/or diet modification as well as regular exercises and weight control.

The other types of the disease include gestational diabetes and the so-called secondary diabetes, also known as "other specific causes" of diabetes. Research has proven that pregnancy causes an increased resistance to insulin by tissue cells (Homko et al., 2001). With this occurrence, pregnant women who fail to produce the hormone to a substantial quantity that would overwhelm this resistance suffer from gestational diabetes. Usually after delivery, blood glucose concentration is expected to normalize. Unfortunately, for some people, the situation may persist even after delivery and therefore progresses to T2DM (Arneson and Brickell, 2007). Other conditions known to cause an increased blood glucose level that fall under "other specific types" include some infections, endocrinopathies, and some genetic defects that disturb the function of the pancreatic beta cells and/or insulin action.

The wonder molecule—Insulin

The pancreas plays a pivotal role in both digestive and endocrine systems. Two major demarcations are present in the structure of the pancreas; the acini and the islets of Langerhans. The acini are associated with the secretion of digestive juices, while the islets are responsible for secreting hormones—insulin, glucagon, and somatostatin. The islets of Langerhans comprise three types of cells, namely, alpha, beta, and delta cells. Alpha and delta cells secrete glucagon and somatostatin, respectively. Beta cells secrete insulin and form the major constituent of the islets of Langerhans consisting of about 60% (Guyton and Hall, 2006).

Though a minute protein with a molecular weight of 5808 (Guyton and Hall, 2006), insulin has profound functions in not only carbohydrate metabolism but also protein and lipid metabolism. It is a dimer consisting of two amino acid chains A and B held together by disulfide linkages. This means that when these linkages are nonexistent, the molecule loses its functional ability (Champe et al., 2005). The amino acids that form these polypeptide chains are 51 in number, 21 for chain A and 30 for chain B (De Meyts, 2004). Its half-life is about 6 min in plasma (Guyton and Hall, 2006).

In the biosynthesis of the hormone, there is initial translation of insulin mRNA into a single-chain 86 amino acid precursor, preproinsulin. After cleavage of the amino terminal signal peptide in the endoplasmic reticulum (ER) from preproinsulin, proinsulin is formed. The hormone is finally formed in the Golgi apparatus after the proinsulin is cleaved into insulin and C-peptide (Berg et al., 2002; Champe et al., 2005). The two components are packaged into secretory granules in the Golgi apparatus and released in equimolar quantity into circulation.

In carbohydrate metabolism, insulin prevents the occurrence of glycogenolysis and gluconeogenesis. It favors glycogen synthesis and also increases uptake of glucose by certain cells. The liver, muscle, and adipose tissues are most important organs in carbohydrate metabolism for which insulin plays a major role (Saini, 2010; Champe et al., 2005). The hormone has long been known as an antagonist to the release of free fatty acids—lipolysis—while supporting protein synthesis/amino acids movement into various body cells.

Mechanisms of insulin secretion

Prior to the release of insulin, glucose (from diet) is transported by facilitated diffusion into the pancreatic beta cells via glucose transporter 2 (GLUT2) after which it is phosphorylated by the enzyme glucokinase, to form glucose-6-phosphate (Fig. 1). The GLUT 2 and glucokinase therefore act as glucosensors of the pancreatic β-cells (Fernandez-Mejia, 2006). The phosphorylated glucose is then oxidized through glycolysis and Krebs cycle to generate adenosine triphosphate (ATP). The oxidation causes the ATP-sensitive K^+ channels to be closed, while the voltage-dependent Ca^{2+} channels are opened. These electrophysiological changes result in depolarization of the plasma membrane of β-cells, leading to the influx of Ca^{2+} and the subsequent release of insulin into circulation after the fusion of the cell membrane and secretory granules containing insulin and C-peptide (Rorsman and Renstrom, 2003). ATP-sensitive K^+ channels are hetero-octamer comprising of four subunits of sulfonylurea 1 receptor (SUR1) and other four subunits of an inward rectifying K^+ channel, Kir6.2. The pharmaceutical drug, sulfonylurea tolbutamide, used to treat T2DM acts via binding to SUR1 to cause similar effect.

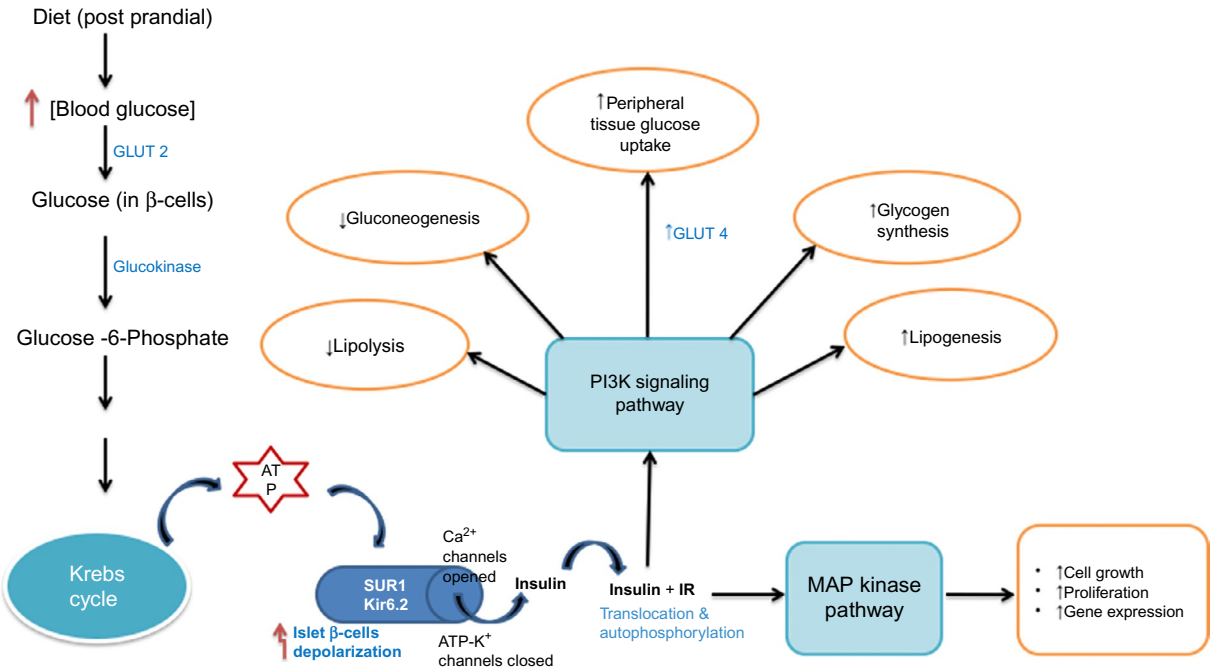

FIG. 1 Mechanism of insulin secretion and action in the regulation of metabolism under normal conditions. In a fed/postprandial state, there is an increased concentration of glucose in the blood. The glucose is transported into pancreatic β-cells via glucose transporter 2 (GLUT 2)-mediated facilitated diffusion. In the β-cells, glucose is phosphorylated by the enzyme glucokinase to form glucose-6-phosphate, which is oxidized via glycolysis and Krebs cycle to generate ATP. The ATP production causes the ATP-sensitive K^+ channels to be closed, while the voltage-dependent Ca^{2+} channels are opened. These result in depolarization of the β-cell plasma membrane, influx of Ca^{2+}, and the subsequent release of insulin into circulation. The insulin released is transported to peripheral tissues where it binds to insulin receptors (IR), resulting in autophosphorylation of these receptors. This in turn activates two main pathways: phosphatidylinositol 3-kinase (PI3K) signaling pathway and mitogen-activated protein (MAP) kinase pathway. PI3K pathway may culminate in the activation of glycogen synthesis, lipogenesis, and peripheral tissue glucose uptake via GLUT 4 and the suppression of gluconeogenesis and lipolysis. Alternatively, the MAP kinase pathway may also be activated leading to cell growth, cell proliferation, and gene expression. Abbreviations: SUR1, sulfonylurea 1 receptor; ATP, adenosine triphosphate; ↑, increase; ↓, decrease.

Other substances including ketoisocaproate, leucine and methyl succinate can also cause insulin secretion.

Mechanisms of insulin signaling and action

When insulin is released, it is transported in the bloodstream to peripheral tissues and binds to insulin receptor (IR) (Fig. 1). These receptors are heterotetrameric membrane glycoproteins and belong to the tyrosine kinase family. The IR is made up of two alpha (α) and two beta (β) subunits. These subunits function in similar manner as allosteric enzymes. The hormone binds to the alpha subunit of the receptor that then causes an activation of the tyrosine kinase in the beta subunit (Saini, 2010) and subsequently autophosphorylation of the receptor. This in turn leads to the phosphorylation of many cellular proteins including members of the insulin receptor substrate (IRS) family, Cbl/Cbl-associated protein (CAP), phosphatidylinositol 3-kinase (PI3K), Akt (protein kinase B), and the transcription factor forkhead box o1 (Foxo1). These phosphorylated proteins serve to activate cascade of reactions that suppress transcription of the genes that encode phosphoenolpyruvate carboxylase (the rate-limiting step in gluconeogenesis), fructose-1, 6-bisphosphatase, and glucose-6-phosphatase (Barthel and Schmoll, 2003). These downregulation mechanisms culminate in the inhibition of gluconeogenesis, glycogenolysis, etc. Again, insulin activates glycogen synthase and inhibits glycogen phosphorylase such that much of the glucose is shunted into glycogenesis. In addition, insulin stimulates lipogenesis by upregulating transcription of sterol regulatory element-binding protein 1c (SREBP-1c) and dephosphorylation (activation) of acetyl-CoA carboxylase (Azzout-Marniche et al., 2000). All these pathways serve to decrease the blood glucose output.

However, in the fasting state, Foxo1 becomes dephosphorylated and confined in the nucleus where it complexes with peroxisome proliferator activator receptor (PPAR) coactivator-1α and CREB binding protein (Cbp)/p300 (Puigserver et al., 2003). Formation of this complex upregulates transcription of the genes encoding phosphoenolpyruvate carboxylase and glucose-6-phosphatase. These enzymes in turn increase blood glucose concentration via gluconeogenesis and glycogenolysis, respectively.

When a desired effect is achieved, the signal from the IR cascade is halted by the action of specific phosphatases and serine/threonine kinases, this is essential in maintaining metabolic control. Examples of these proteins include protein tyrosine phosphatase 1B (PTP1B), phosphatase and tensin homolog (PTEN), and inositol polyphosphate 5-phosphatase (SHIP2) (Zick, 2004).

Biochemical regulation of diabetes mellitus

In diabetic state, insulin is unable to exert its physiological effects described earlier (Section "Mechanisms of insulin signaling and action"), and hence, glucose homeostasis is disrupted. Particularly, the entry of glucose into cells is impaired, and the cells become starved of energy despite high concentration of glucose in the blood. The relatively high glucagon/insulin ratio observed in diabetes decreases fructose-2,6-bisphosphate (allosteric inhibitor of fructose-1,6-bisphosphatase of the gluconeogenic pathway and activator of

phosphofructokinase of glycolytic pathway) concentration in the liver. Thus, gluconeogenesis is stimulated, while glycolysis is inhibited. The high glucagon levels also promote glycogen breakdown via the activation of glycogen phosphorylase and inhibition of glycogen synthase. These pathways result in hyperglycemia and subsequent excretion of glucose in the urine (glycosuria) when the renal reabsorptive threshold (~10 mmol/L) is exceeded. In addition, the "starved cells" make use of other fuel alternatives, primarily lipids, for their metabolic needs. Prolonged use of the stored lipids leads to the generation of ketones bodies via ketogenesis. The ketones bodies can cause metabolic acidosis—diabetic ketoacidosis (DKA)—and slow down most of the body's metabolic processes. Coma and even death may ensue if the metabolic derangement is left untreated (Siddiqui et al., 2013).

Diagnosis of diabetes mellitus

The diagnosis of DM has evolved slightly over the years. In all cases of the disease, polyphagia, polydipsia, polyuria, and weight loss are clinically paramount. Laboratory investigations of blood and urine are key in the diagnosis. The WHO and ADA have played a great role in defining cutoffs for the various laboratory tests. These tests include fasting and random plasma glucose, glycated hemoglobin (HBA1c), and oral glucose tolerance test (OGTT). Fasting plasma glucose concentration or 2-h plasma glucose concentration of $\geq$7.0 and $\geq$11.1 mmol/L, respectively, on two or more occasions confirms the diagnosis of the disease (Fundukian, 2011). Glycated hemoglobin takes into consideration the life span of red blood cells. Red cells usually have some of their hemoglobin A attached to a glucose residue that forms about 5% of circulating hemoglobin. This complex remains till the life span of the red cell is over. The more the plasma glucose, the more the fraction of this glycated hemoglobin. For this reason, it is known to give a better assessment of a patient's blood glucose level. Levels greater or equal to 6.5% on two or more occasions confirm the diagnosis of DM (Fundukian, 2011). In the OGTT, a glucose load of 75 g for adults or 1.75 g/kg for children dissolved in water is given after which the 2-h blood glucose concentration is subsequently measured. It is expected that nondiabetic individuals should be able to use this glucose such that the glucose concentration after 2 h declines to levels below 11.1 mmol/L. Also, urine dipstick test can be performed to detect the presence of glucose in the urine after exceeding the renal threshold value.

Type 1 diabetes mellitus

Type 1 diabetes mellitus emanates from T cell-mediated autoimmune destruction of the pancreatic beta cells that are responsible of for the production of insulin (Fig. 2). This leads to absolute insulin deficiency. The role of the major histocompatibility complex (MHC) or human leucocyte antigen (HLA) and the presence of insulitis or inflammatory products (mononuclear immune cells like dendritic cells, macrophages, and T cells) and islet cell autoantibodies in the pancreas confirm the involvement of autoimmune components in the pathogenesis of the disease (Notkins, 2002).

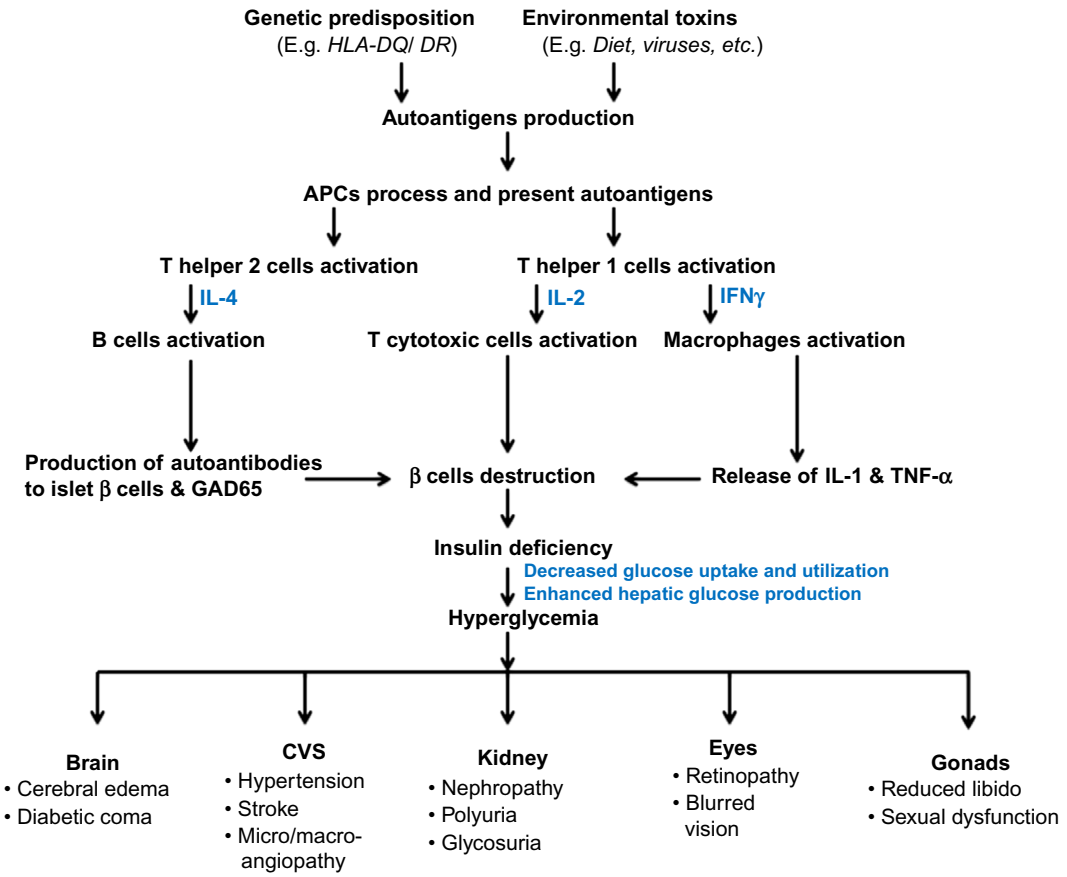

FIG. 2 Pathophysiology of type 1 diabetes mellitus: Type 1 diabetes mellitus results from T cell-mediated autoimmune destruction of the pancreatic β-cells. Genetic and/or environmental factors initiate the formation of autoantigens that are processed and presented by antigen-presenting cells (APCs) to cause the activation of T helper 1 (Th1) and T helper 2 (Th2) lymphocytes. Activated Th1 cells secrete interluekin-2 (IL-2) and interferon gamma (IFNγ). IL-2 activates autoantigen-specific cytotoxic T cells to secrete toxic perforins and granzymes, which destroy β-cells. IFNγ activates macrophages to release inflammatory cytokines such as IL-1 and tumor necrosis factor alpha (TNFα) that cause further destruction to β-cells. The activated Th2 cells also produce IL-4 to activate B lymphocytes to proliferate and produce autoantibodies to β-cells and glutamic acid decarboxylase (GAD65) leading to β-cell destruction. These destructive mechanisms of pancreatic β-cells consequently lead to absolute insulin deficiency that inhibits glucose uptake and utilization by tissue cells resulting in hyperglycemia. The uncontrolled hyperglycemia consequently causes microvascular and macrovascular complications in many organs of the body including the brain, kidney, eyes, sex organs, and the cardiovascular system (CVS).

Mechanisms of beta cell destruction

Autoimmune etiology

Selective destruction of the beta cells of the pancreas by one's immune system is the main factor in the etiology of T1DM. Through immunofluorescence and other molecular techniques, three major autoantigens have been identified in the pancreatic islets: insulin autoantigen; glutamic acid decarboxylase (GAD65); and tyrosine phosphatase, also known as insulinoma antigen (IA-2). Autoantibody directed against these self-antigens is one of the mechanisms of beta-cell destruction (Kelly et al., 2003).

The insulin autoantigen (IA) consists of 51 amino acids and is encoded by a gene located on chromosome 11p15. Its corresponding autoantibody usually appears in the serum of patients at the initial prediabetic state, and about 30%–50% of young children diagnosed of T1DM usually have it (Atkinson and Eisenbarth, 2001).

GAD65 antigen contains 585 amino acids, and its gene is found on chromosome 10p11. GAD65 protein not only is restricted to the pancreatic islet but also may be found in the central nervous system and the testes (Baekkeskov et al., 1990). It is estimated that nearly 70% of newly diagnosed T1DM patients have GAD65 autoantibodies (GADA) (Paschou et al., 2018).

Tyrosine phosphatase, also known as ICA512, is one of the major autoantigens involved in the pathophysiology of T1DM. The antigen consists of 979 amino acids, and its gene is located on the locus of chromosome 2q35. Autoantibodies to tyrosine phosphatase are present in about 60% of T1DM patients. However, these autoantibodies relatively appear later in the serum of T1DM patients, as compared with IAA and GADA, and may indicate progression of the disease (Paschou et al., 2018).

Besides these autoantigens, a fourth autoantigen that has been identified recently is zinc transporter (ZnT8). Autoantibodies to ZnT8 antigen, though present in about 60%–80% of the newly diagnosed patients, happen to disappear shortly after the clinical onset of the disease (Wenzlau et al., 2010). In addition, a study by Salonen et al. (2013) showed that the anti-ZnT8 titers are dependent on the age, HLA genotype, and metabolic status of the patient at the time of diagnosis.

Studies have shown that many months or perhaps years elapse between the appearance of these autoantibodies and the onset of clinical diabetes. The autoantibodies could therefore be used as a predictive marker for T1DM during the prodromal phase of the disease (LaGasse et al., 2002). However, concurrent expression of multiple antibodies (i.e., the presence of more than one autoantibodies rather than the titer of the autoantibodies) is proved to be the more appropriate predictive marker in terms of disease progression. For instance, among first-degree relatives of diabetic patients, the risk of developing T1DM within 5 years is about 10%, 50%, and 60%–80% in the presence of one, two, and three autoantibodies, respectively (Notkins and Lernmark, 2001). Nevertheless, there are some individuals who may test positive to one or more of these autoantibodies but never develop the clinical disease (Kelly et al., 2003).

Cellular immunity

The destruction of the pancreatic beta cells is mainly attributed to cellular immune reactions as compared with the humoral arm of the immune system that is believed to play only a minor role (Fernandez-Mejia, 2006). Generally, immune-mediated (cellular) beta-cell

destruction occurs by apoptosis (programmed cell death) via the activation of caspases or cysteine-asparaginase. Two subpopulations of T lymphocytes are involved: $CD8^+$ cytotoxic T lymphocytes, which recognize processed antigens bound to MHC class I molecules, and $CD4^+$ helper T lymphocytes, which recognize processed antigens bound to MHC class II molecules on the surface of antigen-presenting cells (APCs). These autoreactive T lymphocytes in the pancreatic islet induce a number of the proinflammatory cytokines, which in turn leads to the activation of the caspase cascade. The $CD8^+$ cytotoxic T lymphocytes interact directly with autoantigens in the islet environment, while $CD4^+$ helper T cells interact indirectly with beta cell-specific autoantigens engulfed and processed by APCs (such as macrophages and dendritic cells). In each case, the T cells become activated and trigger the release of a number of proinflammatory cytokines including interleukin-1 (IL-1), interferon gamma (INFγ), tumor necrosis factor alpha (TNFα), and free radicals. These molecules, aside from being toxic to the beta cells, upon binding to complementary receptors on the beta cell activate several enzymatic pathways (e.g., mitogen-activated protein kinase [MAP-Kinase], Fas/Fas ligand [Fas/FasL], and perforin/granzyme) and transcription factors like signal transducer and activator of transcription-1 (STAT-1) and nuclear factor kappa-light-chain enhancer of activated B cells (NF-kappaB) (Pirot et al., 2008; Rabinovitch and Surarez-Pinzon, 1998). All these pathways result in functional impairment, ER stress, atrophic inflammation (insulitis), and eventually apoptosis of the beta cells. Thus, the $CD4^+$ helper T cells (indirect/bystander cell killing) and $CD8^+$ cytotoxic T cells (direct/cell-to-cell killing) synergistically kill the beta cells and ultimately culminates in insulin deficiency.

Selective destruction of the beta cells together with insulitis first results in prediabetes or symptomatic hyperglycemia, which then progresses to overt diabetes (after a long latency period). At this stage, a large percentage (about 80%–90%) of β-cells are destroyed (Notkins, 2002), and the patient become prone to many other autoimmune diseases including Addison's disease, Hashimoto's thyroiditis, celiac disease, and myasthenia gravis. (Siddiqui et al., 2013; Eisenbarth and Gottlieb, 2004).

Susceptibility determinants of type 1 diabetes

Under normal circumstances, a person's T lymphocytes are immunologically anergic to autoantigens (Anjos and Polychronakos, 2004). In other words, they are strictly controlled such that they are not destructive to self-antigens. However, under certain conditions, these dormant immune components become activated and are directed against the self-antigens. The question, therefore, is what factors actually trigger the immune activation? Over the years, research has shown that combination of *genetic*, *humoral*, and *environmental factors* are the main agents implicated in the selective destruction of the pancreatic beta cells.

Genetic factors

Genetic factors are one of the main susceptibility determinants to T1DM. Thus, different genetic components come into play in the etiology of T1DM, which is evidenced by the clustering of the disease within families. In fact, there is about 5%–6% risk of developing T1DM if a first-degree relative is affected in comparison with the general population of 0.4% prevalence rate (Redondo et al., 1999). The risk ratio is higher if a father (rather than a mother)

is the diabetic patient—approximately 12% (Steck et al., 2005). Furthermore, in identical twins, the concordance rate for T1DM is approximately 30%–40% unlike in fraternal twins that is about 6% (Redondo et al., 2001). These confirm the influence of genetic factors in the pathogenesis of the disease. With regard to these factors, two major genes or genetic locus have been implicated. These are the *human leucocyte antigen locus* and the *insulin gene*.

Human leucocyte antigen

The human leucocyte antigen (HLA) or major histocompatibility complex (MHC) is located on chromosome 6p21 and contains more than 200 genes (Kelly et al., 2003). It is a heterodimer consisting of α and β chains that enable it to perform its functional role. The MCH/HLA gene loci encode two groups of molecules: MHC class I and MHC class II molecules. The class I molecules are found on all nucleated cells in the body and present processed antigen to receptors of cytotoxic ($CD8^+$) T lymphocytes. On the other hand, class II molecules are expressed on antigen-presenting cells (APCs) such as dendritic cells, mononuclear phagocytes, and B cells and are essential for the recognition of antigens by helper ($CD4^+$) T cells.

The HLA class I and II genes are highly polymorphic and consist of many different alleles. HLA complex (class II region) polymorphic alleles account for about 30%–50% of the genetic susceptibility of developing T1DM (Noble et al., 1996). While certain alleles or haplotypes show a strong predisposition to the disease, others show a weak or even protective association. For example, the high risk DQ/DR halotypes, DR3-DQA1*0501-DQB1*0201 (DR3), DR4-DQA1*0301-DQB1*0302 (DR4), DQA1*0301-DQB1*0302 (DQ8), and DQA1*0501-DQB1*0201(DQ2), have diabetogenic properties, and therefore, individuals with these haplotypes have increased susceptibility to the T1DM. The DR3/DR4 halotypes may be found in about 90% of T1DM patients as opposed to 40% of the healthy individuals (Paschou et al., 2018). On the hand, the halotypes like DQB1*0602, DRB1*0403, DPB1*0402, and DQA1*0102 have been shown to confer some protection, and as such individuals with these alleles rarely develop T1DM, even when the high-susceptibility allele, DQB1*0302 is also inherited (Paschou et al., 2018).

Through recombinant DNA, it has also been proven that some critical amino acid at position 57 of DQ β-chain and at position 52 of DQ α-chain both influence disease susceptibility. For example, the predisposing DQ halotypes have an uncharged amino acid residue (instead of aspartate) at position 57 on the β-chain (DQ-β-57 Asp-) and arginine at position 52 on the α-chain (DQ-α-52 Arg+) (Deschamps et al., 1991).

Insulin gene

The insulin gene (INS gene) has its locus on 11p15. Although this locus does not itself encode a protein, it plays a functional role in the transcription process of insulin (Paschou et al., 2018). The insulin gene variable number tandem repeat (INS-VNTR) is shown to influence susceptibility to diabetes. The VNTR region usually contains 14–15 bp tandem repeat sequences and occurs at the insulin promoter, about 0.5 kbp or 596 bp upstream the start codon (Steck and Rewers, 2011). The polymorphism is present in two forms: the short/small class I VNTR consisting of about 26–63 repeats and the long/big class III made up of about 140–243 repeats (Esposito et al., 1998). The class III VNTR is shown to be involved in the expression of insulin mRNA in the fetal thymus and has a protective function of negative selection and

deletion of autoreactive T cells involved in β-cell destruction (Barratt et al., 2004). On the contrary, the small class I allelic variant correlates with risk to T1DM (Pugliese et al., 1997). The pleiotropic effect of this polymorphism accounts for nearly 10% of the genetic predisposition to T1DM (Steck and Rewers, 2011).

Other genetic associations

Cytotoxic T-lymphocyte associated protein 4 gene The cytotoxic T lymphocyte-associated protein 4 (CTLA-4) gene, located on chromosome 2q33, encodes costimulatory molecules that maintain anergy in T cells. In other words, it functions as a negative regulator by transmitting signals that downregulates the proliferation of T lymphocytes and other proinflammatory products. A microsatellite polymorphism in CTLA-4 gene (such as CTL-4 $Ala^{17}Thr$ or CTLA-4 A49G) impairs its functional role, and the affected subjects become predisposed to uncontrolled immune response and autoimmune diseases like T1DM, Addison's disease, and Graves' disease (Kayvoura and Ioannidis, 2005).

Protein tyrosine phosphatase, nonreceptor type 22 Gene Recent studies have also shown an association between the protein tyrosine phosphatase nonreceptor type 22 (PTPN22) gene and T1DM (Botini et al., 2006). The PTPN 22 that is located on chromosome 1p13 encodes lymphoid protein tyrosine phosphatase (LYP). The LYP also maintains anergic state of T lymphocytes via dephosphorylation and deactivation of the intracellular protein, C-terminal Src kinase (CSK). A single nucleotide polymorphism (C1858T [Arg620Trp]) in the PTPN 22 gene abrogates the binding affinity of LYP to CSK. This results in unrestrained T-cell activation, and the affected individuals become prone to several autoimmune diseases including T1DM, Graves' disease, and systemic lupus erythematosus (Begovich et al., 2004).

Autoimmune regulator The autoimmune regulator (AIRE) protein is a transcription factor found mainly in the thymic epithelial and dendritic cells where it functions in negative selection of autoreactive T cells. It is encoded by the AIRE gene, located on the long arm of the chromosome 21 (21q22). A rare disorder called autoimmune polyendocrinopathy syndrome type 1 (APS1) results from mutation in the AIRE gene (DeVoss and Anderson, 2007). It is estimated that about 15% of patients with APS1 are also presented with autoimmune diabetes (Pugliese and Skyler, 2013).

Other non-HLA genes that have been found to influence the susceptibility to T1DM also include FoxP3, STAT3, interferon induced with helicase C domain 1 (IFIH1), Erb-B2 receptor tyrosine kinase 3 (ERBB3), and interleukin-2 receptor alpha (IL2RA). Mutation in the FoxP3 genes (found on chromosome X) causes a mild syndrome (IPEX syndrome) comprised of T1DM, allergies, enteropathies, and eczema (Wildin and Freitas, 2005). The FoxP3 is a transcription factor that plays a major function in the development of regulatory T cells. Also a point mutation in the STAT3 transcription factor has been associated with autoimmune diseases like T1DM and autoimmune thyroid dysfunction (Flanagan et al., 2014). Furthermore, several single nucleotide polymorphisms (sNPs) (especially rs2292239) of the ERBB3 gene happen to induce cytokine-mediated apoptosis of pancreatic β-cells, with the end result being insulin deficiency or T1DM (Størling and Pociot, 2017). The IFIH1 gene, located on chromosome 2q24, encodes the melanoma differentiation-associated protein 5 involved in the activation of antiviral immune responses. Nonsynonymous sNPs in the IFIH1 gene such as

rs1990760 have been associated with the development of T1DM, while other variants are protective against the disease (Downes et al., 2010).

Environmental factors

Research has shown that T1DM is a chronic autoimmune disease and that one or more environmental inputs may play a key role in the development of the disease (Eisenbarth et al., 2004). This is supported by the relatively low concordance rate among monozygotic twins. Thus the incidence of the disease in these "closely related" siblings is around 50% and never reaches 100% (Beyan et al., 2012; Redondo et al., 1999). In addition, there is a positive correlation between diabetes and seasons and geographical locations. With regard to seasonal variations, incidence of T1DM tends to peak during autumn and winter (Moltchanova et al., 2009). Also a higher incidence of the disease was reported among French and Jewish children living in Canada than in their counterparts living in France or Israel (Kelly et al., 2003). All these serve to aver the causal role of environmental agents in the pathophysiology of T1DM and the fact that not all genetically susceptible individuals will manifest the clinical diabetes. The environment agents implicated in diabetes include *viruses*, *diet*, *toxins* (e.g., nitrosamines), and gut microbiota. However, the precise mechanism by which some of these environmental insults trigger β-cell autoimmunity still remains a controversy.

Viruses

Certain microbes have been implicated in T1DM by either increasing susceptibility to the disease or conferring some protection against the disease. Rubella virus, Coxsackie B4 virus, and enteroviruses are some of the common viruses that are correlated with high incidence of autoimmune disease like T1DM. Others include mumps, Epstein-Barr virus, varicella zoster virus, rotavirus, and H1N1 influenza virus (Shaheen, 2017).

For example, Coxsackie B4 virus may trigger T1DM by replicating in the thymic epithelial cells and thymocytes, thereby disrupting the maturation and differentiation of T lymphocytes. (Jaïdane et al., 2010; Kim et al., 2016). The encephalomyocarditis virus has also been shown to replicate in the pancreatic beta cells and destroy them directly. In addition, enteroviruses may contribute to the initial stage of the disease through its role in activating the innate immune response (Hober and Sauter, 2010). In spite of this, there are some findings that indicate the role of enteroviral infections in protecting "genetically susceptible" individuals against diabetes under certain conditions (Jaïdane et al., 2010).

Still, other viruses act through molecular mimicry. This theory proposes that some of the autoantigens implicated in T1DM have similar conformation to certain viral proteins. Hence, immune response is sometimes mistakenly directed against these self-antigens, instead of only the viral protein. A typical example is the P2-C protein of the Coxsackie B4 virus, which has a similar amino acid sequence to that of GAD65 autoantigen found in the pancreas (Paschou et al., 2018).

Diet

Another controversy with regard to the etiology of T1DM is the role of dietary factors such as dietary proteins, omega-3 fatty acids, and vitamin D in the development of T1DM in genetically predisposed subjects. Several studies in both man and animal models have indicated that early exposure (within the first 3 months of life) of a child to cow milk-based infant

formulas (containing milk protein like A1-β-casein and whey) or short-term breastfeeding is associated with increased risk of T1DM (Vaarala et al., 2002). Although the direct mechanism of these milk proteins is unclear, it is believed they may act as mimicry epitopes that may induce autoimmunity (Wasmuth and Kolb, 2000). In a study by Vaarala et al. (1999), it was found that the titer of IgG-antibody binding to bovine insulin was higher in ≤3 months infants fed on cow milk than those exclusively breast-fed. Moreover, these antibodies also cross-reacted with human insulin. Gluten is another protein that has been shown to influence the development of T1DM in susceptible individuals apart from its causal role in coeliac disease. Gluten may affect diabetes development by influencing proportional changes in immune cell populations or by modifying the cytokine pattern towards an inflammatory profile (Han et al., 2018). Adlercreutz et al. (2014) reported that gluten-free diet increased the numbers of Tregs but decreased the level of NKG2D receptor and its ligand expression in NOD mice. These in turn altered immune response and cytokine pattern such that a lower incidence of T1DM was observed in these mice in contrast to those fed on gluten-containing diets.

In humans, also, several studies have yielded similar findings. For example, when infants of ≤6 months old are fed on cereal-based or gluten-containing diets, there is a high risk of developing islet autoimmunity and T1DM. Also, in infants of ≥6 months old, there is a high probability of testing positive to autoantibodies to GAD and IA-2 (Elenberg and Shaoul, 2014). The influence of gluten in the development of T1DM (be it diabetogenic vs protective) is, however, found to be dependent on the amount/dose, timing, and mode of introduction (Hanninen and Toivonen, 2015).

Besides the foregoing, low serum concentrations of vitamin D (Raab et al., 2014), insufficient intake of omega-3 fatty acids (Norris et al., 2007) and zinc deficiency (Florowska et al., 2016) have also been associated with the development of T1DM through modulation of inflammatory response and impact on the metabolic regulatory mechanisms.

Toxins and gut microbiota

The gastrointestinal tract harbors a great diversity of both pathogenic and nonpathogenic microbes that may influence the integrity of mucosal barrier and intestinal immune response. Recent studies have showed that alteration in this microbiome composition (dysbiosis) has a marked implication on the pathogenesis of numerous diseases, including heart failure, kidney disease, and diabetes mellitus (Han et al., 2018). These alterations in the gut microbiota have been mainly attributed to diet and/or antibiotic administration.

A study by Knip and Siljander (2016) involving both T1DM children and healthy children as case and control subjects, respectively, revealed a significant variance in the gut microbiota of the two groups. While the T1DM subjects had greater numbers of *Clostridium*, *Bacteroides*, and *Veillonella*, the healthy subjects contained high proportion of *Lactobacillus*, *Bifidobacterium*, *Prevotella*, and *Eubacterium rectale*. Also, vitamin A deficiency has been associated with high intestinal *Firmicutes*/*Bacteroidetes* ratio and reduced levels of butyrate-producing bacteria. These changes are thought to modulate the host's intestinal immune response with subsequent protection against several chronic diseases, including diabetes (Liu and Chen, 2018).

Type 2 diabetes mellitus

The main causes of T2DM are *insulin resistance* and *poor insulin secretion*, which are influenced by several factors.

Insulin resistance and type 2 diabetes

Insulin resistance occurs when insulin signaling becomes redundant (Fernandez-Mejia, 2006; Cerf, 2013). This means a greater concentration of insulin than normal would be needed to keep normal ranged blood glucose concentration. Insulin resistance has long been found to be an underlying cause of metabolic syndrome including T2DM (DeFronzo and Ferrannini, 1991).

Initially in T2DM, insulin is able to respond to hyperglycemia and deal with it at an increased concentration especially when glucose fails to be lowered in the presence of normal insulin concentration. The situation aggravates when consistently hyperglycemia is resolved by hyperinsulinemia. Subsequently normoglycemia becomes unattainable even with hyperinsulinemia in fasting or fed state (Fernandez-Mejia, 2006). At its peak, glucose uptake to peripheral tissues and glycogen synthesis dwindles. Gluconeogenesis is much apparent since there is a generation of free fatty acids in adipose tissue (Boden and Shulman, 2002). Glycogen synthase enzyme has a diminished activity, and hepatic glucose output is not well regulated (Shulman, 2000).

Insulin resistance is a multifaceted syndrome that is influenced by several molecular factors. A lot of research is still being conducted on molecules that contribute to insulin resistance in T2DM. Processing of signals produced by insulin is a complex mechanism involving certain proteins. Much information has been gathered on some of these proteins such as protein kinase B, IRS-2, and foxo1a. Insulin resistance is highly a factor of inadequate function of these proteins (Saini, 2010). Mutations and increased serine phosphorylation of proteins like IRS-1 proteins are also known to cause insulin resistance (Whitehead et al., 1998). Malfunctioning of protein kinase C (PKC), PI3, and the other signaling pathway proteins have been postulated to contribute to insulin resistance, and much work is being carried out on them (Ueki et al., 2002).

Peroxisome proliferator activator receptors (PPARs) regulate transcription of a number of genes with PPAR-γ type noted to be a regulator of insulin action through the regulation of phosphoenolpyruvate carboxykinase (PEPCK) and SREBP-1c. The PPAR activation also inhibits some cytokines that may induce insulin resistance (Xing et al., 1997). Hence, mutations of the PPARs may play a role in insulin resistance as seen in some type 2 diabetic families (Barroso et al., 1999).

Mitochondrial dysfunction has been implicated in insulin resistance and T2DM (Lowell and Shulman, 2005). Mitochondrion is the powerhouse of the cell with regard to fuel oxidation. Studies have suggested that aging is associated with loss or decreased mitochondrial function, which leads to intramyocellular lipid accumulation (Morino et al., 2006). This phenomenon is associated with insulin resistance. Findings from type 2 diabetic patients have also proven that there is a decreased gene expression of an important transcriptional factor, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1) that plays a

critical role in gene regulation in the process of oxidative phosphorylation (Lowell and Shulman, 2005). This finding has been linked to insulin resistance since a defective oxidative phosphorylation leads to a buildup of metabolites such as diacylglycerol that interrupts the process of insulin signaling (Lowell and Shulman, 2005).

Adipose tissue is one of the three main organs of glucose deposition. It stores fat and releases energy when the body requires it. Free fatty acids released by the adipose tissue have long been known to be culprits of insulin resistance and T2DM (Russo et al., 2014; Cerf, 2013). The action of free fatty acid released is such that IRS-1 activation through PI3-kinase in skeletal muscles is rendered inactive and subsequently contributes to poor insulin signaling. Adipocytes also produce a number of cytokines collectively called adipokines that induce insulin resistance leading to T2DM. Interleukin-6, TNF-α, and resistin are well-known adipokines with insulin-resistant activities (Schinner et al., 2005). Leptin and adiponectin, however, have been found to contribute to insulin sensitivity in the body (Saini, 2010). In the mechanism of insulin resistance, interleukin-6 has been found to decrease tyrosine phosphorylation of IRS-1 (Fernandez-Mejia, 2006). TNF-α downregulates GLUT 4 expression and favors serine phosphorylation of IRS-1, while resistin is known to cause an increase in fasting blood glucose concentration and hepatic glucose production (Schinner et al., 2005; Moon et al., 2003).

Obesity is also associated with insulin resistance. Excessive buildup of fats poses a serious threat to the body. Obesity causes the release of certain proinflammatory cytokines as a result of a protracted inflammation primarily due to macrophage infiltration in the adipocytes. Consequently, adipocytes fail to respond adequately to insulin, which progresses to firm resistance, beta cell dysfunction, and T2DM (Cerf, 2013).

Other causes of insulin resistance include pregnancy, hemochromatosis, autoantibodies that are produced against the insulin receptor, and conditions such as acromegaly (increased growth hormone) and Cushing's syndrome (increased glucocorticoids) (Guyton and Hall, 2006).

Poor insulin secretion and T2DM

The pancreatic beta cells' failure to produce required quantities of insulin due to dysfunction is one of the main factors related to poor insulin secretion. Concurrently, a reduced beta-cell mass has been implicated in poor insulin secretion as well. At any point in time, one of these factors or both are perpetrators (Russo et al., 2014; Halban et al., 2014).

Insulin secretion is greatly impaired in beta-cell dysfunction making its severity surpasses that of insulin resistance (Ashcroft and Rorsman, 2012). Although reduced beta-cell mass is a necessary factor in T2DM etiology, it has been established that the beta cells are tough to the extent that they can make up for the body's crave for insulin even with the reduced mass (Cerf, 2013). Hence, secretory function of the beta cells is a supreme factor in hampered insulin secretion and its progress to T2DM. Regulation of beta-cell function depends largely on glucose even in transcription and translation (Henquin et al., 2006; Schuit et al., 2002). This explains why beta cells wear out in steady hyperglycemia. Again in situations where the beta cells are not kindled to function on regular basis, they gradually lose their ability to promptly deal with hyperglycemia when the need arises (Cerf, 2013). This may occur in people who usually have low glycemic states, for example, in people who starve (Cerf, 2013).

TABLE 2 MODY genes, their locations, and state of hyperglycemia

Type	Symbol of gene	Chromosomal location	State of hyperglycemia
MODY1	HNF4A	20q13.12	Mild to severe
MODY2	GCK	7p13	Mild
MODY3	HNF1A/TCF1	12q24.31	Mild to severe—progresses over time
MODY4	IPF1	13q12.2	Mild
MODY5	HNF1B/TCF2	17q12	Mild to severe
MODY6	NeuroD1	2q31.3	Mild to severe

HNF4A, hepatocyte nuclear factor 4 alpha; *GCK,* glucokinase; *HNF1A/TCF1,* hepatocyte nuclear factor 1 alpha/T-cell factor 1; *IPF1,* insulin promoter factor 1; *HNF1B/TCF2,* hepatocyte nuclear factor 1 beta/T-cell factor 2; *NeuroD1,* neuronal differentiation 1.

Beta-cell dysfunction arises from many factors such as oxidative stress, inflammatory stress, ER stress, stressed islet integrity, and cytokines (Halban et al., 2014). Glucotoxicity and glucolipotoxicity lead to persistent hyperglycemia, which causes oxidative stress that in turn leads to variation in insulin gene expression. The mitochondria may be injured so as the ER causing the ER stress and stressed islet integrity. With hyperglycemia, hyperlipidemia, and obesity at play, inflammation is sure to persist. This is because proinflammatory cytokines and other cytokines permeate the pancreas. The sum of the processes earlier leads to beta-cell destruction, which ultimately causes beta-cell dysfunction and reduced beta-cell mass. Saturated fat and free fatty acids are also known to be a factor of beta-cell dysfunction (Cerf, 2013).

Genetic defects also play a part in beta-cell dysfunction. This is evident in the monogenic forms of diabetes usually referred to as maturity onset diabetes of the young (MODY) (Table 2). MODY is typified by primary defect in beta-cell function, autosomal dominant inheritance, and an early onset of T2DM (Fajans, 1990). Mutations of not less than six different genes have been implicated in MODY. These genes are responsible for gene regulation in the beta cells and in the hepatocytes. MODY glucokinase gene defect causes MODY2. Glucokinase is a necessary enzyme factor for glucose uptake in the hepatocytes and regulates insulin secretion in response to glucose (Matschinsky, 1996). Hepatic nuclear factors (HNFs) control insulin expression and proteins that function in glucose transport, mitochondrial metabolism, and glycolysis (Ryffel, 2001). Defects in HNF4A, HNF1A, and HNF1B cause MODY1, MODY3, and MODY5, respectively. Neuronal differentiation 1 (NeuroD1) is involved in pancreatic islet development and also plays part in regulating the transcription mechanism of the insulin gene (Chu et al., 2001). Its defect is known to cause MODY6. Insulin promoter factor-1 (IPF-1) regulates GLUT-2, insulin, and glucokinase transcription, which are all vital beta-cell genes (Edlund, 1998). Defect in IPF-1 causes MODY4.

Conclusion

Diabetes mellitus is a multifactorial condition that results from the interplay between different genetic and/or environmental susceptibility determinants. The global burden of the

disease, in terms of its morbidity and mortality, cannot be underemphasized as it continues to plague millions of people in the world. This underscores the need to put in place measures to bring the dreaded condition under control. Also, considering the far-reaching facts that surround the molecular mechanisms and biochemical regulation of the condition, more research together with massive health education, novel management, and treatment strategies are recommended and should be aimed at the preventing the development of overt form of the disease.

Glossary

Adipokines Adipokines (also called adipocytokines) are cell-signaling molecules (cytokines) produced by the adipose tissue that play functional roles in energy/metabolic status of the body, inflammation, obesity, etc. Notable examples of adipokines include leptin, adiponectin, resistin, interleukin-6, and tissue necrosis factor.

Anergy Anergy refers to a state of unresponsiveness, immune tolerance, or the lack of immune response to a self-antigen or even a foreign antigen. Thus, under normal conditions, an individual's T cells and B cells are immunologically anergic to autoantigens, and this prevents the occurrence of autoimmune diseases.

Autoimmunity Autoimmunity is defined as the initiation of immune response that is directed against self-antigens (autoantigens) or the body's tissues and cells.

Concordance rate Concordance rate, in genetics, refers to the probability or the extent to which a pair/group of individuals will all possess a particular trait present in one of the pair/group. Concordance rate is very essential in determining the influence of genetic factors in the development a certain phenotype or trait, for example, disease susceptibility.

Facilitated diffusion Facilitated diffusion is the passive movement of solutes, molecules, or ions across a biological membrane mediated by carrier (transmembrane) proteins. In facilitated diffusion, the molecules are usually transported along a concentration gradient without energy input or expenditure.

Gestational diabetes Gestational diabetes is a condition that is characterized by insulin resistance or glucose intolerance, with onset or first diagnosis at pregnancy (gestation). The resistance or tolerance is attributed to increased production of placental hormones like human placental lactogen, progesterone, cortisol, growth hormone, and prolactin. The condition mostly occurs after second trimester of pregnancy and usually goes away after delivery.

Gluconeogenesis Gluconeogenesis is an enzyme-controlled metabolic pathway in which glucose is synthesized from noncarbohydrate sources or substrates such as amino acids, lactate, glycerol, and pyruvate

Haplotype Haplotype is a group of alleles found at a particular locus of a chromosome that are inherited together.

Insulin resistance Insulin resistance is a state in which there is diminished sensitivity or response to insulin action, especially in peripheral tissues like the skeletal muscle, adipose, and liver. Under this condition, entry of glucose into these cells is impaired with subsequent increase in blood glucose concentration.

Molecular mimicry Molecular mimicry refers to the structural or functional similarities between the epitope (antigenic determinant) of a foreign protein/antigen and that of a self-antigen, resulting in a cross-reactivity. It is one of the proposed mechanisms underlying the etiology of several autoimmune diseases like type 1 diabetes.

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CHAPTER

4

Glycoprotein folding

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OUTLINE

SUMMARY POINTS

- This chapter focuses on the role of *N*-glycosylation in the folding quality control and degradation of glycoproteins in the endoplasmic reticulum.
- *N*-glycans are exploited as an information code of the folding status and age of glycoproteins.
- UGGT is a folding sensor that reglucosylates misfolded glycoproteins or unassembled oligomers, thus triggering their retention in the ER by the lectins calreticulin and calnexin.
- Lectin-associated enzymes assist in the correct formation of disulfide bridges and in the isomerization of prolyl bonds.
- Glycoproteins that fail to fold successfully are delivered to a specialized subcompartment where mannose residues are trimmed.
- Mannose trimming is a slow process that marks glycoproteins for their retrotranslocation in the cytosol, where they are degraded by the proteasome.

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Key facts

- About one-third of the eukaryotic proteome belongs to the secretory pathway.
- Most secretory pathway proteins are *N*-glycosylated at the lateral chain of Asn residues displayed in the context Asn-X-Ser/Thr (X cannot be Pro).
- *N*-glycans are bulky and hydrophilic moieties that prevent nonspecific protein-protein contacts and were subjected to a strong selection pressure.
- A series of enzymes and lectins modify and recognized specific *N*-glycans to assist in the folding process.
- Monoglucosylated *N*-glycans indicate the presence of folding defects and mannose trimming marks glycoproteins for degradation.

Introduction

Approximately one-third of eukaryotic proteins belong to the secretory pathway. They enter the endoplasmic reticulum (ER) either co- or posttranslationally through the Sec61 αβγ translocon complex. In the ER, these proteins are chemically modified and acquire their native tertiary and quaternary structures. The most common modifications are the attachment of *N*-glycans and the formation of disulfide bridges. Nearly 80% of secretory pathway proteins are *N*-glycosylated at the lateral chain of asparagine residues displayed in the context Asn-X-Ser/Thr (X cannot be proline), a motif known as *N*-glycosylation sequon. In some cases, other consensus sequences such as Asn-X-Cys/Gly/Val can be employed. *N*-glycans fulfill several biological and structural roles. They are bulky and hydrophilic moieties that can be considered as covalently attached "chemical chaperones," preventing nonspecific contacts and modulating the stability of glycoproteins (Helenius and Aebi, 2004). *N*-glycans also favor the acquisition of secondary structural elements such as turns and provide protection toward proteolysis. On the other hand, the great diversity of mature *N*-glycans plays a central role in numerous molecular recognition events, modulating process such as migration, differentiation, and proliferation. *N*-glycans present transiently in the ER are exploited as a platform that encodes information about the folding status and age of glycoproteins, thus allowing the action of specialized folding machinery.

The endoplasmic reticulum

The ER is the second most common place for protein synthesis in eukaryotes. The lumen of the ER is topologically equivalent to the cell exterior, and from an evolutionary perspective, it is analogous to the periplasmic space of bacteria. On average the ER occupies approximately 10% of the cell volume, being much bigger in cells specialized in protein secretion such as activated B lymphocytes or pancreatic beta cells. The chemical composition of the ER is quite different compared with the cytosol, with a higher calcium concentration (∼mM), an oxidizing redox potential and high macromolecular crowding levels. Besides the ER has an ample

battery of chaperones and folding assisting enzymes, many of them homologous to those present in the cytosol. In fact the most abundant protein of the ER is BiP, a member of the HSP70 family. The ER also has a homologous of HSP90s called GRP94. Interestingly, the ER lacks any member of the HSP60s family. A particular feature of the ER is the *N*-glycosylation of proteins, which allowed the evolution of specialized folding systems.

N-glycan transfer

Higher eukaryotes transfer *en bloc* a preassembled oligosaccharide composed of two *N*-acetyl glucosamines, nine mannoses, and three glucoses ($Glc_3Man_9GlcNAc_2$, Fig. 1A). Some organisms such as trypanosomatids employ shorter versions of this compound that may lack glucoses and some mannoses (Parodi, 1993). *N*-glycosylation is performed by the multiprotein complex oligosaccharyltransferase (OST), which can associate with the Sec61

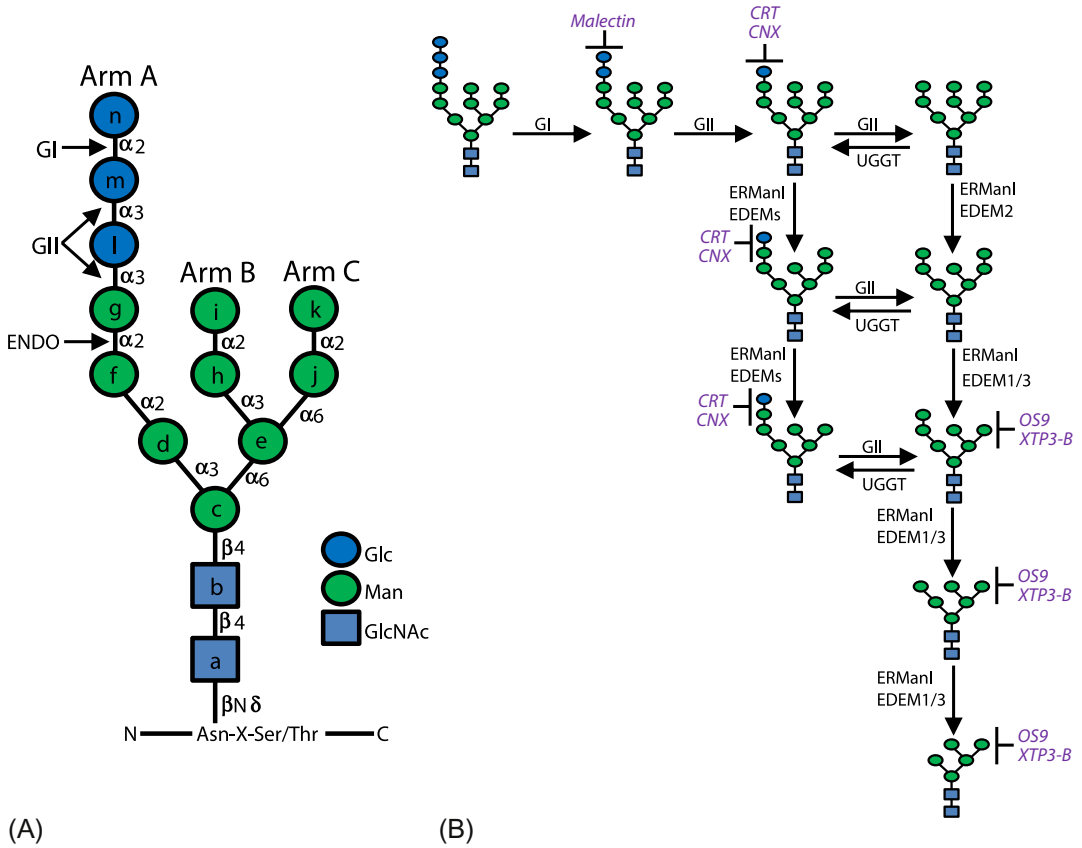

FIG. 1 *N*-glycan processing in the early secretory pathway. (A) Structure of the $Glc_3Man_9GlcNAc_2$ oligosaccharide transferred by the oligosaccharyltransferase complex. (B) Processing and recognition of *N*-glycans in the early secretory pathway.

αβγ translocon (Harada et al., 2009). OST is composed of multiple subunits, being the enzymatic activity located in the STT3 subunit (Kelleher and Gilmore, 2006; Wild et al., 2018). Bacteria and some lower eukaryotes only have this subunit (Hese et al., 2009; Kelleher and Gilmore, 2006). Higher eukaryotes express two paralogues of STT3, STT3A and STT3B. Complexes with STT3A are associated with the translocon and work mainly during the entrance of acceptors into the ER lumen (Ruiz-Canada et al., 2009). This complex is responsible for the about 80% of sequon occupation. STT3A is prone so skip sequons close to the cleavage site of the signal peptide (Ruiz-Canada et al., 2009), closely spaced or suboptimal sequons (Shrimal and Gilmore, 2013) and sites close to cysteine residues (Cherepanova et al., 2014). STT3B-containing complexes are not bound to Sec61 and can occupy sequons that remained vacant after the translocation. This redundancy favors a high occupancy of many sequons. In particular, sequons near the C-terminal end of proteins are poorly recognized by STT3A, presumably because they pass fast through its catalytic site, and their occupation is usually performed by STT3B. In spite of this redundancy, some sequons display partial occupancy, a phenomenon known as macroheterogeneity (Zacchi and Schulz, 2016). Factors controlling this behavior are partially understood. For instance, sequons with Thr in the third position are better acceptors than those with Ser (Kasturi et al., 1995), and residues at the second and fourth positions also influence sequon usage (Mellquist et al., 1998; Shakin-Eshleman et al., 1996). In addition, protein folding can compete with *N*-glycosylation due to steric hindrances to access the catalytic site of STT3s (Holst et al., 1996). Not surprisingly, sequons are subjected to strong selection pressures. Their frequency is highly incremented in solvent exposed site of secretory pathway proteins, notably in segments of secondary structure change (Petrescu et al., 2004), while in parallel they are almost absent at buried positions of these proteins (Medus et al., 2017).

Glycoprotein folding quality control

The multiple biological roles of *N*-glycans are consequence of their great chemical diversity. Nevertheless, in mature glycoproteins, the only remaining portion of the originally transferred oligosaccharide usually is the core glycan ($Man_3GlcNAc_2$). All glucoses and most of the mannose residues are removed at early stages of the secretory pathway. After this processing, a series of glycosyltransferases located mainly in the Golgi apparatus build the mature structures following a nonlineal and complex process. At variance with the initial transfer, these modifications take place one monosaccharide at the time, using nucleotide sugars as donors. The transient high-mannose glycans present in the ER play an important role in the biogenesis of glycoproteins. They are used as an information platform of the folding status and age of these proteins. This information is generated and interpreted by a series of glycosidases, glycosyltransferases, and lectins located in the early secretory pathway. Two processes are central to these mechanisms: (a) the generation of monoglucosylated glycans ($GlcMan_{7–9}GlcNAc_2$) and (b) the progressive loss of mannose residues. The former are hallmarks in the glycoprotein folding quality control system (QC) that indicate the presence of conformational defects, while the loss of mannoses is a central signal for the ER-associated degradation (ERAD) mechanism.

Immediately after the transference, the outermost glucose is cleaved by glucosidase I (GI), a type II membrane protein associated with the Sec61 αβγ translocon and its catalytic domain faces the ER lumen (Fig. 2) (Dejgaard et al., 2010). This location allows its action during protein synthesis, generating very rapidly the intermediate diglucosylated species $Glc_2Man_9GlcNAc_2$. The half lifetime of the initial glycan is very short ($t_{1/2}$ of ~2 min or less). The diglucosylated glycan can be recognized by malectin, a type I membrane protein associated with the OST subunit ribophorin I (Qin et al., 2012). Malectin expression is incremented during ER stress (Galli et al., 2011), and presumably, it works as an initial triage system for misfolded proteins, regulating the flux of glycoproteins. Next, the following glucose is removed by glucosidase II (GII), thus generating the monoglucosylated intermediate $Glc_1Man_9GlcNAc_2$ (G1M9). This reaction is usually also rapid ($t_{1/2}$ of ~5 min). GII is a soluble heterodimeric protein composed of a catalytic subunit (GIIα) and an accessory subunit (GIIβ). The latter is responsible for the retention of GII in the ER and through its C-terminal mannose 6-phosphate receptor homologous (MRH) domain modulates the activity of GIIα (Stigliano et al., 2009). Monoglucosylated glycoproteins generated by GII are recognized by two ER-resident lectin chaperones, calreticulin (CRT) and calnexin (CNX). Afterward, the remaining glucose is also cleaved by GII in a more slow reaction ($t_{1/2}$ of ~20 min), thus dissociating the complexes with the lectins. At this point, properly folded proteins or assembled oligomers can pursue to their final destination. On the other hand, misfolded glycoproteins or orphan complex subunits are recognized by the UDP-Glc:glycoprotein glucosyltransferase (UGGT). This enzyme adds back the last glucose removed by GII, triggering a new round of interaction with CRT/CNX (Fig. 2). Proteins enter the QC cycle until they acquire their native conformation or, alternatively, they are marked for degradation in the cytosol by the proteasome.

The QC lectins

CRT is a soluble ER-resident protein (~46 kDa) with a signal peptide and a C-terminal ER retention/retrieval motif. In spite of that, CRT has also been found associated with the plasma membrane, in secretory granules and in the nucleus and cytosol, fulfilling diverse biological roles (Caramelo and Parodi, 2015). CRT is composed of three structural domains. The N-terminal domain (residues 18–197 in human) is a globular β-sandwich homologous to leguminous lectins that binds monoglucosylated glycans. The proline-rich domain (P-domain, residues 198–308 in human) forms a flexible and long arm that protrudes from the N-terminal domain. Finally the C-terminal domain (residues 309–417 in human) is enriched in negatively charged residues. This domain binds high amounts of calcium with low affinity (K_d ~ 1–2 mM) and is responsible for the calcium buffering activity of CRT. About half of the ER calcium content is bound to this domain, whose intrinsically disordered structure is modulated by calcium (Villamil Giraldo et al., 2010). In addition, this structural effect regulates the retro translocation of CRT into the cytosol (Labriola et al., 2010). CNX is a type I integral membrane protein (~66 kDa) that shares ~40% sequence identity with CRT. Its domain organization is similar to CRT, with the addition of a transmembrane segment intervening between the P- and C-terminal domains. The transmembrane segment has a conserved cysteine that can be palmitoylated, thus targeting CNX to the mitochondria-associated membranes.

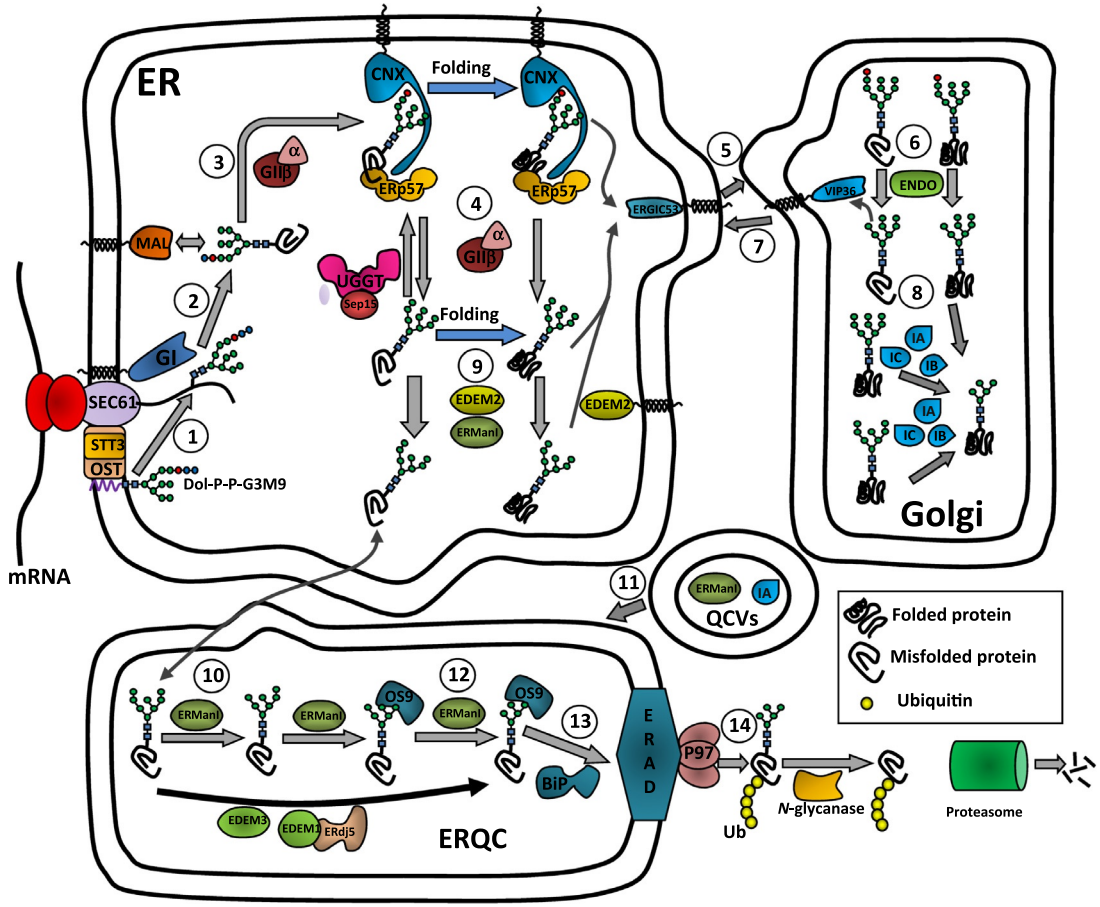

FIG. 2 Glycoprotein folding quality control and degradation. Glycoprotein folding quality control and degradation in the early secretory pathway. (1) G3M9 transfer to nascent proteins by the oligosaccharyltransferase complex (OST). (2) First glucose trimming by GI and recognition by malectin (MAL) of the G2M9 glycoform. (3) *N*-glycans are further processed by the heterodimeric glucosidase II (GIIα-GIIβ) to generate the monoglucosylated intermediates that are bound by the ER lectins calnexin and calreticulin (not shown). (4) Cleavage of the last glucose by GII and reglucosylation of misfolded species by UGGT associated with Sep15. (5) High-mannose glycoproteins leave the ER to the Golgi by bulk flow or associated with sorting receptors such as ERGIC-53. (6) In the Golgi, monoglucosylated species that erroneously escaped the CNX/CRT cycle can be deglucosylated by Golgi endomannosidase (ENDO). (7) Misfolded species can be delivered back to the ER by the sorting receptor VIP36, (8) while properly folded species are further processed to the M5 glycoform by Golgi mannosidases Ia, Ib, and Ic. (9) Mannose trimming initiates by the cleavage of the mannose residue at branch B by EDEM-2 and ER α1,2-mannosidase I (ERManI). (10) Further mannose trimming takes place in the ER or in the ERQC. This process is mainly in charge of ERManI and EDEMs 1 and 3. EDEM 1 associates with the PDI J-domain containing protein ERdj5, which reduces the disulfide bridges of misfolded species. (11) Some ERAD components such as ERManI and mannosidase Ia are stored in quality control vesicles, which upon stress migrate to the ERQC. (12) Uncovering of the α1,6-Man residue allows the interaction of the M6 and M5 glycans with the lectin OS-9 and XTP3-B (not shown) and (13) in conjunction with BiP delivers them to the ERAD retrotranslocation and ubiquitination machinery. (14) Ubiquitinated glycoprotein movement into the cytosol is assisted by the AAA ATPase P97, where they are degraded by the proteasome after being deglycosylated by the cytosolic peptidyl *N*-glycanase.

This modification adds a new level of regulation, since it balances the distribution of CNX between the QC cycle and its role in the Ca^{2+} cross talk between the ER and the mitochondria (Lynes et al., 2013). Even though sugar recognition by these lectins is identical, they bind to partial overlapping sets of glycoproteins in vivo. This behavior is due to their different topologies and can be partially interconverted by adding a transmembrane segment to CRT or by expressing a soluble version of CNX (Danilczyk et al., 2000). Interestingly, some organisms only have one of these lectins. For instance, *Trypanosoma cruzi* express only CRT, while *Saccharomyces cerevisiae* only have CNX.

The P-domain is a unique feature of these lectins. It functions as a platform to interact with folding assisting proteins such as ERp57 (a member of the protein disulfide isomerase family (PDI)), ERp29 (homologous to PDIs without redox activity), and cyclophilin B (CypB, a peptidyl prolyl isomerase) (Kozlov et al., 2017). A patch of positively charge residues on CypB mediates the interaction with the tip of the P-domain. Therefore, glycoprotein association to CRT/CNX not only prevents the exit from the ER and their aggregation but also actively favors a productive folding by facilitating the activity of associated chaperones and enzymes. The ER harbors many PDIs, which are characterized by the presence of up to four thioredoxin domains. These proteins can display four potential activities: reductase, oxidase, isomerase, and chaperone. The active center is the redox motif C-X-Z-C, being CGHC the most frequent. Domain organization of PDI and ERp57 is similar, with two redox active domains (a, a′) and two inactive domains (b, b′), arranged following an abb′a′ pattern. While in PDI, a hydrophobic pocket in the b and b′ domains interacts directly with misfolded proteins, in ERp57, these domains bind to the tip of the P-domain of CRT/CNX (Frickel et al., 2002), which presents the substrates to ERp57 (Jessop et al., 2009). The flexibility of the P-domain presumably allows ERp57 to isomerase disulfide bridges located at various positions. In fact, disulfide bridge maturation is seriously impaired when glycoprotein association with CRT is precluded (Labriola et al., 2011).

The glycoprotein folding sensor

UGGT detects folding defects in glycoproteins. It is an interesting case of a protein that combines the specificity of a classical chaperone with the activity of a glycosyltransferase. UGGT is a large protein (∼170 kDa) that resides in the lumen of the ER lumen. It uses UDP-Glc as donor and senses a bipartite signal on its substrates, a high-mannose oligosaccharide and exposed hydrophobic residues. UGGT recognizes more efficiently advanced folding intermediates, such as molten globules (Caramelo et al., 2003). It also recognizes properly folded subunits of unassembled complexes, provided that they exposed a hydrophobic patch (Keith et al., 2005). For this reason, UGGT participates also in the quality control of oligomer assembly. In addition, it is also active toward hydrophobic nonproteinaceous moieties bound to high-mannose glycans (Ito et al., 2015). The crystal structure of UGGT from the thermophilic yeast *Chaetomium thermophilum* reveals a complex topology (Roversi et al., 2017). It is formed by seven domains: four redox-inactive thioredoxin-like domains (TRXL1–4), two β-sandwiches (βS1 and βS2), and a C-terminal catalytic domain. Domains TRXL4 and βS2 are encoded by nonconsecutive segments of the sequence. TRXL domains form an arrangement similar to a cradle, with a high degree of interdomain flexibility. The structure of the third TRXL domain reveals a hydrophobic patch bound to a detergent molecule, which is

exposed upon the displacement of an adjacent alpha helix (Zhu et al., 2014). This patch could contribute to the recognition of misfolded proteins. The catalytic domain belongs to the glycosyltransferase 24 family, characterized by a conserved DQD motif that binds UDP-Glc. The activity of the enzyme depends on high calcium concentration (∼mM), compatible with that found in the ER lumen. From an evolutionary point of view, UGGT probably arises from the fusion of a glycosyltransferase with a PDI. Almost all eukaryotes have an active UGGT, with the notable exception of *S. cerevisiae*. In addition, most vertebrates have two homologues of the enzyme, UGGT1 and UGGT2, that share ∼55% sequence identity. UGGT1 is more active in vitro than UGGT2 (Takeda et al., 2014). UGGT1 expression is upregulated by ER stress (Blanco-Herrera et al., 2015), and its deletion is embryonically lethal in mice, although cells derived from this organism are viable (Molinari et al., 2005). Both UGGTs form a tight 1:1 complex ($K_d \sim 20$ nM) with the selenocysteine-containing oxidoreductase Sep15 (Korotkov et al., 2001). This association enhances the activity of UGGT1 and UGGT2, being this effect more acute for the former (Takeda et al., 2014). In addition, the redox activity of Sep15 could provide an active mechanism to assist a productive folding.

As we mentioned, UGGT recognizes exposed hydrophobic residues, a common landmark of misfolded proteins. The exact mechanism for this recognition is not fully understood, although its interdomain flexibility plays an important role (Roversi et al., 2017). This would allow the recognition of folding defects located a various distances from the *N*-glycan (Hachisu et al., 2016; Ritter and Helenius, 2000; Taylor et al., 2004). UGGT can also participate in the assembly of protein oligomers, both in vitro and in vivo. For instance, UGGT reglucosylates four of the six subunits of the T-cell receptor until correct disulfide bridges are formed and the complete receptor is properly assembled (Gardner and Kearse, 1999). In addition, UGGT's ability to detect minor structural differences has been exploited to regulate diverse biological processes. For example, UGGT preferentially reglucosylates class I MHC that are empty (Neerincx et al., 2017) or are loaded with suboptimal peptides (Zhang et al., 2011), thus triggering their retention in the ER. This mechanism ensures that only complexes loaded with high-affinity peptides reach the plasma membrane. Nevertheless, this ability is not without risks. An overzealous sensor could trigger the retention of proteins that could be functional if they were allowed to leave the ER. For example, UGGT1 has been found to be associated with the △F508 form of CFTR, the most common mutation in cystic fibrosis (Pankow et al., 2015).

In addition to GII, there is a backup mechanism for protein deglucosylation provided by Golgi *endo*-α-mannosidase (Zuber et al., 2000). This enzyme cleaves between residues g and f of the oligosaccharide (Fig. 1A), definitively releasing the glycoproteins from the CRT/CNX cycle. At variance with UGGT, the activity of this enzyme is independent of the folding status of its substrates (Dedola et al., 2016; Torossi et al., 2006). The biological implications of this property may be dual, since it can promote the trafficking of glycoproteins through the secretory pathway (Fujimoto and Kornfeld, 1991) and it can also facilitate the ERAD mechanism (Kukushkin et al., 2011).

Glycoprotein exit from the ER

Transit of glycoproteins between the ER and the Golgi is mediated by ERGIC-53, VIP36, and VIPL. These proteins are type I transmembrane L-type lectins with a bipartite localization

signal on their short cytosolic tail (Kappeler et al., 1997). VIP36 and VIPL recognize the deglucosylated trimannose in branch A (Fig. 1A). ERGI53 shows a broader recognition pattern with lower affinity and can also bind monoglucosylated species (Kamiya et al., 2008). This difference is due to a shallower binding site of ERGIC53, where the mutation of an Asp to Gly makes room to accommodate the terminal glucose (Satoh et al., 2014). Interestingly, binding to VIPL and ERGIC53 is impaired under acidic conditions, a feature that regulates their affinity along the secretory pathway (Appenzeller-Herzog et al., 2004). The biological roles of these lectins are likely to be different. For instance, VIP36 can facilitate the retrograde transport from the Golgi to the ER, suggesting a role in a post-ER QC mechanism (Reiterer et al., 2010). In addition, mutations in ERGIC-53 disrupt the exit of a subset of glycoproteins, such as coagulation factors V and VIII.

Mannose trimming and degradation

Terminally misfolded proteins are retained in the ER, and eventually, they are tagged for ERAD (Fig. 2). This process involves a series of mannosidases that cleaves α1,2-linked mannoses. Presumably, the low intrinsic activity of these enzymes is central to discriminate between proteins that are on a productive folding pathway from those irreparable cases. Of major importance is the removal of the external mannose in branch A, since it eliminates the acceptor site recognized by UGGT. The first trimming is carried out by ER mannosidase I (ERMan1/Man1B1), which cleaves the α1,2-linked mannose of the B branch, yielding the $Man_8GlcNAc_2$ isomer B. This enzyme is located in specialized quality control vesicles (QCVs), and upon ER stress, it concentrates in a subcompartment of the ER specialized in QC (ERQC) (Benyair et al., 2015b). In addition, mannosidase 1A (Man1A) follows a similar pattern (Ogen-Shtern et al., 2016). Both proteins are type II membrane proteins that belong to the glycoside hydrolase family 47 (GH47). Their concentration in the ERQC presumably allows trimming of three or four mannoses, yielding $Man_{5–6}GlcNAc_2$ (Benyair et al., 2015a). Interestingly, the activity of ERman1 is higher toward denatured glycoproteins (Aikawa et al., 2012), regardless of the presence of glucose residues (Aikawa et al., 2014). This feature could add a selection filter to the ERAD machinery, focusing its activity on misfolded species. In addition, three enzymes called ER degradation-enhancing alpha-mannosidase-like proteins (EDEM1–3) collaborate to mannose trimming. They also belong to the GH47 family, but at variance with ERMan1, their expression is upregulated during ER stress by the IRE1 arm of the unfolded protein response (Lee et al., 2003). In the absence of stress, EDEM1 and ERMan1 are short-lived proteins, thus keeping the ERAD machinery at bay. EDEM1 has a dual topology due to a suboptimal processing of its signal peptide. The uncleaved form is an integral membrane protein, while the cleaved one is soluble. Membrane-bound EDEM1 interacts with SEL1, a scaffold protein of the ERAD machinery, while soluble EDEM1 interacts with ERdj5, a J-domain containing PDI with strong reductase activity. This complex opens the disulfide bridges of ERAD substrates and delivers them to BiP, which assists in their translocation into the cytosol (Fig. 2) (Ushioda et al., 2008). By contrast, EDEM2 and EDEM3 are both soluble proteins. Similarly to ERManI, the activity of EDEM1 and EDEM2 is incremented when *N*-glycans are displayed on misfolded glycoproteins (Shenkman et al., 2018). Of particular importance is the cleavage of residue f, which

exposes the terminal α1,6 linked mannose of branch C (Fig. 1A). These structures are recognized by the MRH domains of the specialized lectins OS-9 and XTP3-B. This recognition is mediated by two contiguous aromatic residues (WW in OS-9, YW in XTP3-B), which makes extensive contact with the exposed trimannose moiety of branch C (Satoh et al., 2010). Afterward, binding of these lectins to the HRD1-SEL1L ubiquitin ligase complex on the ER membrane delivers the misfolded glycoproteins to the ERAD machinery.

The ER is a highly dynamic organelle that modulates its structure according to the folding demands of the cell. As we mentioned earlier, there are subcompartments that escape the classical division between a rough ER dedicated to protein synthesis and a smooth ER focused on lipid synthesis (Benyair et al., 2015a). New experiments have revealed at least three specialized regions: First, protein exit to the Golgi takes places in ER exit sites (ERES), where the lectins dedicated to this process are located (ERGI53, VIP-36, and VIPL). On the other hand, some of the ERAD and QC components concentrate in a perinuclear area named ER quality control compartment (ERQC) or in QCVs. This segregation seems to regulate the ERAD response in two ways. In resting conditions, QCVs take apart key components of the ERAD machinery, in particular ERManI and Man1A. Presumably, this segregation dampers degradation processes. On the contrary, under stress conditions, misfolded proteins concentrate in the perinuclear ERQC along with the ERAD and QC machineries, thus enhancing the capacity of the cell to cope with this situation.

In summary, *N*-glycosylation is a central process in the biogenesis of secretory pathway proteins. Their great diversity displayed in mature glycoproteins makes them key components of numerous recognitions events. On the other hand, their processing in the early secretory pathway is central for the successful folding and assembly of numerous glycoproteins.

Glossary

Glycoprotein folding quality control (QC) QC is the process by which the ER senses the presence of folding defects in glycoproteins and triggers their retention. Of central importance to this process is the enzyme UGGT, which recognizes the exposition of hydrophobic residues.

Endoplasmic reticulum-associated degradation (ERAD) ERAD is a mechanism that recognizes and labels misfolded proteins and mediates their retrotranslocation into the cytosol to be degraded by the proteasome.

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CHAPTER

5

Potential link between sugar consumption and ectopic fat

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OUTLINE

SUMMARY POINTS

- Sugar-sweetened beverages are a major source of added sugar in the diet.
- Fructose is the sugar that is suspected to be particularly harmful to health.
- In observational studies, people who drink more sugary beverages have greater fat around their liver and abdominal organs.
- Summarizing the evidence from randomized controlled trials on the impact of sugars on ectopic fat is challenging due to variation in intervention study designs, study participant characteristics, and sample size.
- In the context of excess energy intake, evidence is relatively consistent that high intakes of sugar in the diet will promote liver fat accumulation.

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Introduction

In 2017, the World Health Organization (WHO) reported that greater than 50% of adults and almost 20% of adolescents and children are overweight or obese (World Health Organization, 2017). In the United States (US), the prevalence is greater, with an alarming 70% of adults and 30% of adolescents and children classified as overweight or obese (Segal et al., 2017). Poor diet and lack of physical activity are recognized as contributors to weight gain. Although no single causative dietary factor leads to obesity, beverage consumption patterns may contribute to this global epidemic. There is great public health concern surrounding the contribution of excess *added sugar*, specifically excess calories derived from sugar-sweetened beverages (SSB), to obesity and its metabolic consequences. The US Department of Agriculture (USDA) defines *added sugars* as "all sugars used as ingredients in processed and prepared foods and sugars eaten separately or added to foods at the table" (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). The most common added sugars in the US food supply are sucrose and high-fructose corn syrup (HFCS), while in European countries, sucrose is the primary added sugar (Varsamis et al., 2017). Both sucrose and HFCS deliver almost equal proportions of the monosaccharides fructose and glucose, and SSBs are a major dietary source of these sugars. Among both children and adults in the US, the major contributors to added sugar intake are soda, fruit-flavored and sports drinks, cakes, and other refined grain desserts (U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015; Welsh et al., 2011). Dietary sources are similar in other countries across the world (Popkin and Hawkes, 2016; Singh et al., 2015; Sluik et al., 2016).

Dietary guidance consistently recommends limiting added sugar consumption. The American Heart Association (AHA) recommends that added sugars be limited to no more than 100 calories per day for women and 150 calories per day for men (six and nine teaspoons, respectively) (Johnson et al., 2009). The World Health Organization (WHO) recommends that intake of "free sugars," which includes added sugars, sugars naturally found in honey, fruit juices, fruit concentrates, and syrups, contribute no more than 10% of total energy intake (World Health Organization, 2015). In addition, WHO suggests that the intake of free sugar be further reduced to less than 5% for additional health benefits. This translates into 100 calories or less per day for a person on a 2000-kcal diet. Despite consistent dietary recommendations to substantially limit added sugar intake, self-reported intakes far exceed recommended amounts and, due to self-reporting measurement error, are likely to be underreported. Current mean intakes fall between 12% and 17% of total energy intake, with the highest mean intakes observed among individuals ages 4–18 and 19–30 (Brand-Miller and Barclay, 2017; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015; Vos et al., 2017; Welsh et al., 2011).

Ectopic fat is defined as the accumulation of lipids (i.e., in the form of triglycerides) in nonadipose lean tissue or the accumulation of adipocytes in adipose tissue (Lettner and Roden, 2008). Using imaging techniques such as magnetic resonance imaging (MRI), fat can be detected not only in subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), but also in the pericardial (heart); intrahepatic (liver); and other region-specific fat depots such as intramuscular, thoracic, periaortic, and renal (Lee et al., 2018). Greater VAT and liver (intrahepatic) fat is "metabolically" harmful, and research has observed that multiple

cardiometabolic risk factors are independently associated with these fat depots (Lee et al., 2018). Most often, VAT and SAT are measured using computed tomography (CT), whereas multiple imaging techniques are used to measure liver fat. For instance, CT or ultrasound are often used to detect liver fat in observational studies, whereas MRI or magnetic resonance spectroscopy (MRS) are used in intervention studies (Seabolt et al., 2015). Excess accumulation of fat around the liver, known as nonalcoholic fatty liver disease (NAFLD), is a disease condition whose prevalence has increased substantially in the past decade (Younossi et al., 2011) to a global prevalence of approximately 24% (Younossi et al., 2018). One hypothesis is that sugar, particularly the fructose moiety, is a key dietary exposure contributing to the rise in this clinical condition (Jensen et al., 2018).

In this chapter, we review the evidence linking added sugar intake to ectopic fat deposition, derived from both observational studies and randomized controlled trials (RCT), and summarize the potential mechanisms whereby sugars may increase ectopic fat.

Observational studies

Emerging evidence suggests that the effect of SSB on cardiometabolic risk may be dependent on the location of the fat accumulation, not just on the absolute amount of body fat. Compared with fat deposition in nonconsumers of SSB, daily SSB intake has been associated with greater VAT (Ma et al., 2014; Odegaard et al., 2012) and a greater increase in the proportion of VAT relative to SAT (Odegaard et al., 2012). In a cross-sectional analysis of 791 non-Hispanic white adults, an increasing frequency of SSB intake was associated with a greater percentage of VAT and proportion of VAT to SAT, with no association observed between SSB intake and either percentage of total body fat or BMI (Odegaard et al., 2012). Similarly, Ma and colleagues, using data from 2596 participants enrolled in the Framingham Heart Study (FHS) cohorts, observed that after statistically accounting for the amount of SAT, daily habitual SSB intake of $\geq$1 serving per day was associated with a greater absolute volume of VAT and VAT/SAT ratio (Ma et al., 2014). Due to the cross-sectional design of this study, the temporality of this relationship was limited; however, Ma and colleagues also *prospectively* analyzed data in a sample of approximately 1000 participants from the same FHS cohort (Ma et al., 2016c). Prospective analyses indicated that after 6 years of follow-up, daily consumers of one or more SSB had a 29% greater increase in the volume of VAT relative to that observed in nonconsumers (Ma et al., 2016c). Consistent with other cross-sectional studies, no relationship was observed between VAT and diet soda (Ma et al., 2014; Odegaard et al., 2012), yet Ma and colleagues did observe a positive association between diet soda, BMI, and SAT. However, no significant prospective association was observed between either SSB or diet soda intake and change in SAT (Ma et al., 2016c). This latter observation is interesting because cross-sectional data in the FHS (Ma et al., 2016a) suggest that higher SSB intake is correlated with lower SAT; however, other cross-sectional studies did not observe this association (Hannou et al., 2018; Shah et al., 2016). This observed cross-sectional association between diet soda, SAT, and body weight may be a consequence of greater diet soda intake among overweight/obese adults. Although prospective studies examining beverage intakes with

longitudinal changes in fat depots are lacking, evidence from long-term prospective studies does not support an association between diet soda and body weight (Malik et al., 2013).

While observational studies have primarily focused on SSB as the exposure of interest, the metabolic consequences of fructose (Hannou et al., 2018; Ouyang et al., 2008) are hypothesized to be a key driver in the observed relationship between SSB and ectopic fat deposition. In a sample of 559 adolescents, Pollock and colleagues (Pollock et al., 2012) found that greater fructose intake, derived from a variety of sugar-sweetened foods and beverages, was associated with greater VAT but not SAT after accounting for age, sex, ethnicity, and lifestyle factors. Given the trajectory of diet behaviors and weight gain tracking from childhood and adolescence into adulthood, understanding the impact of beverage consumption and body fat deposition is important in younger populations. Only a handful of small cross-sectional studies have examined the relationship between SSB intake and VAT in children and adolescents. In a cross-sectional analysis of data from a small sample ($n=60$) of overweight/obese non-Hispanic black and Hispanic adolescents aged 14–18 years, VAT was 7% greater among those drinking two or more SSB per day compared with less than one per day (Shearrer et al., 2016). In contrast, no statistically significant relationship was observed with either dietary fructose intake or total sugar intake, or with the ratio of VAT/SAT in a another small sample ($n=64$) of overweight adolescents after adjustment for age, sex, ethnicity, energy intake, BMI z-score and cardiorespiratory fitness (Mollard et al., 2014). These disparate findings are likely attributed to variation in the characteristics of the sample and the small sample size. However, targeting the reduction of sugary beverages to curb gains in weight and adiposity is supported by evidence from larger prospective cohort studies among children and adolescents (Luger et al., 2017).

In 2007, Zelber-Sagi et al. conducted the first observational study examining the association between carbohydrate consumption from sweet foods and the presence of NAFLD in 349 Israeli adults (Zelber-Sagi et al., 2007). The authors observed that those determined to have NAFLD through abdominal ultrasound tended to consume more sweet foods compared with those who appeared to have normal liver. Since then, several cross-sectional studies have examined the relationship between SSB and liver fat (hepatic fat) in adults (Shah et al., 2016), and others have observed that soft drink or SSB consumption is high among patients diagnosed with NAFLD (Abid et al., 2009; Assy et al., 2008; Zelber-Sagi et al., 2007). Ouyang and colleagues (Ouyang et al., 2008) conducted a small case–control study using data from 73 patients attending a liver clinic and observed that patients with NAFLD tended to consume more energy from fructose-sweetened beverages than the controls. They estimated that consumption of fructose was at least double in NAFLD patients compared with controls (365 vs 170 kcal). Assy et al. observed that the prevalence of excessive soft drink consumption (>50 g/day of added sugar from soft drinks) was alarmingly high in 31 adults with NAFLD relative to age- and sex-matched controls (80% vs 20%) (Assy et al., 2008). It is challenging to draw strong conclusions from these three studies (Assy et al., 2008; Ouyang et al., 2008; Zelber-Sagi et al., 2007) since they were not able to control for other lifestyle factors, such as other dietary factors, alcohol intake, physical activity, and smoking habits, that may be confounding any observed associations.

Ma and colleagues (Ma et al., 2015) conducted the largest study to date examining the cross-sectional association between SSB consumption and measures of liver fat derived by multidetector computed tomography (MDCT), in addition to circulating levels of alanine

aminotransferase (ALT) (a crude marker of fatty liver disease) in 2634 US adults. After accounting for a variety of demographic and lifestyle characteristics, compared with nonconsumers, daily consumers of one or more SSB had a 56% greater risk of fatty liver (based on a liver to phantom ratio [LPR] of <33.0). In addition, a positive dose-response relationship was observed with SSB intake and ALT, while no relationship was observed between diet soda, NAFLD, or ALT levels in this cohort.

In adolescence, the prevalence of NAFLD is on the rise; however, to date, few observational studies have related excess added sugar intake to this condition. One small cross-sectional analysis among 68 overweight Canadian adolescents at risk of T2D did not observe a significant association between SSB intake and the presence of hepatic steatosis while controlling for demographic and lifestyle factors (Mollard et al., 2014). The findings of these emerging observational studies have important health implications as elevated visceral, in contrast to subcutaneous fat, is more strongly associated with increased T2D and CVD (Hwang et al., 2016). In addition, not all overweight or obese individuals display equivalent cardiometabolic risk, and this may be attributed to body fat deposition in ectopic depots (Elffers et al., 2017; Hübers et al., 2017). The growing prevalence of NAFLD and a high degree of abdominal adiposity in adolescents is of great public health concern, and reducing sugary beverages is one dietary modification that can be achieved by replacing these drinks with other beverages.

Evidence from randomized controlled trials

Abdominal VAT and SAT

Data generated from RCT suggest that an increase in SSB consumption may increase body weight in both children and adults (de Ruyter et al., 2012; Stanhope et al., 2015). However, as summarized in Table 1, few RCT have directly tested the hypothesis that sugar intake leads to greater gains in abdominal adiposity or in the distribution of fat depots (Maersk et al., 2012).

Most RCT have small sample sizes and are generally conducted over a short intervention period (<10 weeks). However, in 2012, Maersk and colleagues conducted an RCT over a 6-month intervention period in which participants consumed 1 L/day of beverages sweetened with sucrose (50% fructose and 50% glucose) (Maersk et al., 2012). This parallel RCT compared the effects of SSB, isocaloric semiskim milk, aspartame-sweetened diet drink, and water on changes in various fat depositions in 47 overweight participants (mean age 39 years; 64% women). SSB and semiskim milk had similar energy content, which provided approximately 20% of daily energy requirements. After completion of this trial, VAT increased by ~23% in participants who were assigned to the SSB arm, whereas VAT decreased by ~8% in the milk arm, and no changes were observed in the diet soda and water arms. The authors concluded that compared with other beverages, regular consumption of SSB could increase fat accumulation in VAT and the tendency of fat stored in VAT relative to abdominal SAT.

In a smaller scale parallel RCT ($n=27$), Campos et al. (2015) examined the effect of replacing regular SSB with diet beverages on adipose tissue over a 12-week intervention (Campos et al., 2015). Participants were overweight adults (48% women) who regularly consumed two or more 22-oz SSB daily. In this study, participants were randomized into one of

TABLE 1 Randomized clinical trials testing the effect of added sugars on visceral adipose tissue and liver fat

Author, year, location, design, PubMed ID	Participants	Treatment	Duration (weeks)	Energy intake (designed)	VAT	Liver fat
Maersk et al. (2012) Denmark Parallel 22205311	$n=10$, 60% men, BMI: 31.3 kg/m^2, Age: 39 years	Sucrose-sweetened cola	26	Hypercaloric (ad libitum diet supplemented with 1L/day of regular cola, ~20% energy)	VAT was increased by 24% in SSB arm, decreased by 8% in milk arm, increased by 1% in diet cola arm, and increased by 2% in water arm	Liver fat was increased by 134% in SSB arm, decreased by 9% in milk arm, decreased by 4% in diet cola arm, and increased by 2% in water arm
	$n=12$, 25% men, BMI: 31.9 kg/m^2, Age: 38 years	Milk		Hypercaloric (ad libitum diet supplemented with 1L/day of semiskim milk, ~20% energy)		
	$n=12$, 25% men, BMI: 32.8 kg/m^2, Age: 39 years	Diet cola		Isocaloric (ad libitum diet supplemented with diet soda)		
	$n=13$, 38% men, BMI: 32.2 kg/m^2, Age: 39 years	Water		Isocaloric (ad libitum diet supplemented with water)		
Campos et al. (2015) Switzerland Parallel 26727115	$n=13$, 56% men, BMI: 30.6 kg/m^2	Sugar-sweetened beverage	12	Isocaloric (habitual diet with habitual regular SSB)	VAT was reduced by 2% in SSB arm and by 15% in diet beverage arm	Liver fat was decreased by 14% in SSB arm and by 40% in diet beverage arm
	$n=14$, 57% men, BMI: 31.5 kg/m^2	Artificially sweetened beverages		Hypocaloric (habitual diet with habitual regular SSB replaced by diet beverages)		
Stanhope et al. (2009)[a] US Parallel 19381015	$n=17$, 53% men, BMI: 29.3 kg/m^2, Age: 53 years	Fructose	10	Hypercaloric (ad libitum diet with 25% energy replaced by either fructose or glucose for 2 weeks, and followed by 8 weeks of ad libitum diet supplemented with either fructose or glucose accounting for 25% energy)	VAT was increased 14% ($P<0.01$) in fructose intervention and 3.2% ($P>0.05$) in glucose intervention; no statistical difference between groups, $P=0.06$ (a marginal significant fructose effect was observed in men, $P=0.048$)	The 16-h area under the curve for *fractional* de novo *lipogenesis* (measured using plasma VLDL-^{13}C palmitate) increased more in fructose arm compared with glucose arm (83% vs 7%)
	$n=15$, 47% men, BMI: 29.4 kg/m^2, Age: 55 years	Glucose				

Silbernagel et al. (2011) Germany Parallel 21396140	$n=10$, 70% men, BMI: 25.5 kg/m^2, Age: 33 years ($n=8$ for VAT) $n=10$, 50% men, BMI: 26.2 kg/m^2, Age: 28 years	Fructose Glucose	4	Hypercaloric (weight-maintenance diet supplemented with either fructose or glucose accounting for 22% energy)	Fructose intervention increased VAT by 0.07 kg, while glucose intervention increased 0.07 kg; no difference between groups, $P=0.98$	Increase of liver fat was 0.45% and 0.52% signal in fructose and glucose interventions, respectively; no difference between groups, $P=0.98$
Hudgins et al. (2011)[a] US Crossover 21252253	$n=15$, 60% men, BMI: 31 kg/m^2, Age: 44 years	Fructose 0.5 g/kg body weight Fructose 0.5 g/kg body weight and glucose 0.5 g/kg body weight Fructose 1 g/kg body weight and glucose 1 g/kg body weight	1	Hypercaloric (habitual diet supplemented with tested sugars)		The percent change in VLDL palmitate (de novo lipogenesis marker) was 0.49 in fructose alone, 1.50 in fructose 0.5 and glucose 0.5, 2.71 in fructose 1 and glucose 1
Lecoultre et al. (2014) Switzerland Crossover 24257718	$n=10$, 100% men, BMI: 22.6 kg/m^2, Age: 23 years	Fructose (with or without 3 different coffee; 4 tests) Control (no fructose supplement)	~1	Hypercaloric (weight-maintenance diet supplemented with 4 g/kg body weight) Isocaloric (weight-maintenance diet)		High-fructose supplements increased liver fat by 102% compared with the control diet
Theytaz et al. (2012) Switzerland Crossover 23034968	$n=9$, 100% men, BMI: 22.6 kg/m^2, Age: 23 years	Fructose	~1	Hypercaloric (weight-maintenance diet supplemented with fructose accounting for 35% of energy)		Fructose intervention increased by 116% and 81% liver fat compared with controls, respectively

Continued

TABLE 1 Randomized clinical trials testing the effect of added sugars on visceral adipose tissue and liver fat—Cont'd

Author, year, location, design, PubMed ID	Participants	Treatment	Duration (weeks)	Energy intake (designed)	VAT	Liver fat
		Fructose +essential amino acids				
		Control		Isocaloric (weight-maintenance diet)		
Lê et al. (2009)[b] Switzerland Crossover 19403641	$n=24$, 100% men, BMI 19–25 kg/m^2, Age: 25 years ($n=16$: type 2 diabetes patients' offspring; $n=8$ normal healthy adults)	Fructose	1	Hypercaloric (weight-maintenance diet supplemented with fructose accounting for 35% energy)		Compared with controls, fructose intervention increased ~50% liver fat
		Control		Isocaloric diet (weight-maintenance diet)		
Ngo Sock et al. (2010) Switzerland Crossover 19930762	$n=11$, 100% men, BMI: 19–25 kg/m^2, Age: 25 years	Fructose	1	Hypercaloric (weight-maintenance diets supplemented with either fructose or glucose accounting for 35% energy)		Compared with controls, fructose intervention increased by 52% and glucose intervention increased by 58%
		Glucose				
		Control		Isocaloric diet (weight-maintenance diet)		

Johnston et al. (2013) UK Parallel 23872500	$n=15$, 100% men, BMI: 30 kg/m^2, Age: 35 years	Fructose	2	Hypercaloric (ad libitum diet supplemented with fructose or glucose accounting for 25% energy)	Compared with baseline, liver fat was increased by ~14% in fructose intervention and ~24% in glucose invention. No significant difference between groups
	$n=17$, 100% men, BMI: 28.9 kg/m^2, Age: 33 years	Glucose			
Schwarz et al. (2015)[b] US Crossover 25825943	$n=7$, 100% men, BMI: 24.4 kg/m^2, Age: 42 years	Fructose	1	Isocaloric diet (fructose provided 25% energy)	Fructose arm had a higher liver fat (median, 137%) compared with controls. No significant difference between groups
		Complex carbohydrate		Isocaloric diet	
Jin et al. (2014) US Parallel 25111123	$n=9$, 33% boy, BMI z-score: 2.25, Age: 14 years	Fructose	4	Isocaloric diet (instructed to consume 3 serving fructose-sweetened beverages (99 g of fructose) to replace habitual regular SSB)	No change in liver fat in both arms
	$n=12$, 67% boy, BMI z-score: 2.15, Age: 13 years	Glucose		Isocaloric diet (instructed to consume 3 serving glucose-sweetened beverages (99 g of glucose) to replace habitual regular SSB)	

[a] *Liver de novo lipogenesis was measured (no imaging data for liver fat).*
[b] *Nonrandomized trial.*

two beverage exposures: (1) diet beverages, which reduced daily energy intake by about 500 kcal/day (approximately 20% energy), or (2) SSB, which reduced daily energy intake by about 100 kcal/day. At the end of the intervention, the authors observed that liver fat was reduced, but no significant change in body weight and VAT was observed. This study's smaller sample size and shorter duration, compared with the study implemented by Maersk et al. (Maersk et al., 2012), may be one reason contributing to the difference in observed effects on VAT and SAT outcomes in the two studies. Furthermore, in the study conducted by Campos et al. (Campos et al., 2015), the compliance to the intervention may have been low because participants were required to follow a hypocaloric diet (by replacing regular SSB with diet beverages). Nevertheless, participants in the diet beverage arm displayed reductions in liver fat at the end of the trial, suggesting that an extended intervention may be needed to detect an effect on abdominal adiposity. Additionally, the change in abdominal adiposity trended in the same direction after both 3-month and 6-month interventions. This suggests that replacing SSB with water or diet beverages reduced waist circumference, a clinical proxy for abdominal adipose tissue.

Controlled intervention studies in humans have been conducted to test the hypothesis that fructose rather than glucose is the key driver relating adiposity to added sugar intake. Stanhope et al. (2009) conducted a 10-week parallel RCT to compare the effect of fructose-sweetened vs glucose-sweetened beverages on VAT and SAT (Stanhope et al., 2009). In this study, a group of overweight and obese participants ($n=32$; mean age 54 years; 50% women) went through an 8-week outpatient intervention to consume either fructose- or glucose-sweetened beverages providing 25% of their usual daily energy requirement with ad libitum diets (i.e., a hypercaloric diet). At the final 2 weeks, participants were invited to stay at a clinical research center and consumed the same beverages during a weight-maintenance diet. After 10 weeks, body weight, waist circumference, and total body fat increased in both study arms. Interestingly, fructose-sweetened beverages significantly increased VAT, whereas glucose-sweetened beverages significantly increased SAT. On average, after 10 weeks, fructose-sweetened beverages increased VAT by 14%, and glucose-sweetened beverages increased SAT by 4.6%. The authors suggest that this differential effect on adipose fat depots may be attributed to the specific effect of fructose on promoting de novo lipogenesis. However, inconsistent evidence exists. An exploratory RCT comparing equivalent doses of fructose and glucose reported that both sugars did not significantly increase VAT or SAT (Silbernagel et al., 2011). In this trial, 20 participants (mean age 31 years; 40% women) were randomized to consume 150 g of either fructose or glucose for 4 weeks. The tested sugars were dissolved in water and accounted for about 22% of the daily energy requirement. At the end of this trial, only glucose drinks increased body weight, and neither sugar significantly altered abdominal adipose tissue.

The different observations may be due to different baseline characteristics, such as whether participants already had increased abdominal adiposity or whether they carried a predisposed genetic risk for increased abdominal adiposity. Epidemiological evidence has shown an interaction between genetic risk score for BMI and SSB consumption in relation to body weight change (Brunkwall et al., 2016; Haslam et al., 2018; Olsen et al., 2016; Qi et al., 2012). However, there is little information regarding which genetic, clinical, lifestyle, and environmental factors may modify the effect of fructose and glucose consumption on abdominal fat accumulation. Many short-term studies have shown that the metabolic

characteristics of fructose are different from glucose. However, direct comparison of fructose and glucose may be difficult because fructose may be converted to glucose (Hannou et al., 2018). In addition, under real-world conditions, individuals consume beverages containing both sucrose and high-fructose corn syrup. Thus studying the effects of these sugars in isolation may shed light on mechanisms, but their clinical relevance may not extend to the combination of both glucose and fructose in SSB.

Liver fat

In the aforementioned parallel RCT ($n = 47$) conducted by Maersk and colleagues, the effect of four types of beverages, including SSB, semiskim milk, diet beverages, and water, on liver fat were compared (Maersk et al., 2012). In brief, study participants were instructed to consume SSB or milk providing approximately 20% of their daily energy requirement on top of their regular diet. In contrast, no additional energy was consumed in the diet beverage and water arms. Participants randomized to the SSB and milk arms were likely to reduce their energy intake from other food sources, thereby compensating for the contributing energy from the test beverages, based on intake estimated from dietary questionnaires. Nonetheless, at the end of this 6-month RCT, participants in the SSB arm had significantly increased liver fat content compared with the other three arms. Campos et al. used a different study design to test the effect of replacing SSB with diet beverages on liver fat. In this study, 27 overweight adults were randomized either to continue consuming their habitual amount of SSB or to drink the same amount of diet beverage to replace their habitual SSB. After 12 weeks of intervention, liver fat was reduced by 26% compared with baseline in the diet beverage arm, whereas no substantial change was observed in the habitual SSB arm. This RCT indicates that replacing calorie-containing beverages with noncaloric beverages may be a feasible strategy for reducing liver fat. Although this design cannot isolate the effects of fructose from energy, it could be suggested that a reduction in fructose intake leads to a reduction in liver fat. One notable strength of these RCTs is that they compare commonly consumed beverages at dosages that reflect regular SSB consumption patterns in "real life."

It has been postulated that increased liver fat is mainly due to excess intake of fructose. In comparison with glucose, fructose administered at the same dose has been found to increase liver fat, and this increase has been attributed to the rapid increase of de novo lipogenesis (Parks et al., 2008). Several RCT have been conducted to demonstrate this mechanism. In one randomized crossover study conducted in 15 overweight participants, Hudgins et al. compared *acute* responses with three sugar regimens, fructose (0.5 g/kg), fructose and glucose (0.5:0.5 g/kg), and fructose and glucose (1:1 g/kg) (Hudgins et al., 2011), dissolved in 12 ounces of water. This study showed fructose is more likely to increase hepatic de novo lipogenesis. In the RCT conducted by Stanhope et al. (study design is described in the section of RCTs for abdominal adiposity), participants randomized into the fructose arm had significantly increased 16-h fractional de novo lipogenesis, compared with those in the glucose arm (Stanhope et al., 2009).

However, liver fat is not only influenced by de novo lipogenesis but also affected by secretion of synthesized lipids from the liver to circulation. As such, rather than using de novo lipogenesis as the sole endpoint, several RCTs were implemented to observe the direct effect

of added sugars on liver fat accumulation. Tappy et al. conducted a series of small crossover RCTs to test the effect of fructose on liver fat (i.e., intrahepatocellular lipids) (Lecoultre et al., 2014; Theytaz et al., 2012). In these studies, fructose, accounting for approximately 35% or 40% daily energy requirement, was consumed as part of a weight-maintaining diet. The fructose hypercaloric diet was generally consumed in 6 days. Compared with a weight-maintaining diet, these studies showed that high-fructose consumption (with a short-term hypercaloric diet) increased liver fat.

Interestingly, in one of the aforementioned crossover RCT conducted by Tappy et al., additional consumption of five essential amino acids (leucine, isoleucine, valine, lysine, and threonine) attenuated the fructose effect on liver fat (Theytaz et al., 2012). This study suggests that baseline consumption of essential amino acids may be an important factor to modify an individual's response to fructose consumption and may need to be considered in future studies. Tappy et al. also tested whether predisposed genetic risk may affect the potential effect of fructose on liver fat in a nonrandomized crossover trial (Lê et al., 2009). This study compared the response of liver fat determined by ^{1}H magnetic resonance spectroscopy with a fructose hypercaloric diet among a second generation of T2D adults and among adults who had no such parental diabetes history. Fructose was consumed for 7 days, which provided an additional 35% of energy per day. At the end of the trial, high-fructose consumption led to greater liver fat accumulation in the offspring of type 2 diabetics compared with controls. These data suggest that adults with a history of parental diabetes may be more prone to liver fat accumulation when challenged with high-fructose intakes compared with those with no parental diabetes history.

Several RCTs have also compared fructose and glucose under *hypercaloric* conditions. For example, Tappy et al. conducted a crossover RCT to compare the effect of high fructose (+35% energy) with high glucose (+35% energy) (Ngo Sock et al., 2010). While both sugars led to an increase in liver fat accumulation, the high-fructose diet did not lead to a greater increase in liver fat relative to a high-glucose diet. Johnson et al. showed similar findings in a parallel RCT (Johnston et al., 2013). In this study, participants consumed either high-fructose or glucose diets in which fructose or glucose provided an additional 25% of energy intake. After a 2-week intervention, liver fat content increased in both arms, and there was no substantial difference between arms.

Two studies have examined the effect of fructose and glucose under *isocaloric* conditions, that is, weight-maintenance diets (Jin et al., 2014; Schwarz et al., 2015). In a crossover study conducted in nine healthy men, Schwarz et al. showed that participants who consumed a high amount of fructose (25% energy) as part of an isocaloric diet had increased liver fat compared with a control diet, which replaced fructose by complex carbohydrate (Schwarz et al., 2015). However, the difference between the two study arms was not statistically significant. In a 4-week parallel RCT conducted in 24 overweight adolescents, Jin et al. also observed no effect of either fructose or glucose on changes in liver fat (Jin et al., 2014).

In summary, drawing conclusions from RCTs designed to evaluate the impact of added sugar on liver fat is challenging due to relatively small sample sizes and the administration of intervention doses in amounts higher than typical intakes among US adults. In addition, it is difficult to design an RCT to compare regularly consumed beverages since blinding may be a challenge (e.g., milk vs cola), and this may bias the study outcome. Nevertheless, evidence from these RCTs suggests that excess sugar intake promotes increases in liver fat

accumulation, particularly in the context of excess energy, and that fructose is particularly harmful (Hannou et al., 2018). Future studies are needed to clarify whether the effect on liver fat is due to the specific effect of sugar intake or the surplus energy intake from excess sugar intake.

Potential mechanisms

Increased added sugar consumption is likely to cause a positive energy balance, that is, energy intake is greater than energy expenditure. This may be due to the calorie content of added sugar per se or the overeating promoted through enhanced overall food palatability. Indeed, as summarized by Johnson et al. (Johnson and Wardle, 2014), prior feeding studies showed palpability could promote a positive energy balance. The consequence is increased body fat mass, including increased fat accumulation in liver and abdominal viscera. Whether added sugars have specific effects on ectopic fat depots that is independent of the state of positive energy balance remains elusive. However, some evidence suggests that added sugars, particularly fructose, may exert such effects through regulating certain biological pathways (Hannou et al., 2018).

Excess fructose intake due to an overload of added sugars in the diet substantially increases de novo lipogenesis (DNL) in the liver (Stanhope et al., 2009). Increased DNL provides excess substrate for lipid synthesis in the liver (Sevastianova et al., 2012). It has been postulated that some intermediate products, such as diacylglycerols (DAG) generated during synthesis or degradation of triglycerides, may trigger hepatic insulin resistance (Badin et al., 2011). DAG may activate protein kinase C and promote serine phosphorylation of insulin receptor and insulin receptor substrates and subsequently impair insulin signaling (Jornayvaz and Shulman, 2012). Hepatic insulin resistance may further enhance DNL. As such, excess fructose intake may trigger a vicious cycle to promote liver fat accumulation (Stanhope, 2016). Fructose overload may also stimulate two key transcription factors in lipogenesis (Hannou et al., 2018), sterol receptor element–binding protein 1c (SREBP-1c) (Nagai et al., 2002) and carbohydrate response element–binding protein (ChREBP) (Uyeda and Repa, 2006). Alternatively, excess fructose intake may inhibit fatty acid catabolism by reducing beta-oxidation in the liver (Hallfrisch, 1990; Roglans et al., 2007). This could lead to increased accumulation of triglycerides in droplets, leading to increased liver fat.

Fructose-triggered hepatic insulin resistance may further induce peripheral insulin resistance (Stanhope et al., 2009). Lipoprotein lipase (LPL) is a key enzyme to regulate the uptake of triglycerides from the circulation to adipose tissues (Mead et al., 2002). In the state of insulin resistance, LPL in VAT is more sensitive to insulin than LPL in SAT (Fried et al., 1993), which promotes more triglyceride storage in VAT (Després, 2006). VAT is rich in glucocorticoid receptors (Fried et al., 1993). Increased VAT has been associated with increased activity of type 1 11b-hydroxysteroid dehydrogenase, a key enzyme catalyzing the local conversion of inert cortisone to active cortisol (Tchernof and Després, 2013). Senesi et al. showed that fructose may impact intracellular activation of glucocorticoids to promote LPL activity (Senesi et al., 2010). Therefore, it is possible that fructose may also directly promote fat accumulation in VAT.

Future studies

As summarized in a review by Ma and colleagues (Ma et al., 2016b), further research is needed to determine whether sugars, specifically fructose relative to glucose, would differentially affect fat accumulation in VAT. In addition, it remains unknown whether added sugar intake may have a different metabolic effect on VAT compared with other macronutrients, such as proteins. Similarly, with respect to liver fat, more studies are needed to investigate whether added sugars have a specific effect that is independent of the positive energy balance and whether fructose is more harmful than glucose. Also, future studies are needed to understand if excess added sugar intake could promote the progression of fatty liver disease, that is, from simple steatosis to more advanced fatty liver disease such as steatohepatitis or cirrhosis.

Glossary

Sugar-sweetened beverage (SSB) Any beverage that contains added sugars; examples include sodas, fruit-flavored drinks, sports drinks, and sweetened coffees and teas.
Visceral adipose tissue (VAT) Fat tissue located deep in the abdomen and around the organs.
Subcutaneous adipose tissue (SAT) Fat tissue located beneath the skin.
Ectopic fat The accumulation of lipids (i.e., in the form of triglycerides) in nonadipose lean tissue or the accumulation of adipocytes in adipose tissue.
Body weight (BW) The weight or mass of an individual.
Waist circumference (WC) A circumference measurement taken around the abdomen at the level of the umbilicus.
Body fat (BF) A normal constituent of the human body that is used as a fuel source and for energy storage, also known as adipose tissue.
Nonalcoholic fatty liver disease (NAFLD) The accumulation of extra fat in the liver cells that is not due to alcohol consumption.
Randomized controlled trial (RCT) The gold standard for a scientific experiment aimed to reduce bias by randomly assigning participants to a treatment group or to a control group.
Observational study A type of study in which individuals are observed and certain characteristics/outcomes are measured over time; no intervention is performed.

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CHAPTER

6

Contribution to molecular nutrition: Carbohydrates

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OUTLINE

SUMMARY POINTS

- This chapter focuses on carbohydrates that serve as energy sources and as essential structural components in organisms.
- The energy stores of most animals and plants are both carbohydrate and lipid in nature; carbohydrates are generally available as an immediate energy source, whereas lipids act as a long-term energy resource and tend to be utilized at a slower rate.
- Although carbohydrates may compose as much as 80% of the total caloric intake in the human diet, for a given diet, the proportion of starch to total carbohydrate is quite variable, depending upon the prevailing customs.

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- Polysaccharides, or glycans, may be classified in a number of ways.
- Homopolysaccharides are defined as polysaccharides formed from only one type of monosaccharide.
- Heteropolysaccharides are defined as polysaccharides containing two or more different types of monosaccharides.

Key facts of carbohydrates

- Carbohydrates are absorbed as monosaccharides (simple sugars such as glucose, fructose, and galactose that cannot be further broken down by hydrolysis) or as disaccharides (carbohydrates such as sucrose, lactose, maltose, and dextrin that can be hydrolyzed to two monosaccharides).
- These simpler molecules, however, must be obtained by the breaking down of polysaccharides, complex carbohydrates that contain many monosaccharides.
- Chief among these is amylose, a starch that accounts for 20% of dietary carbohydrate.
- Amylose consists of a straight chain of glucose molecules bound to their neighbors by oxygen links.
- The bulk of the starch is amylopectin, which has a branch chain linked in after every 25 molecules of glucose on the main chain.

Carbohydrate is a class of naturally occurring compounds and derivatives formed from them. In the early part of the 19th century, substances such as wood, starch, and linen were found to be composed mainly of molecules containing atoms of carbon (C), hydrogen (H), and oxygen (O), and to have the general formula $C_6H_{12}O_6$; other organic molecules with similar formulas were found to have a similar ratio of hydrogen to oxygen. The general formula $C_x(H_2O)_y$ is commonly used to represent many carbohydrates, which means "watered carbon" (Morales-Hernández et al., 2019).

Carbohydrates are probably the most abundant and widespread organic substances in nature, and they are essential constituents of all living things. Carbohydrates are formed by green plants from carbon dioxide and water during the process of photosynthesis. Carbohydrates serve as energy sources and as essential structural components in organisms; in addition, part of the structure of nucleic acids, which contain genetic information, consists of carbohydrate (Brunetti et al., 2019).

General features

Classification and nomenclature

Although a number of classification schemes have been devised for carbohydrates, the division into four major groups—monosaccharides, disaccharides, oligosaccharides, and polysaccharides—used here is among the most common (Cummings and Stephen, 2007). Most monosaccharides, or simple sugars, are found in fruits, especially in grapes, and honey. Although they can contain from three to nine carbon atoms, the most common representatives consist of five or six joined together to form a chainlike molecule. Three of the most important simple sugars—glucose (also known as dextrose, grape sugar, and corn sugar), fructose

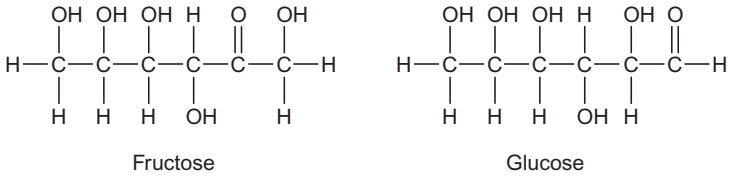


FIG. 1 Fructose and glucose.

(fruit sugar), and galactose—have the same molecular formula, ($C_6H_{12}O_6$), but because their atoms have different structural arrangements, the sugars have different characteristics; that is, they are isomers (Fig. 1) (Joosten and Lütteke, 2017).

Slight changes in structural arrangements are detectable by living things and influence the biological significance of isomeric compounds. It is known, for example, that the degree of sweetness of various sugars differs according to the arrangement of the hydroxyl groups (—OH) that compose part of the molecular structure (Davies and Williams, 2016). A direct correlation that may exist between taste and any specific structural arrangement, however, has not yet been established; that is, it is not yet possible to predict the taste of a sugar by knowing its specific structural arrangement. The energy in the chemical bonds of glucose indirectly supplies most living things with a major part of the energy that is necessary for them to carry on their activities. Galactose, which is rarely found as a simple sugar, is usually combined with other simple sugars to form larger molecules.

Two molecules of a simple sugar that are linked to each other form a disaccharide, or double sugar. The disaccharide sucrose, or table sugar, consists of one molecule of glucose and one molecule of fructose; the most familiar sources of sucrose are sugar beets and cane sugar. Milk sugar, or lactose, and maltose are also disaccharides. Before the energy in disaccharides can be utilized by living things, the molecules must be broken down into their respective monosaccharides. Oligosaccharides, which consist of three to six monosaccharide units, are rather infrequently found in natural sources, although a few plant derivatives have been identified.

Polysaccharides (the term means many sugars) represent most of the structural and energy-reserve carbohydrates found in nature. Large molecules that may consist of as many as 10,000 monosaccharide units linked together, polysaccharides vary considerably in size, in structural complexity, and in sugar content; several hundred distinct types have thus far been identified. Cellulose, the principal structural component of plants, is a complex polysaccharide comprising many glucose units linked together; it is the most common polysaccharide. The starch found in plants and the glycogen found in animals also are complex glucose polysaccharides. Starch (from the Old English word *stercan*, meaning "to stiffen") is found mostly in seeds, roots, and stems, where it is stored as an available energy source for plants. Plant starch may be processed into foods such as bread, or it may be consumed directly—as in potatoes, for instance. Glycogen, which consists of branching chains of glucose molecules, is formed in the liver and muscles of higher animals and is stored as an energy source.

The generic nomenclature ending for the monosaccharides is *-ose*; thus the term *pentose* (*pent* = five) is used for monosaccharides containing five carbon atoms, and *hexose* (*hex* = six) is used for those containing six. In addition, because the monosaccharides contain a chemically reactive group that is either an aldehyde group or a keto group, they are frequently referred to as aldopentoses or ketopentoses or aldohexoses or ketohexoses (Fig. 2) (Cummings and Stephen, 2007). The aldehyde group can occur at position 1 of an

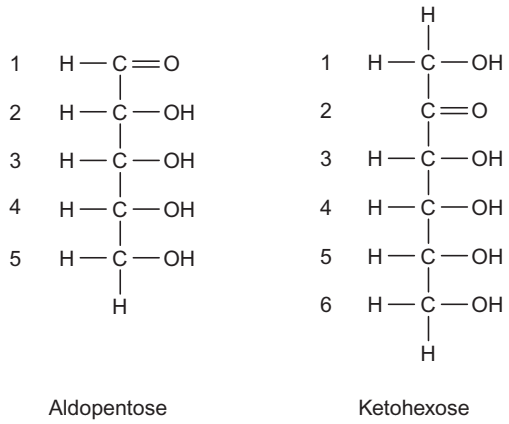


FIG. 2 Aldopentose and ketohexose.

aldopentose, and the keto group can occur at a further position (e.g., 2) within a ketohexose. Glucose is an aldohexose—that is, it contains six carbon atoms, and the chemically reactive group is an aldehyde group.

Biological significance

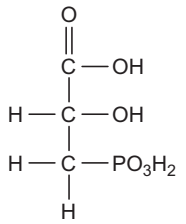
The importance of carbohydrates to living things can hardly be overemphasized. The energy stores of most animals and plants are both carbohydrate and lipid in nature; carbohydrates are generally available as an immediate energy source, whereas lipids act as a long-term energy resource and tend to be utilized at a slower rate. Glucose, the prevalent uncombined, or free, sugar circulating in the blood of higher animals, is essential to cell function. The proper regulation of glucose metabolism is of paramount importance to survival (Montgomery et al., 2017).

The ability of ruminants, such as cattle, sheep, and goats, to convert the polysaccharides present in grass and similar feeds into protein provides a major source of protein for humans. A number of medically important antibiotics, such as streptomycin, are carbohydrate derivatives. The cellulose in plants is used to manufacture paper, wood for construction, and fabrics.

Role in the biosphere

The essential process in the biosphere, the portion of Earth in which life can occur, that has permitted the evolution of life as it now exists is the conversion by green plants of carbon dioxide from the atmosphere into carbohydrates, using light energy from the Sun. This process, called photosynthesis, results in both the release of oxygen gas into the atmosphere and the transformation of light energy into the chemical energy of carbohydrates. The energy stored by plants during the formation of carbohydrates is used by animals to carry out mechanical work and to perform biosynthetic activities (Goddard-Borger and Williams, 2017).

During photosynthesis, an immediate phosphorous-containing product known as 3-phosphoglyceric acid is formed (Fig. 3).



3-Phosphoglyceric acid

FIG. 3 3-Phosphoglyceric acid.

This compound then is transformed into cell wall components such as cellulose, varying amounts of sucrose, and starch—depending on the plant type—and a wide variety of polysaccharides, other than cellulose and starch, that function as essential structural components. For a detailed discussion of the process of photosynthesis, see photosynthesis (Tripathi et al., 2018).

Role in human nutrition

The total caloric, or energy, requirement for an individual depends on age, occupation, and other factors but generally ranges between 2000 and 4000 cal per 24-h period (one calorie, as this term is used in nutrition, is the amount of heat necessary to raise the temperature of 1000 g of water from 15°C to 16°C (from 59°F to 61°F); in other contexts, this amount of heat is called the kilocalorie). Carbohydrate that can be used by humans produces four calories per gram as opposed to nine calories per gram of fat and four per gram of protein. In areas of the world where nutrition is marginal, a high proportion (approximately one to two pounds) of an individual's daily energy requirement may be supplied by carbohydrate, with most of the remainder coming from a variety of fat sources (Lovegrove et al., 2017).

Although carbohydrates may compose as much as 80% of the total caloric intake in the human diet, for a given diet, the proportion of starch to total carbohydrate is quite variable, depending upon the prevailing customs. In East Asia and in areas of Africa, for example, where rice or tubers such as manioc provide a major food source, starch may account for as much as 80% of the total carbohydrate intake. In a typical Western diet, 33%–50% of the caloric intake is in the form of carbohydrate (Delarue, 2019). Approximately half (i.e., 17%–25%) is represented by starch; another third by table sugar (sucrose) and milk sugar (lactose); and smaller percentages by monosaccharides such as glucose and fructose, which are common in fruits, honey, syrups, and certain vegetables such as artichokes, onions, and sugar beets. The small remainder consists of bulk, or indigestible carbohydrate, which comprises primarily the cellulosic outer covering of seeds and the stalks and leaves of vegetables.

Role in energy storage

Starches, the major plant-energy-reserve polysaccharides used by humans, are stored in plants in the form of nearly spherical granules that vary in diameter from about three to 100 μm (from about 0.0001 to 0.004 in.). Most plant starches consist of a mixture of two components: amylose and amylopectin. The glucose molecules composing amylose have a

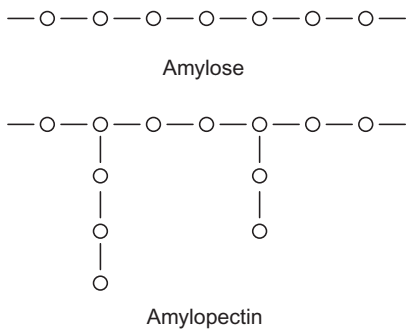


FIG. 4 Amylose and amylopectin.

straight-chain, or linear, structure. Amylopectin has a branched-chain structure and is a somewhat more compact molecule. Several thousand glucose units may be present in a single starch molecule. (In Fig. 4, each small circle represents one glucose molecule.)

In addition to granules, many plants have large numbers of specialized cells, called parenchyma cells, the principal function of which is the storage of starch; examples of plants with these cells include root vegetables and tubers. The starch content of plants varies considerably; the highest concentrations are found in seeds and in cereal grains, which contain up to 80% of their total carbohydrate as starch (Smith et al., 2017). The amylose and amylopectin components of starch occur in variable proportions; most plant species store approximately 25% of their starch as amylose and 75% as amylopectin. This proportion can be altered, however, by selective-breeding techniques, and some varieties of corn have been developed that produce up to 70% of their starch as amylose, which is more easily digested by humans than is amylopectin (Aguirre et al., 2018).

In addition to the starches, some plants (e.g., the Jerusalem artichoke and the leaves of certain grasses, particularly rye grass) form storage polysaccharides composed of fructose units rather than glucose (Yang et al., 2019). Although the fructose polysaccharides can be broken down and used to prepare syrups, they cannot be digested by higher animals.

Starches are not formed by animals; instead, they form a closely related polysaccharide, glycogen. Virtually all vertebrate and invertebrate animal cells, as well as those of numerous fungi and protozoans, contain some glycogen; particularly high concentrations of this substance are found in the liver and muscle cells of higher animals. The overall structure of glycogen, which is a highly branched molecule consisting of glucose units, has a superficial resemblance to that of the amylopectin component of starch, although the structural details of glycogen are significantly different. Under conditions of stress or muscular activity in animals, glycogen is rapidly broken down to glucose, which is subsequently used as an energy source (Smith et al., 2017). In this manner, glycogen acts as an immediate carbohydrate reserve. Furthermore, the amount of glycogen present at any given time, especially in the liver, directly reflects an animal's nutritional state. When adequate food supplies are available, both glycogen and fat reserves of the body increase, but when food supplies decrease or when the food intake falls below the minimum energy requirements, the glycogen reserves are depleted quite rapidly, while those of fat are used at a slower rate (Aguirre et al., 2018).

Role in plant and animal structure

Whereas starches and glycogen represent the major reserve polysaccharides of living things, most of the carbohydrate found in nature occurs as structural components in the cell walls of plants. Carbohydrates in plant cell walls generally consist of several distinct layers, one of which contains a higher concentration of cellulose than the others (de Godoy et al., 2013). The physical and chemical properties of cellulose are strikingly different from those of the amylose component of starch.

In most plants, the cell wall is about 0.5-μm thick and contains a mixture of cellulose, pentose-containing polysaccharides (pentosans), and an inert (chemically unreactive) plastic-like material called lignin (Tarasov et al., 2018). The amounts of cellulose and pentosan may vary; most plants contain between 40% and 60% cellulose, although higher amounts are present in the cotton fiber.

Polysaccharides also function as major structural components in animals. Chitin, which is similar to cellulose, is found in insects and other arthropods. Other complex polysaccharides predominate in the structural tissues of higher animals.

Structural arrangements and properties

Configuration

Molecules, such as the isomers of glyceraldehyde—the atoms of which can have different structural arrangements—are known as asymmetrical molecules. The number of possible structural arrangements for an asymmetrical molecule depends on the number of centers of asymmetry; that is, for n (any given number of) centers of asymmetry, 2^n different isomers of a molecule are possible (Shen et al., 2019). An asymmetrical center in the case of carbon is defined as a carbon atom to which four different groups are attached (Fig. 5). In the three-carbon aldose sugar, glyceraldehyde, the asymmetrical center is located at the central carbon atom.

The position of the hydroxyl group (—OH) attached to the central carbon atom—that is, whether —OH projects from the left or the right—determines whether the molecule rotates the plane of polarized light to the left or to the right. Since glyceraldehyde has one asymmetrical center, n is one in the relationship 2^n, and there thus are two possible glyceraldehyde isomers. Sugars containing four carbon atoms have two asymmetrical centers; hence, there are four possible isomers (2^2). Similarly, sugars with five carbon atoms have three asymmetrical centers and thus have eight possible isomers (2^3). Keto sugars have one less asymmetrical center for a given number of carbon atoms than do aldehyde sugars (Menichetti et al., 2019).

A convention of nomenclature, devised in 1906, states that the form of glyceraldehyde whose asymmetrical carbon atom has a hydroxyl group projecting to the right is designated

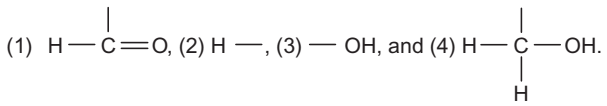


FIG. 5 Asymmetrical center of cabon.

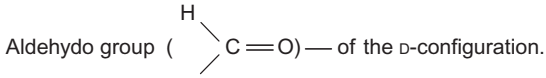


FIG. 6 Aldehydo group configuration.

TABLE 1 Representative disaccharides and oligosaccharides

Common name	Component sugars	Linkages	Sources
Cellobiose	Glucose, glucose	β1 → 4[a]	Hydrolysis of cellulose
Gentiobiose	Glucose, glucose	β1 → 6	Plant glycosides, amygdalin
Isomaltose	Glucose, glucose	α1 → 6	Hydrolysis of glycogen, amylopectin
Raffinose[b]	Galactose, glucose, fructose	α1 → 6, α1 → 2	Sugarcane, beets, seeds
Stachyose[b]	Galactose, galactose, glucose, fructose	α1 → 6, α1 → 6, α1 → 2	Soybeans, jasmine, twigs, lentils

[a] *The linkage joins carbon atom 1 (in the β configuration) of one glucose molecule and carbon atom 4 of the second glucose molecule; the linkage may also be abbreviated β-1,4.*
[b] *Note that raffinose and stachyose are galactosyl sucroses.*

as of the D-configuration; that form, whose asymmetrical carbon atom has a hydroxyl group projecting to the left, is designated as l. All sugars that can be derived from D-glyceraldehyde—that is, hydroxyl group attached to the asymmetrical carbon atom most remote from the aldehyde or keto end of the molecule projects to the right—are said to be of the D-configuration; those sugars derived from L-glyceraldehyde are said to be of the L-configuration (Fig. 6; Table 1) (Popkin and Hawkes, 2016).

The configurational notation d or l is independent of the sign of the optical rotation of a sugar in solution. It is common, therefore, to designate both, as, for example, D-(*L*)-fructose or D-(*D*)-glucose; that is, both have a D-configuration at the center of asymmetry most remote from the aldehyde end (in glucose) or keto end (in fructose) of the molecule, but fructose is levorotatory and glucose is dextrorotatory—hence the latter has been given the alternative name dextrose. Although the initial assignments of configuration for the glyceraldehydes were made on purely arbitrary grounds, studies that were carried out nearly half a century later established them as correct in an absolute spatial sense. In biological systems, only the d or l form may be utilized (Newens and Walton, 2016).

When more than one asymmetrical center is present in a molecule, as is the case with sugars having four or more carbon atoms, a series of dl pairs exists, and they are functionally, physically, and chemically distinct. Thus, although D-xylose and D-lyxose both have five carbon atoms and are of the D-configuration, the spatial arrangement of the asymmetrical centers (at carbon atoms 2, 3, and 4) is such that they are not mirror images.

Hemiacetal and hemiketal forms

Although optical rotation has been one of the most frequently determined characteristics of carbohydrates since its recognition in the late 19th century, the rotational behavior of freshly

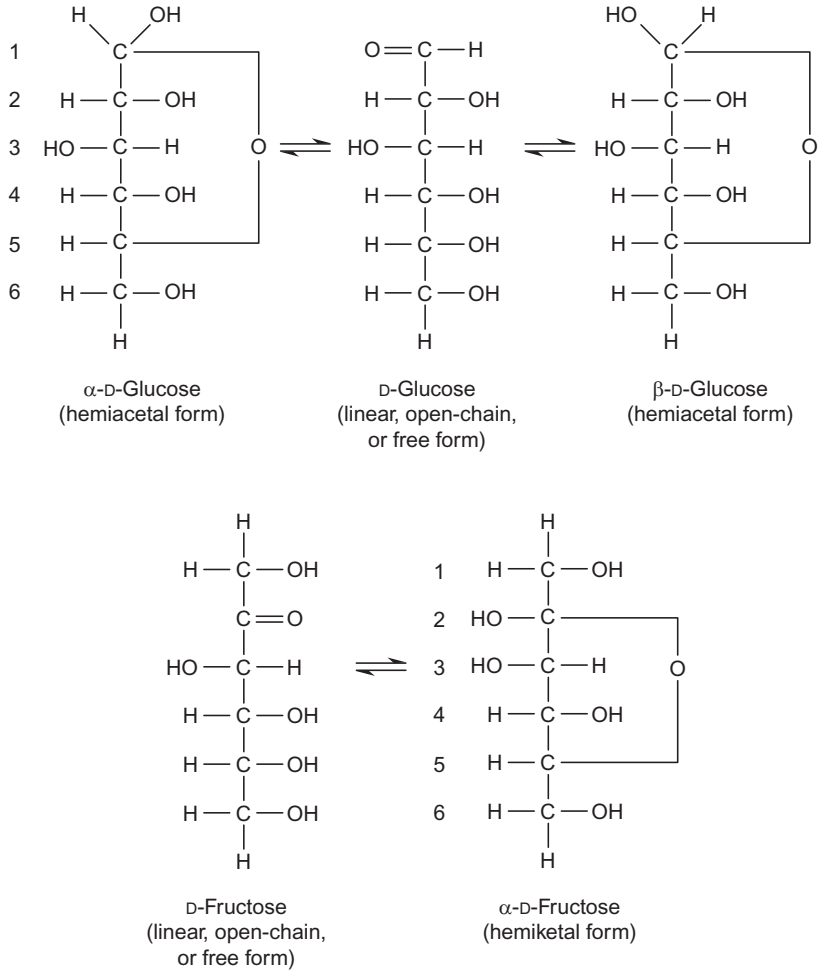


FIG. 7 Hemiacetal and hemiketal form.

prepared solutions of many sugars differs from that of solutions that have been allowed to stand (Rabus et al., 2018). This phenomenon, known as mutarotation, is demonstrable even with apparently identical sugars and is caused by a type of stereoisomerism involving formation of an asymmetrical center at the first carbon atom (aldehyde carbon) in aldoses and the second one (keto carbon) in ketoses (Fig. 7).

Most pentose and hexose sugars, therefore, do not exist as linear, or open-chain, structures in solution but form cyclic, or ring, structures in hemiacetal or hemiketal forms, respectively. As illustrated for glucose and fructose, the cyclic structures are formed by the addition of the hydroxyl group (—OH) from either the fourth, fifth, or sixth carbon atom to the carbonyl group at position 1 in glucose or 2 in fructose (Fig. 8). In the case of five-membered cyclic ketohexose or six-membered cyclic aldohexose, the cyclic forms are in equilibrium with

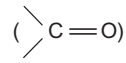


FIG. 8 Hydroxyl group (—OH) from either the fourth, fifth, or sixth carbon atom to the carbonyl group.

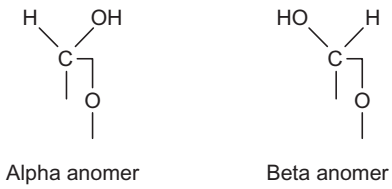


FIG. 9 Alfa and beta anomer.

(i.e., the rate of conversion from one form to another is stable) the open-chain structure—a free aldehyde if the solution contains glucose, a free ketone if it contains fructose; each form has a different optical rotation value. Since the forms are in equilibrium with each other, a constant value of optical rotation is measurable; the two cyclic forms represent more than 99.9% of the sugar in the case of a glucose solution.

By definition, the carbon atom containing the aldehyde or keto group is called the anomeric carbon atom; similarly, carbohydrate stereoisomers that differ in configuration only at this carbon atom are called anomers. When a cyclic hemiacetal or hemiketal structure forms, the structure with the new hydroxyl group projecting on the same side as that of the oxygen involved in forming the ring is called the alpha anomer; that with the hydroxyl group projecting on the opposite side from that of the oxygen ring is called the beta anomer (Fig. 9) (Kraimi et al., 2017).

Classes of carbohydrates

Monosaccharides

Sources

The most common naturally occurring monosaccharides are D-glucose, D-mannose, D-fructose, and D-galactose among the hexoses and D-xylose and L-arabinose among the pentoses. In a special sense, D-ribose and 2-deoxy-D-ribose are ubiquitous because they form the carbohydrate component of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), respectively; these sugars are present in all cells as components of nucleic acids (Table 2).

D-Xylose, found in most plants in the form of a polysaccharide called xylan, is prepared from corncobs, cottonseed hulls, or straw by chemical breakdown of xylan. D-Galactose, a common constituent of both oligosaccharides and polysaccharides, also occurs in carbohydrate-containing lipids, called glycolipids, which are found in the brain and other nervous tissues of most animals (Kwak et al., 2019; Wen et al., 2019). Galactose is generally prepared by acid hydrolysis (breakdown involving water) of lactose, which is composed of galactose and glucose. Since the biosynthesis of galactose in animals occurs through intermediate compounds derived directly from glucose, animals do not require galactose in the diet. In fact, in most human populations, the majority of people do not retain the ability to

TABLE 2 Some naturally occurring monosaccharides

Sugar	Sources
L-Arabinose	Mesquite gum, wheat bran
D-Ribose	All living cells; as component of ribonucleic acid
D-Xylose	Corncobs, seed hulls, straw
D-Ribulose	As an intermediate in photosynthesis
2-Deoxy-D-ribose	As constituent of deoxyribonucleic acid
D-Galactose	Lactose, agar, gum arabic, brain glycolipids
D-Glucose	Sucrose, cellulose, starch, glycogen
D-Mannose	Seeds, ivory nut
D-Fructose	Sucrose, artichokes, honey
L-Fucose	Marine algae, seaweed
L-Rhamnose	Poison-ivy blossom, oak bark
D-Mannoheptulose	Avocado
D-Altroheptulose	Numerous plants

manufacture the enzyme necessary to metabolize galactose after they reach the age of four, and many individuals possess a hereditary defect known as galactosemia and never have the ability to metabolize galactose (Coelho et al., 2015).

D-Glucose (from the Greek word *glykys*, meaning "sweet"), the naturally occurring form, is found in fruits, honey, blood, and, under abnormal conditions, urine. It is also a constituent of the two most common naturally found disaccharides, sucrose and lactose, and the exclusive structural unit of the polysaccharides cellulose, starch, and glycogen. Generally, D-glucose is prepared from either potato starch or cornstarch (Mano, 2019).

D-Fructose, a ketohexose, is one of the constituents of the disaccharide sucrose and is also found in uncombined form in honey, apples, and tomatoes. Fructose, generally considered the sweetest monosaccharide, is prepared by sucrose hydrolysis and is metabolized by humans (Xiao et al., 2019).

Chemical reactions

The reactions of the monosaccharides can be conveniently subdivided into those associated with the aldehyde or keto group and those associated with the hydroxyl groups.

The relative ease with which sugars containing a free or potentially free aldehyde or keto group can be oxidized to form products has been known for a considerable time and once was the basis for the detection of these so-called reducing sugars in a variety of sources. For many years, analyses of blood glucose and urinary glucose were carried out by a procedure involving the use of an alkaline copper compound. Because the reaction has undesirable features—extensive destruction of carbohydrate structure occurs, and the reaction is not very specific (i.e., sugars other than glucose give similar results) and does not result in the formation of

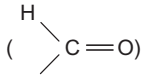


FIG. 10 Aldehyde group of glucose.

readily identifiable products—blood and urinary glucose now are analyzed by using the enzyme glucose oxidase, which catalyzes the oxidation of glucose to products that include hydrogen peroxide. The hydrogen peroxide then is used to oxidize a dye present in the reaction mixture; the intensity of the color is directly proportional to the amount of glucose initially present. The enzyme, glucose oxidase, is highly specific for β-D-glucose.

In another reaction, the aldehyde group of glucose reacts with alkaline iodine to form a class of compounds called aldonic acids (Fig. 10).

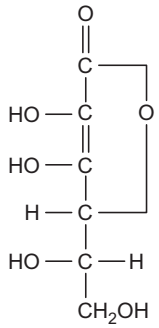
One important aldonic acid is ascorbic acid (vitamin C), an essential dietary component for humans and guinea pigs. The formation of similar acid derivatives does not occur with the keto sugars (Fig. 11).

Either the aldehyde or the keto group of a sugar may be reduced (i.e., hydrogen added) to form an alcohol; compounds formed in this way are called alditols, or sugar alcohols (Koh et al., 2018). The product formed as a result of the reduction of the aldehyde carbon of D-glucose is called sorbitol (D-glucitol). D-Glucitol also is formed when L-sorbose is reduced. The reduction of mannose results in mannitol and that of galactose in dulcitol.

Sugar alcohols that are of commercial importance include sorbitol (D-glucitol), which is commonly used as a sweetening agent, and D-mannitol, which is also used as a sweetener, particularly in chewing gums, because it has a limited water solubility and remains powdery and granular on long storage.

Formation of glycosides

The hydroxyl group that is attached to the anomeric carbon atom (i.e., the carbon containing the aldehyde or keto group) of carbohydrates in solution has unusual reactivity,



Ascorbic acid,
vitamin C
(L-gulonolactone-
2,3-enediol)

FIG. 11 Ascorbic acid, vitamin C.

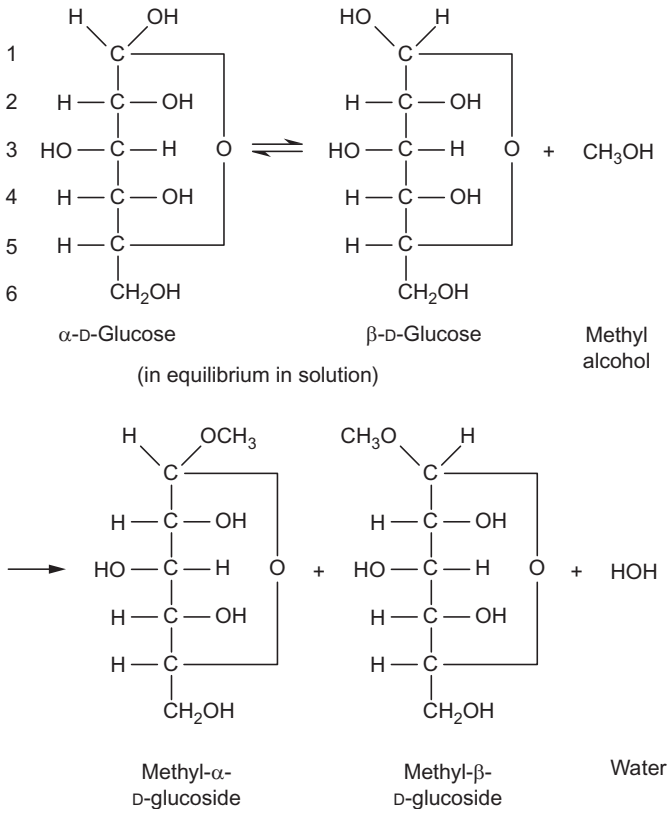
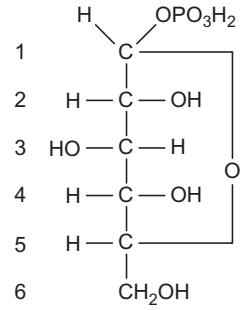


FIG. 12 Hydroxyl group (—OH) of the anomeric carbon atom of both α and β forms of D-glucose—α and β forms of D-glucose.

and derivatives, called glycosides, can be formed; glycosides formed from glucose are called glucosides. It is not possible for equilibration between the α- and β-anomers of a glycoside in solution (i.e., mutarotation) to occur. The reaction by which a glycoside is formed involves the hydroxyl group (—OH) of the anomeric carbon atom (numbered 1) of both α and β forms of D-glucose—α and β forms of D-glucose are shown in equilibrium in the reaction sequence—and the hydroxyl group of an alcohol (methyl alcohol in the reaction sequence); methyl α-D-glucosides and β-D-glucosides are formed as products, as is water (Fig. 12) (Gucchait and Misra, 2019).

Among the wide variety of naturally occurring glycosides are a number of plant pigments, particularly those red, violet, and blue in color; these pigments are found in flowers and consist of a pigment molecule attached to a sugar molecule, frequently glucose. Plant indican (from *Indigofera* species), composed of glucose and the pigment indoxyl, was important in the preparation of indigo dye before synthetic dyes became prevalent. Of a number of heart muscle stimulants that occur as glycosides, digitalis is still used. Other naturally occurring glycosides include vanillin, which is found in the vanilla bean, and amygdalin (oil of bitter almonds); a variety of glycosides found in mustard have a sulfur atom at position 1 rather than oxygen.



α-D-Glucose-1-phosphate

FIG. 13 α-D-Glucose-1-phosphate.

A number of important antibiotics are glycosides; among the best known are streptomycin and erythromycin. Glucosides—that is, glycosides formed from glucose—in which the anomeric carbon atom (at position 1) has phosphoric acid linked to it, are extremely important biological compounds. For example, α-D-glucose-1-phosphate is an intermediate product in the biosynthesis of cellulose, starch, and glycogen; similar glycosidic phosphate derivatives of other monosaccharides participate in the formation of naturally occurring glycosides and polysaccharides (Fig. 13) (Hayes and Pietruszka, 2017).

The hydroxyl groups other than the one at the anomeric carbon atom can undergo a variety of reactions. Esterification, which consists of reacting the hydroxyl groups with an appropriate acidic compound, results in the formation of a class of compounds called sugar esters. Among the common ones are the sugar acetates in which the acid is acetic acid. Esters of phosphoric acid and sulfuric acid are important biological compounds; glucose-6-phosphate, for example, plays a central role in the energy metabolism of most living cells, and D-ribulose 1,5-diphosphate is important in photosynthesis.

Formation of methyl ethers

Treatment of a carbohydrate with methyl iodide or similar agents under appropriate conditions results in the formation of compounds in which the hydroxyl groups are converted to methyl groups (—CH_3) (de Gonzalo et al., 2019). Called methyl ethers, these compounds are employed in structural studies of oligosaccharides and polysaccharides because their formation does not break the bonds, called glycosidic bonds, that link adjacent monosaccharide units. An example is the etherification of a starch molecule carried out using methyl iodide in which methyl groups become attached to the glucose molecules, forming a methylated segment in the starch molecule; note that the glycosidic bonds are not broken by the reaction with methyl iodide. When the methylated starch molecule then is broken down (hydrolyzed), hydroxyl groups are located at the positions in the molecule previously involved in linking one sugar molecule to another, and a methylated glucose, in this case named 2,3,6 tri-*O*-methyl-D-glucose, forms (Fig. 14). The linkage positions (which are not methylated) in a complex carbohydrate can be established by analyzing the locations of the methyl groups in the

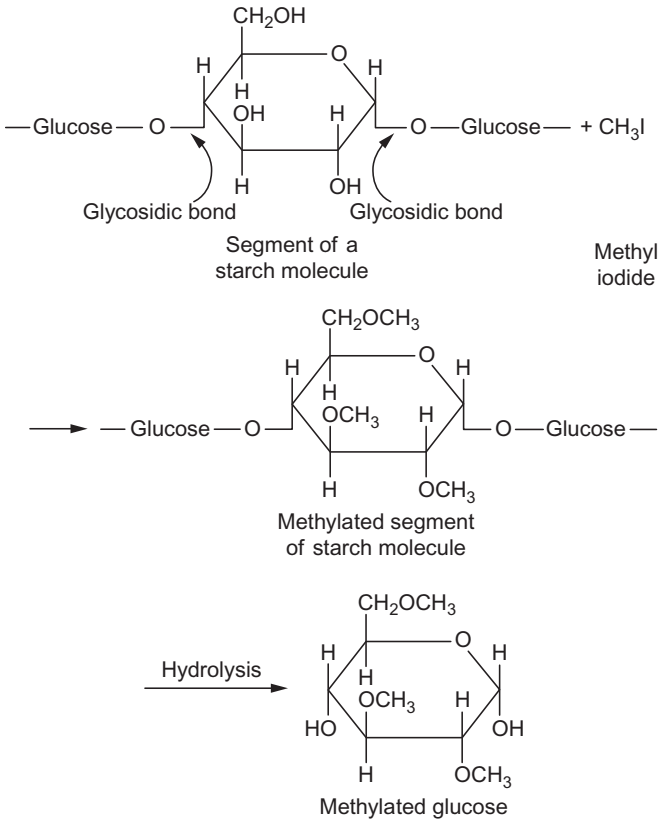


FIG. 14 Segment of a starch molecule, methylated segment of starch molecule, and methylated glucose.

monosaccharides. This technique is useful in determining the structural details of polysaccharides, particularly since the various methylated sugars are easily separated by techniques involving gas chromatography in which a moving gas stream carries a mixture through a column of a stationary liquid or solid, the components thus being resolved.

When the terminal group (CH_2OH) of a monosaccharide is oxidized chemically or biologically, a product called uronic acid is formed. Glycosides that are derived from D-glucuronic acid (the uronic acid formed from D-glucose) and fatty substances called steroids appear in the urine of animals as normal metabolic products; in addition, foreign toxic substances are frequently converted in the liver to glucuronides before excretion in the urine. D-Glucuronic acid also is a major component of connective tissue polysaccharides, and D-galacturonic acid and D-mannuronic acid, formed from D-galactose and D-mannose, respectively, are found in several plant sources.

Other compounds formed from monosaccharides include those in which one hydroxyl group, usually at the carbon at position 2, is replaced by an amino group ($—NH_2$); these compounds, called amino sugars, are widely distributed in nature. The two most important ones

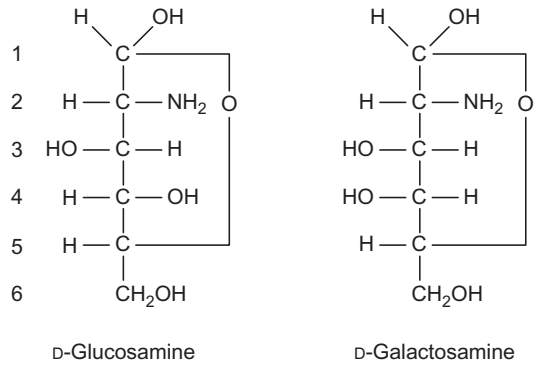


FIG. 15 D-Glucosamine and D-galactosamine.

are glucosamine (2-amino-2-deoxy-D-glucose) and galactosamine (2-amino-2-deoxy-D-galactose) (Fig. 15).

Neither amino sugar is found in the uncombined form. Both occur in animals as components of glycolipids or polysaccharides: for example, the primary structural polysaccharide (chitin) of insect outer skeletons and various blood group substances.

In a number of naturally occurring sugars, known as deoxy sugars, the hydroxyl group at a particular position is replaced by a hydrogen atom. By far the most important representative is 2-deoxy-D-ribose, the pentose sugar found in deoxyribonucleic acid (DNA); the hydroxyl group at the carbon atom at position 2 has been replaced by a hydrogen atom (Fig. 16).

Other naturally occurring deoxy sugars are hexoses of which L-rhamnose (6-deoxy-L-mannose) and L-fucose (6-deoxy-L-galactose) are the most common; the latter, for example, is present in the carbohydrate portion of blood group substances and on the outer surface of red blood cells.

Disaccharides and oligosaccharides

Disaccharides are a specialized type of glycoside in which the anomeric hydroxyl group of one sugar has combined with the hydroxyl group of a second sugar with the elimination of the elements of water. Although an enormous number of disaccharide structures are possible, only a limited number are of commercial or biological significance.

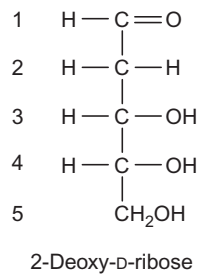


FIG. 16 2-Deoxy-D-ribose.

Sucrose and trehalose

Sucrose, or common table sugar, is a major commodity worldwide. By the second decade of the 21st century, its world production had amounted to more than 170 million tons annually (Figueroa and Lunn, 2016). The unusual type of linkage between the two anomeric hydroxyl groups of glucose and fructose means that neither a free aldehyde group (on the glucose moiety) nor a free keto group (on the fructose moiety) is available to react unless the linkage between the monosaccharides is destroyed; for this reason, sucrose is known as a nonreducing sugar. Sucrose solutions do not exhibit mutarotation, which involves formation of an asymmetrical center at the aldehyde or keto group. If the linkage between the monosaccharides composing sucrose is broken, the optical rotation value of sucrose changes from positive to negative; the new value reflects the composite rotation values for D-glucose, which is dextrorotatory (+52°), and D-fructose, which is levorotatory (−92°). The change in the sign of optical rotation from positive to negative is the reason sucrose is sometimes called invert sugar (Fig. 17).

The commercial preparation of sucrose takes advantage of the alkaline stability of the sugar, and a variety of impurities are removed from crude sugarcane extracts by treatment with alkali. After this step, syrup preparations are crystallized to form table sugar. Successive "crops" of sucrose crystals are "harvested," and the later ones are known as brown sugar. The residual syrupy material is called either cane final molasses or blackstrap molasses; both are used in the preparation of antibiotics, as sweetening agents, and in the production of alcohol by yeast fermentation. Sucrose is formed following photosynthesis in plants by a reaction in which sucrose phosphate first is formed.

The disaccharide trehalose is similar in many respects to sucrose but is much less widely distributed (Zhang and DeBosch, 2019). It is composed of two molecules of α-D-glucose and is also a nonreducing sugar. Trehalose is present in young mushrooms and in the resurrection plant (*Selaginella*); it is of considerable biological interest because it is also found in the circulating fluid (hemolymph) of many insects. Since trehalose can be converted to a glucose phosphate compound by an enzyme-catalyzed reaction that does not require energy, its function in hemolymph may be to provide an immediate energy source, a role similar to that of the carbohydrate storage forms (i.e., glycogen) found in higher animals. Trehalose and its analogues are promising cardiometabolic therapeutic agents with pleiotropic effects across tissue types.

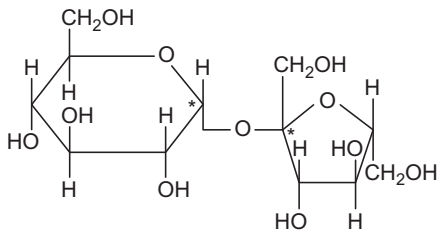


FIG. 17 Sucrose.

Lactose and maltose

Lactose is one of the sugars (sucrose is another) found most commonly in human diets throughout the world; it constitutes about 7% of human milk and about 4%–5% of the milk of mammals such as cows, goats, and sheep. Lactose consists of two aldohexoses—β-D-galactose and glucose—linked so that the aldehyde group at the anomeric carbon of glucose is free to react; that is, lactose is a reducing sugar (Fig. 18) (Ugidos-Rodríguez et al., 2018).

A variety of metabolic disorders related to lactose may occur in infants; in some cases, they are the result of a failure to metabolize properly the galactose portion of the molecule.

Although not found in uncombined form in nature, the disaccharide maltose is biologically important because it is a product of the enzymatic breakdown of starches during digestion. Maltose consists of α-D-glucose linked to a second glucose unit in such a way that maltose is a reducing sugar. Maltose, which is readily hydrolyzed to glucose and can be metabolized by animals, is employed as a sweetening agent and as a food for infants whose tolerance for lactose is limited (Dupas-Langlet et al., 2019).

Polysaccharides

Polysaccharides, or glycans, may be classified in a number of ways; the following scheme is frequently used. Homopolysaccharides are defined as polysaccharides formed from only one type of monosaccharide (Fonseca et al., 2019). Homopolysaccharides may be further subdivided into straight-chain and branched-chain representatives, depending upon the arrangement of the monosaccharide units. Heteropolysaccharides are defined as polysaccharides containing two or more different types of monosaccharides; they may also occur in both straight-chain and branched-chain forms. In general, extensive variation of linkage types does not occur within a polysaccharide structure, nor are there many polysaccharides composed of more than three or four different monosaccharides; most contain one or two.

Homopolysaccharides

In general, homopolysaccharides have a well-defined chemical structure, although the molecular weight of an individual amylose or xylan molecule may vary within a particular range, depending on the source; molecules from a single source also may vary in size, because

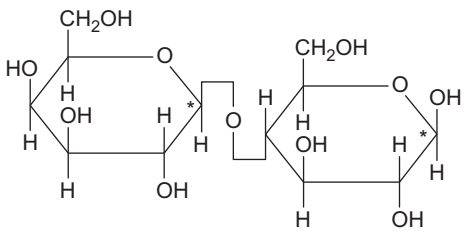


FIG. 18 β-Lactose.

TABLE 3 Representative homopolysaccharides

Homopolysaccharide	Sugar component	Linkage	Function	Sources
Cellulose	Glucose	β, 1 → 4	Structural	Throughout plant kingdom
Amylose	Glucose	α, 1 → 4	Food storage	Starches, especially corn, potatoes, rice
Chitin	*N*-Acetylglucosamine	β, 1 → 4	Structural	Insect and crustacean skeleton
Inulin	Fructose	β, 2 → 1	Food storage	Artichokes, chicory
Xylan	Xylose	β, 1 → 4	structural	All land plants
Glycogen	Glucose	α, 1 → 4, 6 ← 1, α	Food storage	Liver and muscle cells of all animals
Amylopectin	Glucose	α, 1 → 4, 6 ← 1, α	Food storage	Starches, especially corn, potatoes, rice
Dextran	Glucose	α, 1 → 6, 4 ← 1, α	Unknown	Primarily bacterial
Agar[a]	Galactose	α, 1 → 3	Structural	Seaweeds

[a] *May contain sulfate groups.*

most polysaccharides are formed biologically by an enzyme-catalyzed process lacking genetic information regarding size (Table 3) (Naseri-Nosar and Ziora, 2018; Ralph et al., 2019; Singh, 2019).

Heteropolysaccharides

In general, heteropolysaccharides (heteroglycans) contain two or more different monosaccharide units (Table 4) (Hsieh and Harris, 2019; Hui et al., 2019; Liu et al., 2018; Servais et al.,

TABLE 4 Representative heteropolysaccharides

Heteropolysaccharide	Component sugars	Functions	Distribution
Hyaluronic acid	D-Glucuronic acid and *N*-acetyl-D-glucosamine	Lubricant, shock absorber, water binding	Connective tissue, skin
Chondroitin-4-sulfate[a]	D-Glucuronic acid and *N*-acetyl-D-galactosamine-4-*O*-sulfate	Calcium accumulation, cartilage and bone formation	Cartilage
Heparin[a]	D-Glucuronic acid, L-iduronic acid, *N*-sulfo-D-glucosamine	Anticoagulant	Mast cells, blood
Gamma globulin[a]	*N*-Acetyl-hexosamine, D-mannose, D-galactose	Antibody	Blood
Blood group substance[a]	D-Glucosamine, D-galactosamine, L-fucose, D-galactose	Blood group specificity	Cell surfaces, especially red blood cells

[a] *Covalently linked to protein; the proportion of protein to carbohydrate in such complex molecules varies from about 10% protein in the case of chondroitin-4-sulfate to better than 95% for gamma globulin.*

2019; da Silva-Leite et al., 2018). Although a few representatives contain three or more different monosaccharides, most naturally occurring heteroglycans contain only two different ones and are closely associated with lipid or protein. The complex nature of these substances has made detailed structural studies extremely difficult. The major heteropolysaccharides include the connective-tissue polysaccharides, the blood group substances, glycoproteins (combinations of carbohydrates and proteins) such as gamma globulin, and glycolipids (combinations of carbohydrates and lipids), particularly those found in the central nervous system of animals and in a wide variety of plant gums.

Disclosure statement

The author declares that there are no conflicts of interest.

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PART 2

Molecular biology of the cell

CHAPTER

7

Peroxisome proliferator receptor gamma and the control of glucose metabolism: Insights from knockout mice

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OUTLINE

SUMMARY POINTS

- Whole-body deletion of all PPARγ isoforms caused embryonic lethality.
- Mice heterozygous for global PPARγ deletion were viable and were protected from high-fat diet-induced insulin resistance.
- Whole-body deletion of the PPARγ2 isoform caused insulin resistance even for mice on a regular diet.
- PPARγ deletion in adipose tissue showed that it is required for adipocyte survival and its loss caused lipodystrophy.
- Deletion in macrophages predisposed mice to diet-induced obesity and insulin resistance.
- When deleted in either the liver or skeletal muscle, animals were prone to glucose intolerance and insulin resistance.
- Deletion in neurons had no effect on glucose homeostasis, although animals were protected from obesity-induced leptin resistance.
- Astrocyte-specific knockout mice had impaired glucose tolerance and hepatic steatosis that did not worsen with HFD.
- The studies reviewed here show that PPARγ acts in many tissues, not only adipose tissue, to regulate glucose metabolism.

Key facts

- PPARγ, a nuclear receptor, is important for adipose tissue differentiation.
- Whole-body PPARγ deletion causes embryonic lethality.
- Tissue-specific deletion of PPARγ revealed actions in many tissues, including the adipose tissue, brain, liver, macrophages, muscle, and pancreas.

PPARγ

Peroxisome proliferator receptor gamma (PPARγ) is a member of the nuclear receptor superfamily. As in other members of the family, PPARγ structure is composed by two activation domains (AF1 and AF2), a DNA-binding domain and a ligand-binding domain. To bind to the DNA, PPARγ heterodimerizes with another transcription factor, retinoic X receptor. PPARγ is constitutively nuclear and bound to DNA and in the unliganded state is associated with transcriptional corepressors to inhibit gene expression. Upon agonist binding, PPARγ undergoes a conformational change to dismiss the corepressor complex and attract transcriptional coactivators to activate gene expression (Lehrke and Lazar, 2005). The composition of the coactivator complexes can vary with the agonist leading to overlapping but distinct gene expression profiles.

In humans, PPARγ gene is located on chromosome 3, and 17 PPARγ mRNA isoforms have been described. Many of these mRNA isoforms differ in noncoding exons in the 5′ UTR, and the relative amounts vary with cell type. Chen et al. described up to seven isoforms expressed

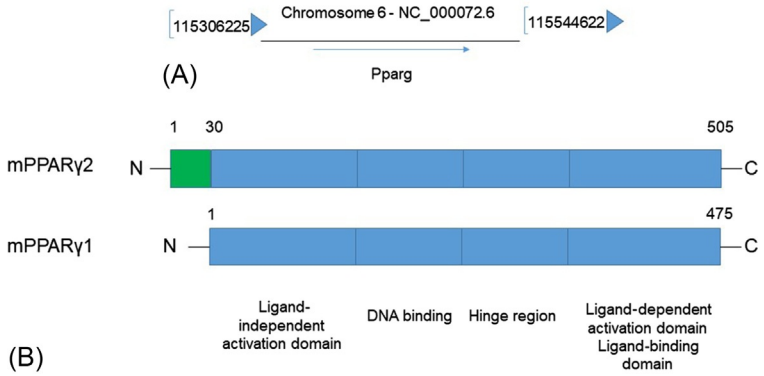

FIG. 1 Mouse PPARγ. (A) Chromosome localization of the *Pparg* gene in the mouse genome. (B) Mouse PPARγ isoforms.

in human TH1 macrophages (Chen et al., 2006). Two major protein isoforms are made PPARγ1 and PPARγ2. PPARγ2 is encoded by a transcript initiated from an internal promoter downstream of the PPARγ1 promoter, and thus the two isoforms differ through the use of alternative 5′ UTRs. The 5′ UTR of the PPARγ 2 mRNA isoform has an in-frame AUG codon and adds 28 additional amino-terminal amino acids. The mouse PPARγ gene is located on chromosome 6, and seven mRNA isoforms have been reported. Like the human gene, these isoforms encode two proteins mPPARγ1 and mPPARγ2 that differ by 30 amino acids at the amino terminus (Fig. 1). PPARγ1 is expressed ubiquitously (Heikkinen et al., 2007) with PPARγ2 expression mostly limited to adipose tissue. Although both isoforms are abundant in adipose tissue, PPARγ2 has the greatest adipogenic activity in vitro, and it is regulated by nutritional status. Under normal physiological conditions, PPARγ2 expression is limited to adipose tissue, but it can be induced in the liver and skeletal muscle in response to obesity (Medina-Gomez et al., 2005; Vidal-Puig et al., 1996).

PPARγ was first identified for its role in adipocyte differentiation (Semple et al., 2006). Its expression is highest in adipose tissue and macrophages, and it is expressed in lower levels in other tissues related to glucose homeostasis, such as the skeletal muscle, liver, and pancreatic β-cells. Interestingly, genetic manipulation of PPARγ has uncovered many unexpected actions in various tissues. In this review, we will focus on the effects of PPARγ deletion on glucose metabolism in mice. We will cover tissues important for glucose homeostasis, such as the adipose tissue, skeletal muscle, liver, and pancreatic β-cells, as well as in the brain, the central regulator of feeding behavior.

Whole-body PPARγ deletion

Genetic deletion of all PPARγ isoforms in the whole body causes embryonic lethality due to a defect in placental vascularization (Barak et al., 1999; Kubota et al., 1999). As a consequence, mice heterozygous for PPARγ were studied (Kubota et al., 1999; Matsui et al., 2004; Miles et al., 2000). Heterozygous PPARγ-deficient mice (*PPARγ* +/−) had improved insulin sensitivity on regular diet and were protected from the development of insulin resistance due to

adipocyte hypertrophy under a high-fat diet (HFD). Interestingly, *PPARγ* +/− mice showed overexpression and hypersecretion of leptin despite having smaller adipocytes and decreased fat mass. It was also shown that glucose-induced insulin secretion in *PPARγ* +/− mice was impaired in vitro. The tissue triglyceride (TG) content of the white adipose tissue, skeletal muscle, and liver was decreased in *PPARγ* +/− mice but increased in pancreatic islets, where the increased TG content was associated with decreased glucose oxidation. The administration of a PPARγ agonist, pioglitazone, corrected these defects, reduced the islet TG content, and ameliorated the impaired insulin secretion in vitro (Matsui et al., 2004).

To overcome the embryonic lethality, a conditional PPARγ knockout (KO) was created using a Mox2-Cre recombinase to delete *Pparg* in the embryo but not the trophoblasts (Duan et al., 2007). The homozygous null mice still showed over 90% postnatal lethality, but some survived to adulthood and were studied metabolically. The surviving animals showed >95% recombination of the floxed *Pparg* allele in all tissues. These mice showed lipodystrophy with dramatically reduced fat mass but organomegaly of many other organs similar to that seen in lipodystrophic models. Not surprisingly the mice had elevated plasma TG and FAs, reduced leptin and adiponectin, and were insulin resistant and glucose intolerant.

In contrast to the complete PPARγ deletion, mice that lack only the mPPARγ2 isoform survive and breed normally (Medina-Gomez et al., 2005). Male mice lacking PPARγ2, but not female mice, were slightly more glucose intolerant by GTT and insulin resistant by euglycemic-hyperinsulinemic clamp study than wild-type animals despite similar weight, body composition, food intake, energy expenditure, and adipose tissue morphology; hence the insulin resistance was not the result of lipodystrophy. The difference between genotypes disappeared, however, when the mice were placed on HFD. These PPARγ2 KO mice exhibited a defect in their rate of adipose tissue lipid storage, and acute overfeeding caused an increase in insulin levels compared with controls (Virtue et al., 2018). Another group created a similar PPARγ2 KO and also reported that male mice were insulin resistant, but these mice showed reduced adipose tissue and adipocyte differentiation. In addition, the skeletal muscle showed decreased expression of insulin receptor substrate 1 and glucose transporter 4. Interestingly, rosiglitazone, a thiazolidinedione PPARγ agonist, was able to normalize the insulin resistance indicating that the remaining PPARγ1 could functionally replace the γ2 isoform given the right ligand (Zhang et al., 2004). When the PPARγ2 KO was crossed to leptin-deficient ob/ob mice, the double KO mice prevented obesity in both male and female mice compared with ob/ob mice but caused severe insulin resistance, hyperglycemia, β-cell failure, and dyslipidemia (Medina-Gomez et al., 2007). These studies highlight the importance of PPARγ2 as a crucial regulator of adipose tissue differentiation and lipid storage.

Due to the embryonic lethality of complete PPARγ deletion and the multiple effects in various tissues, many investigators have used conditional tissue-specific deletions to analyze PPARγ effects on glucose metabolism.

PPARγ deletion in adipose tissue

Due to PPARγ's role in adipogenesis (Vidal-Puig et al., 1997), it is not surprising that adipocyte PPARγ KO mice were some of the earliest mice generated. Several labs have deleted PPARγ in the adipose tissue, and their findings are summarized in Table 1.

TABLE 1 Adipose-specific PPARγ knockout

Ref	Cre	Phenotype
He et al. (2003)	aP2-Cre. Cre under the promoter of adipocyte protein 2 (fatty acid-binding protein)	Adipocyte hypocellularity and hypertrophy, elevated plasma free fatty acids and triglyceride, and decreased plasma leptin and ACRP30. Adult KO mice were more susceptible to high-fat diet-induced steatosis, hyperinsulinemia, and insulin resistance
Imai et al. (2004)	aP2-Cre-ER. Tamoxifen-inducible Cre under the control of aP2	Mature PPARγ null white and brown adipocytes die within a few days and are replaced by newly formed PPARγ-positive adipocytes
Jones et al. (2005)	aP2-Cre. aP2 promoter-enhancer	Marked abnormalities in the formation and function of both brown and white adipose tissues. When fed a high-fat diet, KO displayed diminished weight gain despite hyperphagia, had diminished serum concentrations of both leptin and adiponectin, and did not develop glucose intolerance or insulin resistance
Wang et al. (2013)	Adipoq-Cre. Cre recombinase under control of the mouse adiponectin (*Adipoq*) promoter/enhancer regions within the BAC transgene	KO had almost no visible brown and white adipose tissue at age 3 months, enlarged pancreatic islets, massive fatty livers, and elevated levels of blood glucose and serum insulin accompanied by insulin resistance. KO also had delayed hair coat formation associated with absence of dermal fat, disrupted mammary gland development with loss of mammary fat pads, and high bone mass with loss of bone marrow fat
Xiong et al. (2018)	UCP-1-Cre. Cre under the control of uncoupling protein-1 (expression in brown adipose tissue)	Development of perivascular adipose tissue and interscapular brown adipose tissue was impaired, genes encoding lipogenic enzymes were reduced in the BA-PPARγ KO mice. Thermogenesis in brown adipose tissue was significantly impaired with reduced expression of thermogenesis genes in brown adipose tissue and compensatory increase in subcutaneous and gonadal white adipose tissues Basal expression of inflammatory genes and macrophage infiltration in PVAT and brown adipose tissue were significantly increased in the BA-PPARγ KO mice

The first KOs used the adipocyte protein 2-Cre (aP2-CRE) mouse to delete PPARγ. Not surprisingly, animals that lack PPARγ in white and brown adipose tissue had impaired adipogenesis, were lipodystrophic, and were also glucose intolerant and insulin resistant. One lab reported that adipocyte PPARγ KO mice were significantly more susceptible to

HFD-induced steatosis, hyperinsulinemia, and insulin resistance (He et al., 2003), while another lab found that KO mice on HFD did not develop glucose intolerance and insulin resistance (Jones et al., 2005). One difference between the studies is that in He et al., mice were placed on HFD at adulthood, while in Jones et al., mice were placed on HFD at weaning. It is possible that HFD feeding during early development may allow compensation as neural pathways are still being established. One difficulty with these models is that the aP2 promoter also expresses in macrophages, so the deletion is not adipocyte specific, so another study used adiponectin promoter to driver Cre expression (Adipoq-Cre) as this promoter shows greater adipocyte specificity. Wang et al. found that these KO animals had no visible brown and white adipose tissue at 3 months consistent with the adipogenic defect in previous aP2-mediated KOs; had enlarged pancreatic islets and fatty livers; and had elevated levels of blood glucose and serum insulin and leptin, accompanied by insulin resistance (Wang et al., 2013). All of these models were complicated by the fact that the promoters used to delete PPARγ were themselves PPARγ target genes, so many of the effects could be developmental. To circumvent this problem a tamoxifen-inducible Cre was used to delete PPARγ from mature adipocytes in adult mice (Imai et al., 2004). Both white and brown adipocytes die within days and are replaced by newly differentiated PPARγ-positive adipocytes, demonstrating that PPARγ is essential for the survival of mature adipose tissue. In a recent paper the uncoupling protein 1 (UCP-1) promoter was used as the driver for Cre to delete PPARγ in brown and beige adipocytes (Xiong et al., 2018). In this study, no effects on whole-body glucose metabolism were noted, but macrophage infiltration and inflammatory gene expression were increased in brown adipose tissue, and atherosclerosis was increased when mice were crossed to ApoE-deficient mice. These studies confirm the importance of PPARγ for adipose tissue development and adipocyte function and survival.

PPARγ deletion in pancreas

As noted previously, in vitro glucose-stimulated insulin secretion was impaired in islets from *PPARγ* +/− mice (Matsui et al., 2004), suggesting that PPARγ is important for islet function. To evaluate PPARγ actions in pancreatic islets, pancreatic PPARγ KO mice were generated using the Cre:loxp technology. Animal models and results are summarized in Table 2.

Results from these studies varied depending on the promoter used to target the β-cell. When the rat insulin promoter was used to drive Cre expression, islet hyperplasia was observed under normal chow, and the expansion in response to HFD was blunted, but no effects were seen on glucose metabolism (Rosen et al., 2003). Rosiglitazone was still able to improve glucose metabolism in these mice indicating that PPARγ in the β-cell is not required for the antidiabetic actions of these drugs. When Pdx1 was used as driver for Cre expression (Gupta et al., 2008), opposite results were observed. There was no difference in islet morphology or β-cell content, but the mice were hyperglycemic and glucose intolerant, and islets showed increased basal insulin secretion and reduced glucose-stimulated insulin secretion in vitro. It is possible that the differences between both models are due to the developmental issues as Gupta et al. showed that the Pdx1 gene itself is a PPARγ target gene, so KO β-cells had lower Pdx1 expression (Gupta et al., 2010). Another group used a tamoxifen-inducible Cre under

TABLE 2 Beta cell-specific PPARγ KO mice

Ref	Cre	Phenotype
Rosen et al. (2003)	Insulin-CRE	PPARγ KO mice had islet hyperplasia on a regular diet. Normal expansion of β-cell mass in response to high-fat feeding is markedly blunted in these animals Despite this alteration in β-cell mass, no effect on glucose homeostasis in PPARγ KO mice was noted
Gupta et al. (2008)	Pdx1-CRE	Male KO mice were hyperglycemic at 8 weeks of age
Welters et al. (2012)	pdx1-CreER: tamoxifen-inducible CRE driven by the PDX-1 enhancer element. PPARγ was deleted in adult β-cells	No significant changes in glucose metabolism or insulin sensitivity when fed normal or high-fat diet, compared with wt mice. No difference in inlet morphology or response to rosiglitazone

the promoter of Pdx1 to delete PPARγ in adult β-cells (Welters et al., 2012). These mice showed no changes in glucose metabolism or insulin sensitivity on a normal diet or on a HFD, no difference in islet morphology, and no difference in the response to rosiglitazone. Overall, these models show that PPARγ does not have a role in glucose homeostasis in the adult mice, but could be involved in pancreatic islet development.

PPARγ deletion in liver

Although PPARγ expression is low in the normal liver, the upregulation of PPARγ expression, particularly PPARγ2, has been reported in steatotic livers raising the possibility that

TABLE 3 Liver-specific PPARγ KO mice

Ref	Cre	Phenotype
Gavrilova et al. (2003)	Alb-CRE, CRE under the promoter of albumin	KO mice developed fat intolerance and had increased adiposity, hyperlipidemia, hyperglycemia, and hyperinsulinemia
Matsusue et al. (2003)	Alb-CRE	In leptin-deficient mice, PPARγ deletion improved the fatty liver condition but worsened the hyperglycemia and insulin resistance
Moran-Salvador et al. (2011)	Alb-CRE	KO are protected from HFD-induced liver steatosis
Kineman et al. (2016)	AAV8-TBGp-Cre: adeno-associated virus injected in the tail vein	Loss of hepatic PPAR blunted the rise in fatty acid translocase/CD36 and monoacylglycerol acyltransferase 1 expression induced by GH receptor knockdown in adult animals

PPARγ is associated with lipid deposition in the fatty liver. To address the role of PPARγ in the liver, a number of labs have deleted PPARγ specifically in hepatocytes, and the results are summarized in Table 3.

Deletion of PPARγ in hepatocytes using albumin-Cre did not alter the body or liver weight, liver or serum TG, serum glucose or insulin, muscle glucose uptake, or suppression of hepatic glucose production and did not prevent the antidiabetic effect of rosiglitazone (Gavrilova et al., 2003). Older mice fed a lipogenic diet, however, showed increased adipose tissue; decreased lipid clearance; and increased serum TG, glucose, and insulin. When the liver PPARγ KO was crossed to the lipoatrophic AZIP mice (Moitra et al., 1998), the PPARγ deletion reduced the hepatic steatosis but worsened the hyperlipidemia, triglyceride clearance, and muscle insulin resistance observed in these animals (Gavrilova et al., 2003). Hepatocyte PPARγ deletion also improved hepatic steatosis in other models of obesity, including the ob/ob mouse (Matsusue et al., 2003), and DIO animals (Moran-Salvador et al., 2011), indicating that PPARγ is an important player in the development of the fatty liver in obese animals. The improved steatosis came with a cost, however, as PPARγ deletion worsened hyperglycemia and insulin resistance in these animals (Gavrilova et al., 2003; Matsusue et al., 2003; Moran-Salvador et al., 2011), suggesting that the loss of PPARγ worsened the liver insulin resistance. Not all steatosis is PPARγ-dependent however, as adult animals with loss of growth hormone signaling in the liver developed hepatic steatosis regardless of PPARγ status (Kineman et al., 2016). These results suggest that, although liver PPARγ might play an important role in obesity-induced steatosis, it might not be involved in other types of steatosis.

PPARγ deletion in macrophages

PPARγ is highly expressed in macrophages, and its actions can regulate many physiological processes. The earliest report used the interferon-inducible MX promoter to drive Cre expression (Akiyama et al., 2002). The PPARγ-deficient macrophages had reduced ability to transfer cholesterol to HDL particles due to reduced expression of LPL, CD36, LXRa, ABCA1, ABCG1, and ApoE. Subsequent studies have used the LysMCre mouse to delete PPARγ in macrophages. Results from these different models are summarized in Table 4. Animals that lacked PPARγ in macrophages were glucose intolerant and showed muscle and hepatic insulin resistance on normal chow, and these phenotypes worsened when fed a HFD (Hevener et al., 2007; Odegaard et al., 2007; Moran-Salvador et al., 2011), although liver steatosis improved. The phenotype was associated with elevated proinflammatory cytokines and decreased antiinflammatory cytokines with a concurrent reduction in insulin signaling in adipocytes, muscle cells, and hepatocytes. One possible explanation for the observed phenotype is that PPARγ is important for macrophage respiration and alternative activation, serving as a guardian against inflammation (Nelson et al., 2018).

TABLE 4 Macrophage-specific PPARγ KO mice

Ref	Cre	Phenotype
Akiyama et al. (2002)	Interferon-inducible MX-CRE	Reduced cholesterol efflux from macrophages
Hevener et al. (2007)	LysMCre: CRE under the control of lysozyme 2 gene. It is used for CRE-induced deletion in the myeloid cell lineage (monocytes, mature macrophages, and granulocytes) and the innate immune response	Macrophage-specific deletion of PPARγ results in the development of significant glucose intolerance plus skeletal muscle and hepatic insulin resistance in lean mice fed a normal diet. The insulin resistance became more severe in mice lacking macrophage PPARγ following high-fat feeding
Odegaard et al. (2007)	LysMCre	Disruption of PPARγ in myeloid cells impairs alternative macrophage activation, thereby predisposing these animals to development of diet-induced obesity, insulin resistance, and glucose intolerance
Moran-Salvador et al. (2011)	LysMCre	KO mice are protected from HFD-induced liver steatosis. Deletion of PPARγ in macrophages led to increased serum glucose levels accompanied by glucose intolerance and upregulation of gluconeogenic genes

PPARγ deletion in skeletal muscle

Skeletal muscle, together with the liver and adipose tissue, is the main insulin-sensitive tissues that control glucose homeostasis. The observation that PPARγ agonists enhance skeletal muscle insulin sensitivity in obesity and in patients with noninsulin-dependent diabetes mellitus suggests that PPARγ expression in skeletal muscle plays a key role in determining tissue sensitivity to insulin (Kruszynska et al., 1998). Therefore, to study the involvement of PPARγ in glucose metabolism and insulin sensitivity, PPARγ was deleted in skeletal muscle in mice (Hevener et al., 2003; Norris et al., 2003). Both papers used MCK (muscle creatine kinase) promoter as the driver for CRE expression. One of the papers (Norris et al., 2003) reported that KO mice developed excess adiposity despite reduced dietary intake. Although insulin-stimulated glucose uptake in muscle was not impaired, KO mice had whole-body insulin resistance primarily due to dramatic impairment in hepatic insulin action. When placed on a HFD, KO mice developed hyperinsulinemia and impaired glucose homeostasis identical to controls. In this study, animals were maintained on a mixed 129/sv, C57BL/6, and FVB background. The second study used pure C57BL/6 animals (Hevener et al., 2003), and they observed that as early as 4 months of age, mice with targeted disruption of PPARγ in muscle showed glucose intolerance and progressive insulin resistance. Differences between phenotypes due to the KO are likely to be due to the different backgrounds. From these results, we can conclude that muscle PPARγ is involved in insulin action, as when PPARγ is deleted from the muscle, the result is systemic insulin resistance, when animals are fed normal diet.

PPARγ deletion in brain

Lastly, although the brain is not a classic organ involved in glucose metabolism, it is the main controller of feeding behavior. It was shown that PPARγ agonists improve blood glucose control and insulin sensitivity in patients with type 2 diabetes mellitus but induce weight gain in humans and rodent models not only via enhanced adipogenesis and edema but also by increasing food intake (Lehrke and Lazar, 2005; Shimizu et al., 1998). This latter effect suggests that PPARγ signaling in the central nervous system may influence energy intake and storage. Consistent with this hypothesis, PPARγ is expressed in key brain areas involved in energy homeostasis and glucose metabolism (Sarruf et al., 2009). It was of great interest to investigate the role of PPARγ in the control of energy expenditure, feeding, and metabolism. The results are summarized in Table 5.

Two papers investigated the effects of PPARγ deletion in neurons using the synapsin-Cre mouse that expresses Cre recombinase in the majority of mature neurons. While male PPARγ KO had reduced food intake and increased energy expenditure, resulting in reduced weight gain when fed a HFD. Interestingly the neuronal PPARγ KO mice did not become leptin resistant with obesity, but were resistant to rosiglitazone effects on insulin sensitivity (Lu et al., 2011). In contrast, female neuronal PPARγ KO mice showed no effect on body weight on normal chow or HFD (Fernandez et al., 2017b) and like the male mice did not develop leptin resistance. These findings were consistent with obesity generating PPARγ agonists that induce SOCS3 expression in the hypothalamus to impair leptin signaling. This is likely mediated by POMC neurons as a PPARγ KO in POMC neurons had a similar leptin-sensitive phenotype

TABLE 5 Neuron and astrocyte-specific PPARγ KO mice

Ref	Cre	Phenotype
Lu et al. (2011)	Syn-CRE: CRE under the control of the promoter for synapsin Neuron-specific	During high-fat diet (HFD) feeding, food intake was reduced, and energy expenditure increased in PPARγ KO male mice compared with controls, resulting in reduced weight gain. PPARγ KO mice were resistant to rosiglitazone-induced hyperphagia and weight gain and to rosiglitazone effects on hepatic insulin sensitivity during HFD feeding
Long et al. (2014)	Pomc-Cre: CRE under the control of the promoter for Pomc Neuron-specific	Leptin sensitivity in obese mice. Increased energy expenditure. Reduced body weight, adipose tissue, and food intake. Protected from HFD-induced glucose intolerance
Fernandez et al. (2017a)	Syn-CRE	Female KO mice showed normal body weight, glucose and insulin tolerance, and leptin levels but were protected from obesity-induced leptin resistance
Fernandez et al. (2017b)	GCTF-CRE: tamoxifen-inducible CRE under the control of the glial fibrillary acidic protein Astrocyte-specific	KO mice had impaired glucose tolerance and hepatic steatosis that did not worsen with HFD. Expression of gluconeogenic genes was elevated in the mouse livers, as was expression of several genes involved in lipogenesis, lipid transport, and storage

(Long et al., 2014). The other major cell type in the brain is the astrocytes that provide metabolic support to neurons and clear and recycle neurotransmitters. Surprisingly, when PPARγ was deleted specifically in astrocytes using a tamoxifen-inducible CRE under the control of the glial fibrillary acidic protein promoter (Fernandez et al., 2017a), mice had impaired glucose tolerance and hepatic steatosis that did not worsen with HFD-induced obesity. So PPARγ's only metabolic role in neurons is to create leptin resistance, but in astrocytes, PPARγ can modulate glucose tolerance and insulin sensitivity. At first glance, this may seem counterintuitive as neurons are known to regulate the liver, muscle, and adipose function through the sympathetic and parasympathetic systems, but astrocytes are not known to signal to the periphery. The results are likely explained by the ability of PPARγ to regulate glutamate transporters in astrocytes to reduce excitotoxicity. The observed results could be explained by the loss of PPARγ increasing glutamatergic signaling and sympathetic output. Overall the results show that PPARγ has distinct role in different cells in the brain with its principal role in neurons to modulate leptin sensitivity, but in astrocytes, PPARγ prevents steatosis and maintains glucose and insulin sensitivity.

Final considerations

In summary the multiple tissue-specific genetic deletion studies of PPARγ have confirmed its important role in adipogenesis and adipocyte survival but have also expanded the range of actions in other tissues to include promoting lipid storage in the liver, modulating the inflammatory response, controlling islet development, and regulating astrocyte to neuronal communication in the brain. Further studies will be needed to fully understand some of these metabolic phenotypes.

Glossary

KO Knockout. This refers to genetically modified mice.
PPARγ Peroxisome proliferator receptor gamma. Nuclear receptor.
UTR Untranslated region of a gene.

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CHAPTER

8

Glucose metabolism in CD4 and CD8 T cells

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OUTLINE

SUMMARY POINTS

- This chapter focusses on glucose metabolism in T cells under normal physiological conditions and in disease states.
- Metabolic reprogramming from oxidative phosphorylation to glycolysis is a key feature of activated and inflammatory CD4 and CD8 T cells.
- In some disease conditions such as HIV

infection, the immune system may be compromised due to "metabolic exhaustion."
- CD4 and CD8 T cells are made up of different cell subsets with not only similar but also unique metabolic signatures.
- Metabolic nodes such as the PI3K-/AKT-/mTOR axis regulate glucose metabolism in T cells.
- Antidiabetic drugs including metformin and natural compounds such as resveratrol, quercetin, and curcumin modulate T cell metabolism and are being exploited as novel antiinflammatory therapies.

Key facts of immunometabolism

- Glucose is the main source of fuel for immune cells. Without glucose, many functions of T cells are compromised.
- The first human experiments to study glucose metabolism in T cells in any disease were conducted in HIV+ patients at the Burnet Institute in Melbourne, Australia—first published in 2014, in the journal AIDS.
- The metabolic profile of activated T cells resembles that of cancer cells.
- Regulators of glucose metabolic processes in T cells are also involved in normal aging processes.

Introduction

Eukaryotic metabolism is the sum of diverse biochemical processes that either produce or consume energy and is responsible for all regulatory mechanisms associated with cell functions. These mechanisms are the result of multiple, overlapping pathways, which contribute to ATP production by breaking down nutrients such as glucose, amino acids, and lipids. Central to the metabolism of CD4 and CD8 T cells are the glycolytic pathway, the tricarboxylic acid and urea cycles, glycogen catabolism, and oxidative phosphorylation (Pearce and Pearce, 2013; Murray et al., 2015; Palmer et al., 2015). Emerging knowledge of immunometabolism has shaped our understanding of the fundamental processes by which nutrient metabolism impact immune cell functions and the degree by which dysregulated metabolic processes in T cells influence disease pathogenesis.

T cell immunometabolism is the specific study of the cellular metabolism of various subsets of T cells and includes primary cell lineages such as naïve, effector, and memory T cells involved in the adaptive immunological response to foreign antigens. Upon initial exposure of naïve T cells to priming cytokines and or the presentation of foreign antigens by antigen presenting cells (APCs), metabolic reprogramming is initiated to meet their bioenergetic demands for activation, differentiation, and effector function (Shehata et al., 2017; Geltink et al., 2018).

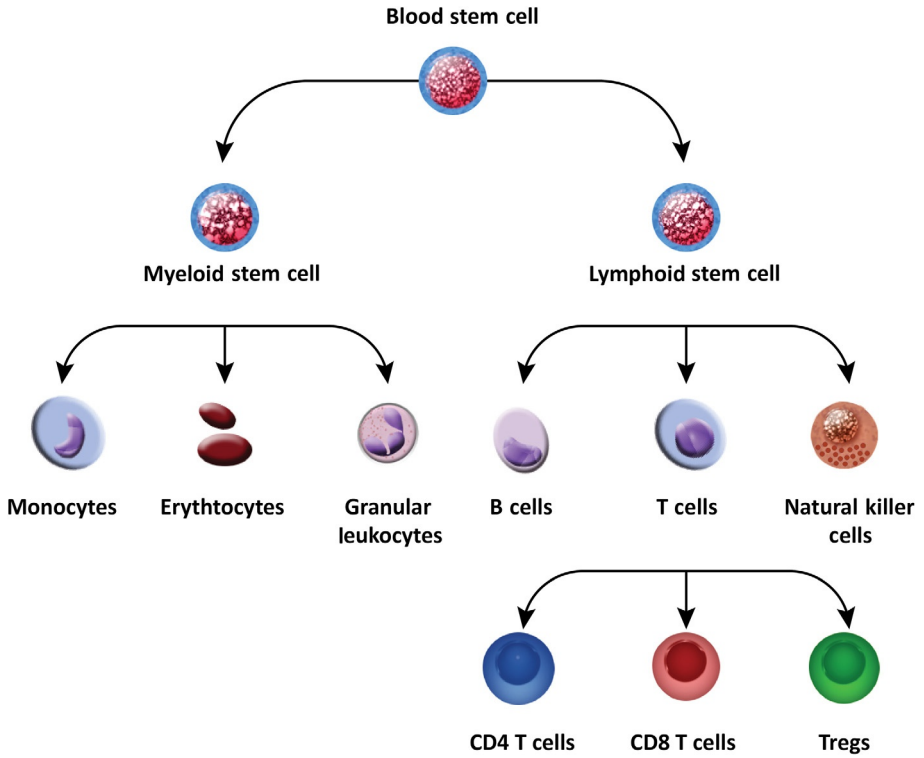

FIG. 1 The differentiated lineages of blood stem cells. From lymphoid stem cells, T cells develop into two major lineages, CD4 and CD8 T cells. These can differentiate into immune suppressor cells called Tregs.

Arising from the lymphoid stem cell line, T cells develop into two major lineages, CD4 and CD8 T cells, named for their surface expression of CD4 (cluster of differentiation 4) and CD8 (cluster of differentiation 8) glycoproteins, respectively (Fig. 1). Also known as helper T cells, CD4 T cells bind the major histocompatibility complex class II (MHCII) molecule present on APCs to initiate the secretion of cytokines, such as those of the interleukin family. This kick starts an immune response that features proliferation of B cells and T cells that bind to specific antigens in a process called clonal selection (Raeber et al., 2018). In contrast, CD8 T cells bind the MHCI complex of APCs, cancer, or parasitic cells, leading to their differentiation into cytotoxic T cells (Raeber et al., 2018). These functional changes constitute the basis of the adaptive immune system, kept in check by T regulatory cells, called Tregs. These Tregs secrete cytokines that regulate T cell responses to restrict overstimulation of the immune system (Biswas et al., 2018).

The emergence of immunometabolism has made it possible to control differentiation, growth, activation, and functions of CD4 and CD8 T cells by manipulating metabolic pathways that regulate nutrient influx and metabolism in these cells. The field has facilitated the development of new theories to understand fundamental processes that drive T cell dysfunction in inflammatory diseases such as cancer, autoimmune disease, obesity, and viral and bacterial infections (Hotamisligil, 2017; Shehata et al., 2017). Furthermore, increased glucose uptake in hepatocytes and CD4 T cells drives metabolic states that favor hepatitis

B virus (HBV) (Masson et al., 2017) and human immunodeficiency virus (HIV) infectivity (Palmer et al., 2017; Shehata et al., 2017), respectively. The intricate link between T cell metabolism, functions, and disease outcomes has increased interest in the development of immunometabolic therapeutics to treat inflammatory-mediated disease states.

The metabolic requirements for T cell activation and effector function

Activation of CD4 and CD8 T cells is associated with an increase in metabolic activity orchestrated by upregulation of glucose transporter Glut1 on their cell surface. Glut1 is the major isoform of glucose transporters on activated T cells and is responsible for the influx of glucose (Cao et al., 2014). While glycolysis predominates in activated T cells, naïve T cells are more reliant on oxidative phosphorylation to generate ATP (O'Neill et al., 2016). Although activated CD4 T cells and CD8 T cells have shared metabolic signatures, the rate in which glycolysis occurs is different between them (Cretenet et al., 2016; Jones et al., 2017).

Naïve CD4 T cells have low basal energy demands and express lower levels of Glut1 than effector CD4 T cells even in the context of a viral infection, such as HIV (Palmer et al., 2014). However, upon activation and differentiation into effector T cells, Glut1 levels are increased on their cell surface. Glut1 is essential for CD4 T cell activation (Macintyre et al., 2014) and is considered a functional metabolic activation marker of CD4 T cells. Higher cell surface Glut1 promotes glucose uptake and increases glycolysis. Glycolytic enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are also known for their involvement in several extra-glycolytic functions based on their cellular locations. GAPDH can bind to the AU-rich elements within the 3′ UTR of mRNA that encodes inflammatory proteins such as IL-2 and IFN-γ and control their expression (Chang et al., 2013).

The tremendous influx of glycolytic metabolites provides necessary precursors for protein, DNA, and lipid synthesis to sustain biomass production for cell growth and subsequent multiple cellular proliferations (Macintyre et al., 2014). Immunometabolic reprogramming of CD4 and CD8 T cells is generally regarded as the switch from oxidative phosphorylation towards a highly glycolytic metabolism even when sufficient oxygen is available. This is referred to as aerobic glycolysis. However, other auxiliary metabolic pathways such as glutaminolysis and hexosamine biosynthesis may be important at different stages of T cell activation (Araujo et al., 2017). Upon T cell activation, glucose can be shuttled into the hexosamine biosynthetic pathway where it is used to produce uridine diphosphate-*N*-acetyl glucosamine (UDP-GlcNAc). UDP-GlcNAc is a substrate critical for protein glycosylation—a posttranslational modification that regulates important biological processes. Thus "metaboglycomics" utilizes metabolic and glycomic technologies to understand how nutrient metabolism influences sugar modification of macromolecules and the significance of these modifications in health and diseases.

Differentiation of naïve T cells into effector T cells also requires uptake and metabolism of amino acids such as glutamine, arginine, and tryptophan for proliferation and cytokine production (O'Neill et al., 2016). T cell activation is linked to glutamine uptake, which is dependent on the levels of the amino acid transporters solute carrier family 1 member 5 (SLC1A5) (Nakaya et al., 2014; Klysz et al., 2015) and solute carrier family 7 member 5 (SLC7A5) (Sinclair et al., 2013) and is essential for activation of the mechanistic target of rapamycin complex 1

TABLE 1 The metabolic and function phenotypes of the CD4 and CD8 T cell

Fuel consumption	CD4		CD8		Tregs
	Effector	Memory	Effector	Memory	
Glucose	High	Low	High	Low	Low
Fatty acid	Low	High	Low	High	High
Amino acid	High	Intermediate	High	Intermediate	Low
Function	Releases cytokines to stimulate cytotoxic T cells	Inactive state that undergoes rapid activation when reencountering APC	Release cytokines into APC to induce apoptosis	Inactive state that undergoes rapid activation when reencountering APC	Secrete antiinflammatory cytokines

(mTORC1) kinase activation (Sinclair et al., 2013; Nakaya et al., 2014), a signaling pathway important for glucose and amino acid metabolism. The mechanistic link between glycolysis and a proinflammatory T cell phenotype (e.g., Th1 and Th17) is coordinated by moonlighting of metabolic enzymes in the nucleus where they promote proinflammatory gene expression (Boukouris et al., 2016). This effect also involves transport of glycolytic metabolites into the nucleus as substrates for epigenetic regulation of genes (Palmer et al., 2015; Boukouris et al., 2016). A comparison of substrate usage by various T cell subsets is shown in Table 1 and Fig. 2. Thus while glucose metabolism is critical in various T cell biological processes, the metabolism of amino acids such as glutamine through glutaminase activity is important for the production of α-ketoglutarate that fuels the TCA cycle. This is particularly important for activated T cells and promotion of Th17 T cell differentiation through α-ketoglutarate regulation of chromatin and expression of inflammatory genes (Johnson et al., 2018).

Fatty acid oxidation and oxidative phosphorylation in memory and regulatory T cells

Following clonal expansion, some T cells become established as long-lived memory cells (Polizzi et al., 2015). Memory cells enter a quiescent state, becoming dependent on fatty acid oxidation, maintaining energy production through OXPHOS (Mathieu et al., 2015; Van der Windt et al., 2012). High mitochondrial content ensures rapid reactivation upon reencountering the pathogen, which initially orchestrated the T cell response (Van der Windt et al., 2012).

While activated T cells increase anabolic pathways such as glycolysis, memory T cells adopt a metabolic configuration that engages catabolic pathways including fatty acid oxidation. In memory T cell glucose is used to fuel fatty acid synthesis, and free fatty acids are imported and stored as triglycerides for utilization by β-oxidation to fuel OXPHOS (Shehata et al., 2017). In mouse CD8 T cells, it has been shown that the development of memory T cells is promoted by inhibiting glycolysis, indicating that the ratio of effector and memory T cells may be determined by the energy source used by T cells (Sukumar et al., 2013; O'Sullivan et al., 2014). Memory T cells have high mitochondrial fusion rates and undergo

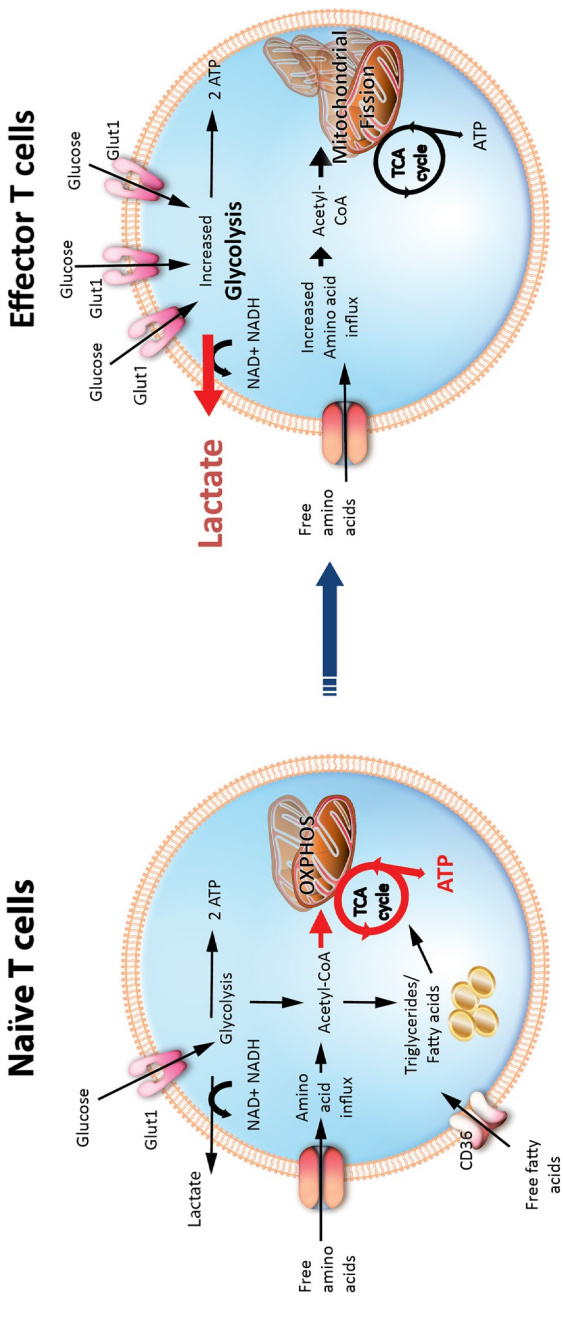

FIG. 2 Metabolic reprogramming during T cells activation and differentiation. Naïve T cells favor oxidative phosphorylation (OXPHOS) to generate ATP from fatty acids and from small amount of glucose and amino acids. During activation, cells are reprogrammed to increase glucose uptake and aerobic glycolysis.

cristae remodeling to enable ATP production (Buck et al., 2016). Higher mitochondrial fusion using hydrazone M1 and the fission inhibitor (Mdivi-1) promotes memory T cell differentiation from effector T cells, while inhibition of mitochondrial fusion is associated with reduced FAO, lower oxygen consumption, and higher rates of glycolysis (Buck et al., 2016).

Unlike proinflammatory T cells, Tregs are immunosuppressive, promoting anti-inflammatory activity. Identified by the cell surface expression of CD25 and the lineage-defining master transcription factor Foxp3, these cells produce an antiinflammatory cytokine TGF-β, a homeostatic regulator, and a series of interleukins, such as IL-10 and IL-35, to control IFN-γ production in T-effector cells (Schmidt et al., 2012; Wei et al., 2017). In order to function efficiently, Tregs synthesize large amounts of AMPK, which control genes involved in energy yielding processes such as fatty acid oxidation (Sojka et al., 2008; Lee and Kim, 2010). Among these is the gene that encodes carnitine palmitoyl transferase (CPT-1), which mediates the influx of fatty acids into the mitochondria (Rinaldo et al., 2002).

Regulators of T cell metabolism

Metabolic remodeling is a critical process in CD4 and CD8 T cell activation. Thus regulation of glycolytic genes including *SLC2A1*, which encodes Glut1, ensures the transition from a quiescence state to T cell expansion and effector functions. Trafficking of Glut1 to the cell surface is coordinated by the phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K-/AKT-/mTOR) axis (Palmer et al., 2015).

Activation of the PI3K-/AKT-/mTOR axis creates a metabolic imbalance towards aerobic glycolysis required for T cell differentiation and function and is controlled by expression of genes that regulate functionally distinct mTOR complexes known as mTORC1 and mTORC2 (Patsoukis et al., 2017). T cells that are unable to express these complexes have enhanced CD8 memory formation and a metabolic phenotype dominated by FAO and elevated AMPK activity (Patsoukis et al., 2017). Although Glut1 cell surface trafficking is regulated posttranslationally in part by the PI3K-/AKT-/mTOR axis, in CD4 T cells, Glut1 expression is tightly coordinated by the ribosomal machinery. Naïve CD4 T cells accumulate untranslated mRNAs that encode key enzymes involved in glycolysis and fatty acid synthesis, providing the translational machinery to position them in a "ready to be activated" state for rapid protein synthesis (Ricciardi et al., 2018). Thus when cued for activation Glut1 is synthesized to facilitate glucose uptake.

Mitogen-activated protein kinase (MAPK) is another important regulator of glucose metabolism in T cells and promotes the stability of hypoxia-inducible factor 1-alpha (HIF-1α), a transcription factor that regulates many glycolytic genes (Seagroves et al., 2001). Increased expression of these metabolic factors generally accompanies glycolysis (Seagroves et al., 2001; Doedens et al., 2013) of which lactate is an end product even in the presence of sufficient oxygen, a metabolic phenomenon called the Warburg effect (Wang et al., 2016). Other critical regulators of T cell activation and differentiation include T-bet, Bcl-6, and p70S6K, which have been comprehensively reviewed (Palmer et al., 2016a).

T cell metabolism in diseases

T cells have remarkable metabolic plasticity that enables them to reprogram metabolism upon sensing environmental and metabolic signals such as changes in oxygen concentrations, inflammatory cytokines, adipokines, and metabolites. This process requires the engagement of molecular modules called "metabolic checkpoints" that allow them to transduce messages to evoke effector responses. These modules represent potential therapeutic targets to treat a host of inflammatory diseases such as obesity, autoimmune diseases, cancer, or as host-directed treatments for infections.

Obesity

Obesity has become a worldwide health problem with significant ramifications for health span and health-care costs. The adipose tissue is not simply a fat depot but is considered a biologically active organ. Adipose tissue secretes bioactive molecules known as adipokines, which have been found to impact the inflammatory status of T cells (Aguilar and Murphy, 2018). Prolonged nutrient overload and obesity result in the release of leptin, a cytokine that promotes T cell glycolysis and effector T cell differentiation (Gerriets and MacIver, 2014). Leptin stimulates glucose uptake by encouraging Glut1 expression and trafficking to the T cell surface. These metabolic changes are abrogated when T cells are challenged with small interfering RNA targeting the leptin receptor, ObR (Han et al., 2018). This highlights a strong link between leptin signaling and glucose metabolism.

Upon activation, ObR expressed at the surface of T cells binds leptin released by adipocytes in the blood and triggers glycolysis (Han et al., 2018). Controlling leptin levels by fasting decreases expression of the critical glycolytic enzyme hexokinase 2 (HK2) in effector T cells (Gerriets et al., 2016). Obesity is generally associated with an upregulation of leptin, proinflammatory cytokines, and chemokines within the adipose tissue (Godfrey et al., 2019). The inflammatory milieu of the adipose environment is exacerbated by the recruitment of activated CD8 and CD4 T cells and IL-17-expressing inflammatory T cells (Alwarawrah et al., 2018). Experiments utilizing mice models have shown that leptin induces proliferation of highly glycolytic CD4 T cells, while leptin restriction through fasting increases proliferation of Tregs and attenuates IFN-γ and IL-17 production by inflammatory T cells (Gerriets et al., 2016). Conversely, adiponectin, an insulin sensitizing adipokine, has positive immunomodulatory effects in diseases and may improve the immune response against viruses such as hepatitis C. The adiponectin receptor 1 (AdipoR1) is expressed on T cells, but it is unknown whether it is involved in glucose metabolism in T cells (Palmer et al., 2008).

The development of chronic low-grade inflammation in obese individuals is multifactorial, resulting from both higher glycolysis in effector cells and higher fatty acid oxidation in memory cells. Obesity has been shown to result in the depletion of naïve CD4 T cells and elevated levels of effector memory-like CD4 T cells. Further, palmitate, a proinflammatory fatty acid, induces PI3K-AKT activation in CD4 T cells. Inhibition of the p110δ subunit of the PI3K and fatty acid oxidation corrects the bias towards the development of the proinflammatory effector memory CD4 T cells supporting the concept of modulating T cell metabolism for translational benefits (Mauro et al., 2017).

Autoimmune disease

Self-reactivity and dysregulated recognition of host tissues is caused by chronic activation of T cells that target host tissues (Raphael et al., 2015; Ding et al., 2016; Prinz, 2018). Synovial fluid from psoriatic arthritis and rheumatoid arthritis patients is enriched with IL-9 producing T cells, and IL-9 by itself can induce PI3K-/AKT-/mTOR-dependent T cell activation (Kundu-Raychaudhuri et al., 2016). In experimental autoimmune encephalomyelitis, T cell activation and differentiation into proinflammatory Th1 and Th17 cells is attenuated by vitamin D supplementation in a PI3K-/AKT-/mTOR-dependent manner. However, the Jak/Stat and Erk/MAPK pathways are also implicated in the vitamin D effect (Zeitelhofer et al., 2017).

The proliferation of antigen-specific self-targeting effector T cells and the upregulation of mTOR RNA imply a link with the mTOR pathway (Ding et al., 2016). In the murine autoimmune disease model K/BxN (KBN), CD4 T cells exhibit high glycolysis and mTOR signaling (Abboud et al., 2018). Furthermore, when glycolysis is inhibited with 2-deoxy-D-glucose (2DG), joint inflammation is shown to be reduced (Abboud et al., 2018) along with the glycolytic enzyme enolase-1 (De Rosa et al., 2015). Suppression of glucose metabolism in follicular helper (T_{FH}) CD4 T cells in a mouse model of systemic lupus erythematosus had favorable immunological effects perhaps by eliminating autoreactive T_{FH} cells (Choi et al., 2018).

Cancer

As the cancer tumor microenvironment is a common site for nutrient deprivation, local T cells may be starved of nutrients needed for their survival and function (Mockler et al., 2014; Jiang and Yan, 2016). Within the tumor environment, CD8 T cells are able to detect glucose deprivation via the energy sensor AMP-activated protein kinase (AMPK) (Rolf et al., 2013). Production of the AMPK subunit AMPKα1 enforces quiescence in T cells to limit activation and differentiation; a metabolic feature also shared by memory T cells (Rolf et al., 2013; Blagih et al., 2015).

As a form of immune system dampening, some cancers can impose nutrient deprivation on T cells by depleting glucose and glutamine from the local environment and attenuating release of cytokines, such as IFN-γ that would normally kill cancer cells (Chang et al., 2015) (Fig. 3). To combat the dysfunctional T cells within the tumor microenvironment, checkpoint blockade therapy has evolved as a promising therapeutic strategy. These blockade therapies that utilize monoclonal antibodies to specifically target coinhibitory ligands can improve T cell metabolism and reinvigorate T cells (Scharping and Delgoffe, 2016).

HIV infection

Remodeling of T cell metabolism is a hallmark of viral infections. The persistence of viruses in humans relies on their ability to evade the innate and adaptive immune system, bypassing the mechanisms designed to detect and eliminate them. The persistence of viral infections further compromises the immune system as a result of immune exhaustion and chronic inflammation, which often compromise other organs such as the liver, heart, kidney, lungs, and brain.

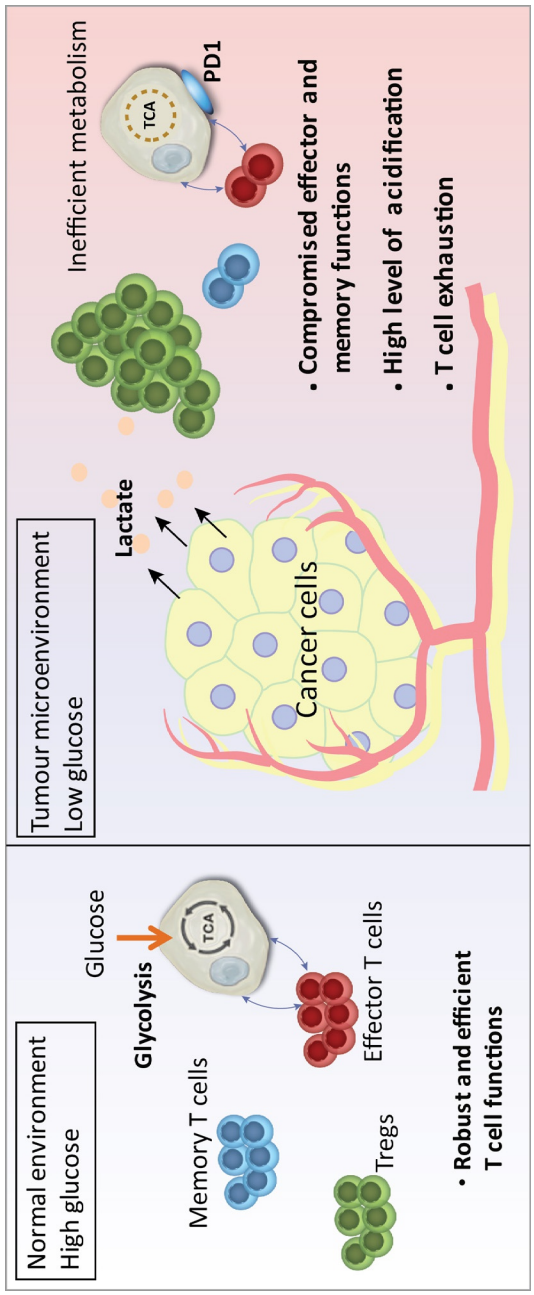

FIG. 3 T cell functions within the tumor microenvironment.

As of 2017 an estimated 37 million people are living with HIV of which 22 million are on life saving antiretroviral treatment. The primary cellular targets of HIV are metabolically active CD4 T cells that have increased cell surface Glut1 (Palmer et al., 2014; Palmer et al., 2017). In HIV+ persons the percentage of CD4 T cells that express Glut1 are inversely correlated with peripheral blood CD4 T cell counts, regardless of whether the individual is virologically suppressed on antiviral therapy (Palmer et al., 2014).

The term "T cell metabolic exhaustion" was coined by Palmer and colleagues to explain the strong relationship between high CD4 T cell glycolytic activity and their high activation and exhaustion status in HIV+ persons (Palmer et al., 2016b; Shehata et al., 2017). HIV infection itself can induce metabolic reprogramming of CD4 T cells towards a proinflammatory glycolytic phenotype (Kavanagh et al., 2018). Furthermore, CD4 T cells from virologically suppressed HIV+ persons on antiretroviral therapy have high PI3K-mTOR activity, marked by elevated OX40 cell surface expression (Palmer et al., 2017). These cells have a very high metabolic activity, which increases their permissiveness to HIV infection. Suppressing glycolysis using an inhibitor of the PI3K isoform p110γ abrogates CD4 T cell infection in cell culture (Palmer et al., 2017). In a hyper metabolically active state and under the influence of reactive oxygen species, CD4 T cell can release extracellular vesicles, which may drive HIF1-α-dependent metabolic and inflammatory responses in monocytes and macrophages via a paracrine effect. This proinflammatory response underpins the development of age-associated comorbidities in HIV+ persons on antiretroviral treatment (Duette et al., 2018; Palmer et al., 2018; Aounallah et al., 2016). The metabolic status of CD4 T cells in HIV+ persons may be partly responsible for the persistence of the HIV reservoir, which has impeded the quest for an elusive cure, (Palmer et al., 2017; Valle-Casuso et al., 2019).

Memory CD4 T cells are the major HIV reservoir cells, and homeostatic proliferation of these cells is a key mechanism by which the HIV reservoir persists in HIV+ persons on effective antiretroviral therapy (Chomont et al., 2009). Memory CD4 T cells that contain integrated HIV DNA, historically described as "resting," are now shown to be metabolically active (Palmer et al., 2014). In this regard the field of immunometabolism has enabled the conceptualization of a novel HIV cure strategy "starving the HIV reservoir," which aims to target the metabolic machinery of HIV reservoir CD4 T cells to suppress homeostatic proliferation and decay the viral reservoir (Palmer et al., 2018). Such a strategy may normalize immune cell metabolism, reverse exhaustion and improve antiviral functionality: recipes for a functional HIV cure or posttreatment control. Since these metabolic processes are coordinated by a myriad of metabolic players and nodes, only combination therapy directed at multiple pathways (glycolysis, hexosamine biosynthesis, and glutaminolysis) and interrogation of mitochondrial dynamics in reservoir cells are likely to be successful at eradicating the reservoir.

Immunotherapeutics

Metabolic dysregulation in immune cells is central to our understanding of inflammatory diseases mediated by prolonged activation of T cells. Therefore targeting the pathways that regulate metabolism in T cells is of considerable commercial interest in the search for host-based therapeutics. Existing drugs used in oncology and transplantation medicine such as

everolimus, ridaforolimus, and temsirolimus can inhibit mTOR and are being used at low concentrations in clinical trials to normalize immune cell metabolism and to improve immunity (Sivendran et al., 2014; Routy et al., 2019). In fact, low-dose combination of mTOR inhibitors does improve immune functions and reduce infections in the elderly (Mannick et al., 2018). Upstream regulators of mTOR such as PI3K and AKT are also targets of therapeutic intervention to treat chronic illnesses and more specifically the PI3K isoforms that are restricted to T cells (Janku et al., 2018).

Inhibition of mTOR in mice using sirolimus in combination with other upstream inhibitors effectively diminishes effector CD4 T cell proliferation and cytokine production while relieving symptoms associated with autoimmune diseases such as arthritis, Crohn's disease, ulcerative colitis, ischemic colitis, allergic reactions, and microscopic colitis (Lin et al., 2012; Wu et al., 2013).

CD4 T cell activation may be attenuated by the glucose analogue and glycolytic inhibitor 2DG in combination with metformin (Shi et al., 2011; Yin et al., 2015; Yin et al., 2016). However, many metabolic inhibitors including 2DG have off-target effects, affecting other auxiliary metabolic pathways such as hexosamine biosynthesis (Shehata et al., 2017). Other approaches include indirect inhibition of mTOR with drugs such as metformin, an AMPK activator used to treat type 2 diabetes. Metformin increases Treg differentiation in mouse models for obesity (Kim et al., 2016) and cancer (Pereira et al., 2018) and is in human clinical trials to treat metabolic syndrome and multiple sclerosis (Negrotto et al., 2016). Other potential targets include nutrient transporters such as Glut1 and enzymes involved in rate limiting steps in nutrient metabolism. A list of metabolic drugs and natural products that modulate cellular metabolism, some of which may be exploited for clinical use, are shown in Table 2. The limited clinical benefits demonstrated with many antiglycolytic natural products may relate to their unfavorable bioavailability and pharmacokinetics. New formulations or chemical modifications such as nanoparticle delivery may be required for optimal benefits.

TABLE 2 Drugs and natural molecules that inhibit key glucose metabolic pathways

Function	Therapy
PI3K inhibitors	Alpelisib (BYL719) ATU027 AZD6482 Buparlisib (BKM120) CH5132799 Copanlisib (BAY80-6946) CUDC-907 Duvelisib Idelalisib (CAL101) GSK2636771 PX-866 Pictilisib (GDC-0941) Pilaralisib (SAR245408) Puquitinib mesylate

TABLE 2 Drugs and natural molecules that inhibit key glucose metabolic pathways—Cont'd

Function	Therapy
	Seletalisib Taselisib
PI3K-/mTOR inhibitors	Apitolisib (GDC-0980) BGT226 Dactolisib (BEZ235) Gedatolisib (PF-05212384) PF-04691502 SF1126 Voxtalisib (XL765) Omipalisib (GSK2126458, GSK458) XH002
AKT inhibitors	Afuresertib (GSK2110183) Archexin (RX-0201) AZD5363 GSK690623 Ipatasertib (RG7440, GDC0068) MK-2206 PBI-05204 Perifosine (KRX-0401) Triciribine phosphate Uprosertib (GSK2141795) Ipatasertib (RG7440, GDC0068) LY2780301
mTOR1 inhibitors	Everolimus (DB01590) Ridaforolimus (Deforolimus) Sirolimus (Rapamycin) Temsirolimus
mTOR1/mTOR2 inhibitors	AZD8055 CC-223 OSI-027 Sapanisertib (INK128) Vistusertib (AZD2014) TAK-228
Glut1 inhibitors	2DG Fasentin Phloretin STF-31 WZB117
Hexokinase inhibitors	3-Bromopyruvate Genistein-27 Benserazide Lonidamine

Continued

TABLE 2 Drugs and natural molecules that inhibit key glucose metabolic pathways—Cont'd

Function	Therapy
AMPK activators	Metformin A-769662 PF-06409577 PF-249 MT63-78 GSK621 MK8722 PF739
Natural PI3K-/AKT-/mTOR inhibitors	Apigenin Curcumin Cryptotanshinone Fisetin Deguelin Genistein Quercetin Resveratrol Tocotrienol

Conclusion

Biochemical pathways that regulate glucose metabolism in T cells are intricately connected to T cell activation, growth, differentiation, and effector functions. Dysregulated immune cell metabolism characterizes cancers, autoimmune diseases, and infections. Metabolic reprogramming of T cells while fulfilling the host response to control pathogens has deleterious consequences for host cells and impacts disease pathogenesis. An understanding of these processes in the settings of infectious diseases and in noncommunicable conditions such as obesity, diabetes, kidney, and cardiovascular diseases and cancers will inform novel therapeutic strategies to treat them.

Glossary

Glucose metabolism This is a series of biochemical processes by which glucose is taken up by cells and utilized to make cell biomass or is broken down to produce ATP. The two main processes by which glucose is broken down are glycolysis and oxidative phosphorylation.

Glutaminolysis A biochemical pathway that breaks down the amino acid glutamine into glutamate, and subsequently α-ketoglutarate, by glutaminase to fuel the tricarboxylic acid cycle (TCA), allowing cells to maintain a degree of oxidative phosphorylation. This usually occurs when glucose is diverted towards glycolysis during T cell activation or cellular stress.

Glycolysis This is the first and major pathway in glucose metabolism. It breaks down glucose to form pyruvate, which is metabolized to lactate under anaerobic conditions. Intermediates produced during this process are used to make amino acids, lipids, and nucleic acids for biomass production. Glycolysis produces a net of 2 ATPs.

Hexosamine biosynthesis An auxiliary pathway that utilizes glucose-derived fructose-6-phosphate, made in the glycolytic pathway to produce key substrate (uridine diphosphate-*N*-acetylglucosamine: UDP-GlcNAc) for protein glycosylation, an important posttranslational modification required for T cell proliferation.

Immunometabolism This is the field that seeks to understand how fundamental metabolic machineries of immune cells affect biological processes such as differentiation and effector functions. It also describes how whole body metabolic homeostasis impacts immune cell functions, generally in metabolic diseases such as obesity and diabetes.

Metaboglycomics Integrative study of metabolism and glycomics to understand how nutrient metabolism influences sugar modification of macromolecules such as proteins, which affect their localization, stability, and or functions.

Metabolic reprogramming This is a process that refers to a switch from oxidative phosphorylation to glycolysis or vice versa or an imbalance towards one or the other. This process may also include other auxiliary pathways such as glutaminolysis or fatty acid oxidation.

Oxidative phosphorylation This is the process by which glucose is broken down completely to produce ATP via the TCA cycle and the electron transport chain in the mitochondria, normally under sufficient oxygen. The net amount of ATP molecules produced by this process is being revised from 36 to 30–32.

T cell metabolic exhaustion A term coined based on the observation of an inverse relationship between CD4 T cell glycolytic activity and their cell count in HIV+ persons. CD4 T cells with higher glycolysis also express higher levels of senescence and aging markers.

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CHAPTER

9

Liquid fructose and liver insulin signaling: Molecular mechanisms controlling hepatic steatosis

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OUTLINE

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SUMMARY POINTS

- Most clinical studies suggest that the consumption of high amounts of fructose reduces the insulin-mediated suppression of hepatic glucose production.
- In contrast, studies performed in animal models show a preserved hepatic response to insulin, involving a reduction in hepatic glucose output.
- Regarding liver fat content, the results of clinical studies are not conclusive, whereas studies in rodents show the importance of the duration of the sugar supplementation period.
- Our short-term studies in female rats show hepatic steatosis that arises from the activation of the ChREBP-KHK and mTORC1-SREBP-1c axes, together with fatty acid β-oxidation inhibition.
- However, in the context of chronic fructose supplementation, these mechanisms may be compensated by the selective activation of IRE-1, resulting in the absence of hepatic steatosis.
- The detrimental consequences of high sugar intake are not only a matter of calories, as our studies in rodents show specific detrimental effects of fructose, compared with glucose, under isocaloric conditions.

Key facts

Key facts on hepatic insulin signaling

- The insulin signaling cascade is initiated by its binding to the insulin receptor and its autophosphorylation, followed by phosphorylation of insulin receptor substrate (IRS) molecules. This activates two pathways: the phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathway.
- PI3K activation leads to the phosphorylation of phosphatidylinositol diphosphate to phosphatidylinositol triphosphate (PIP_3). PIP_3 stimulates the activity of 3-phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates Akt at Thr308. To be fully activated, Akt must also be phosphorylated by the mammalian (mechanistic) target of rapamycin complex-2 (mTORC2) at Ser473.
- Once activated, Akt controls carbohydrate and lipid metabolism through interaction with several targets: (i) inhibits by phosphorylation peroxisome proliferator-activated receptor α coactivator 1 (PGC1α) and forkhead box protein O1 (FoxO1) and, as a consequence, downregulates the expression of the key gluconeogenic genes glucose-6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK), thus reducing hepatic glucose production; (ii) inhibits glycogen synthase kinase 3 β (GSK3β), and as a consequence the inhibition of glycogen synthase (GS) is released, and therefore glycogen synthesis is enhanced; (iii) by direct and indirect mechanisms (through mTORC1), it activates the transcription factor sterol response element–binding protein 1-c (SREBP1-c) to upregulate key lipogenic enzymes thus promoting hepatic de novo lipogenesis (DNL).

Key facts on hepatic insulin resistance

- Reduced sensitivity of insulin-target tissues to the actions of insulin is a hallmark of several metabolic derangements such as obesity, prediabetes, and type 2 diabetes.

- Insulin resistance in white adipose tissue increases lipolytic activity, the subsequent release of free fatty acids to the plasma, and its hepatic uptake. This results in higher substrate availability for triglyceride synthesis and therefore can contribute to hepatic fat accumulation and nonalcoholic fatty liver disease (NAFLD).
- An insulin-resistant liver can further lead to triglyceride accumulation due to selective insulin resistance in this tissue. This means that insulin signaling through FoxO1 is impaired, so that gluconeogenic gene expression is not reduced, but at the same time, insulin signaling through the mTORC1-SREBP-1C axis is preserved, and therefore hepatic de novo lipogenesis is not suppressed.
- In addition, most studies in rodents and humans point to NAFLD as a causal agent of hepatic insulin resistance, through the buildup of diacylglycerol and ceramides (which activate atypical protein kinases Cε and ζ), the activation of proinflammatory pathways, or the altered secretion of hepatokines.

Key facts on fructose and fructose metabolism

- Fructose provides the same amount of calories as glucose, but it has higher sweetening power and a lower glycemic index than glucose and does not induce insulin secretion.
- In contrast to glucose, fructose is mainly metabolized in the liver, where it induces its own metabolism by increasing the expression of the enzyme fructokinase. Moreover, fructose hepatic metabolism bypasses the main rate-controlling enzyme of glycolysis, phosphofructokinase.
- Therefore fructose has a higher lipogenic potential than glucose: a high flux of fructose to the liver results in a marked increase in lipogenesis, synthesis of very-low-density lipoproteins, and hypertriglyceridemia.

Introduction

Fructose, a monosaccharide, is one of the components of the two main sweeteners used by the food and drink industry: high-fructose corn syrup (HFCS, which contains between 42% and 55% of fructose, and is used mainly in the United States) and sucrose (containing 50% glucose and 50% fructose, preferred in European countries). In the recent decades, there has been a change in dietary patterns toward the consumption of hypercaloric diets that are rich in processed foods and sugar-sweetened beverages (SSB) containing sucrose or HFCS. As a result, the consumption of fructose has increased dramatically: estimates from the Nationwide Food Consumption Survey (NFCS) and the National Health and Nutrition Examination Survey (NHANES) showed that fructose intake in the United States increased from 37 g/day in 1978 to 49 g/day in 2004 (Marriott et al., 2009). The increase in fructose intake has largely been attributed to the elevated consumption of SSB such as soft, energy, and fruit drinks, which are currently the main source of added sugars in the diet (Malik and Hu, 2015). SSB consumption is slowing down overall in North America, Australia, and Western Europe, but at the same time, it is increasing in low- and mid-income areas (Popkin and Hawkes, 2016). Moreover, despite the reduction observed in some countries, the consumption of sugars in the United States continues to exceed the recommendations from the U.S. Department of Health and Human Services and U.S. Department of Agriculture (2015), which suggests that added sugars should not exceed 10% of total energy intake; even

more restrictive, the guidelines from the World Health Organization (WHO) recommend that added sugars should account for at most 5% of total calorie consumption (Rippe and Angelopoulos, 2016).

Concerns about excessive contents of added sugars in the diet derive from observations that the rise in their consumption has been paralleled by an increased prevalence of cardiometabolic alterations including obesity, insulin resistance, and nonalcoholic fatty liver disease (NAFLD) (Dekker et al., 2010). Moreover, it has been recognized that consuming sugars in liquid form, that is, fructose in SSBs, is more harmful for human health than their consumption in solid form, as the ingestion of liquid carbohydrates does not elicit a proportional compensatory reduction in food intake, resulting in a positive energy balance (DiMeglio and Mattes, 2000). However, the detrimental consequences of high SSB intake do not seem to be only a matter of calories but may also depend on the type of sugar providing these calories, as it has been shown that not all of the carbohydrates are equal regarding their metabolic effects (Schaefer et al., 2009; Stanhope et al., 2009). In this review, we discuss the role of liquid fructose in the development of hepatic insulin resistance and fatty liver, summarizing the evidence obtained in recent epidemiological and intervention studies in humans, as well as from studies performed in animal models, in order to define the molecular mechanisms potentially involved.

Clinical studies on fructose consumption and hepatic insulin resistance

Insulin is a pleiotropic hormone that regulates lipid and carbohydrate metabolism, as well as energy homeostasis, through a signaling cascade in target tissues (mainly the adipose tissue, skeletal muscle, and liver, Fig. 1). The disruption of insulin signaling, leading to reduced sensitivity of insulin-target tissues to the actions of insulin, is a hallmark of several metabolic derangements such as obesity, prediabetes, and type 2 diabetes (T2D) (Bazotte et al., 2014). Insulin resistance in white adipose tissue causes, among other effects, increased lipolytic activity, the subsequent release of free fatty acids from this tissue to the plasma, and their uptake by the liver leading to hepatic steatosis (Fig. 2).

Since our last reviews about the effects of fructose on liver metabolism (Alegret et al., 2011; Rebollo et al., 2012), several studies have been performed in humans to investigate the effects of excessive fructose consumption on insulin sensitivity (Table 1). One of these studies (Aeberli et al., 2013) was specifically designed to assess the effects of SSB consumption using the gold standard method to evaluate hepatic insulin sensitivity, the euglycemic-hyperinsulinemic clamp. The study was performed in young, normal weight males, who received beverages sweetened with fructose (40 or 80 g/day), glucose, or sucrose (80 g/day) for 3 weeks. The results showed that the hepatic suppression of glucose production during the clamp was significantly lower after drinking the high-fructose sweetened beverage compared with the group consuming the same amount of glucose. However, the glucose clearance rate did not differ significantly between groups. These results indicate that the consumption of fructose in liquid form, even for short periods of time, impairs hepatic insulin sensitivity to a greater extent than glucose-sweetened beverages, whereas whole-body insulin sensitivity (mainly related to skeletal muscle glucose uptake), is not affected. In contrast, a study performed in healthy but centrally overweight men (Johnston et al., 2013) showed that

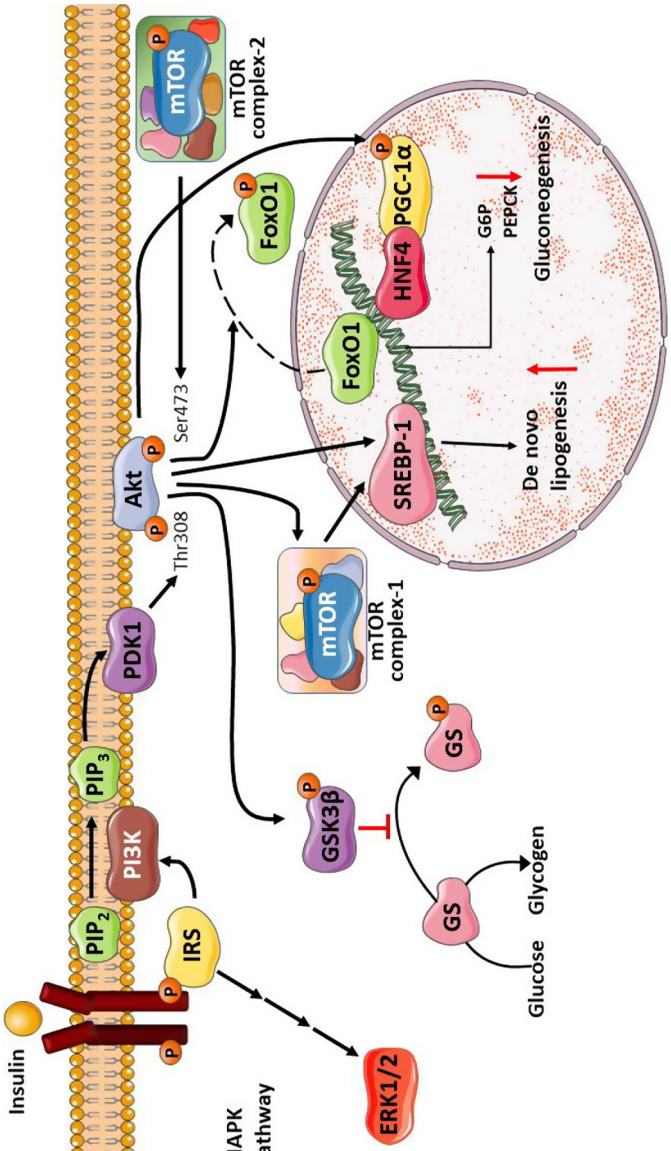

FIG. 1 Insulin signaling in the liver. The molecular events occurring in the hepatocyte after the binding of insulin to its receptor are shown. As a final result of this cascade of events, hepatic gluconeogenesis is repressed, and de novo lipogenesis is induced. *Akt*, v-Akt murine thymoma viral oncogene homologue/protein kinase B; *ERK*, extracellular signal-regulated kinase; *FoxO1*, forkhead box protein O1; *G6P*, glucose-6-phosphatase; *GS*, glycogen synthase; *GSK3β*, glycogen synthase kinase 3β; *HNF4*, hepatic nuclear factor 4; *IRS*, insulin receptor substrate; *mTOR*, mammalian (mechanistic) target of rapamycin; *PDK1*, 3-phosphoinositide-dependent kinase 1; *PEPCK*, phosphoenolpyruvate carboxykinase; *PGC-1α*, peroxisome proliferator-activated receptor γ coactivator 1; *PI3K*, phosphoinositide 3-kinase; PIP_2, phosphatidylinositol diphosphate; PIP_3, phosphatidylinositol triphosphate.

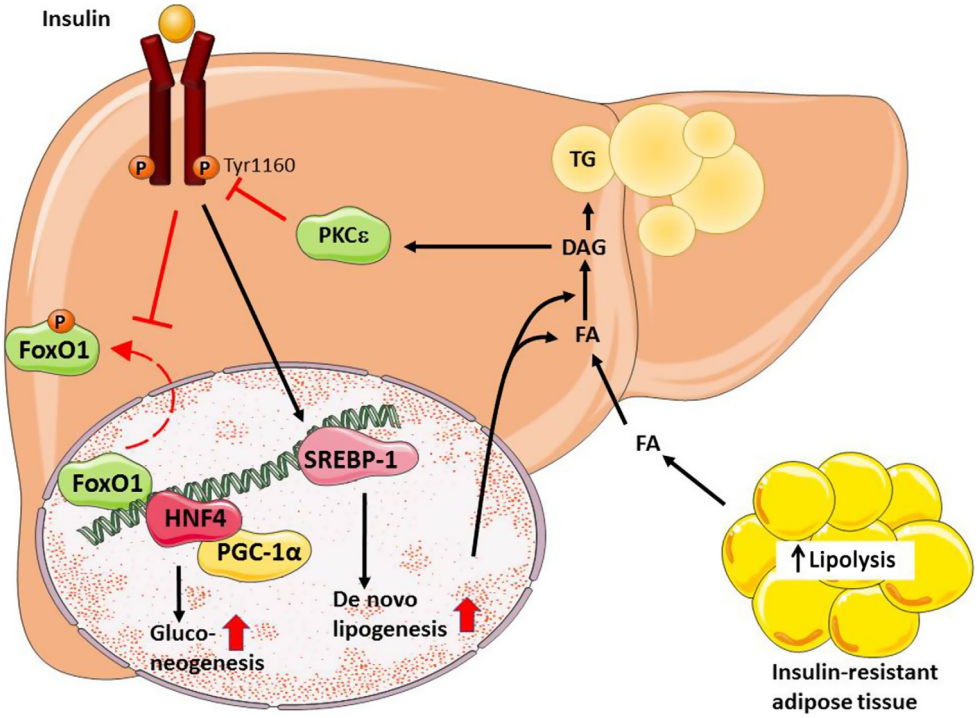

FIG. 2 Hepatic insulin resistance and steatosis. This figure depicts the potential links between insulin resistance and hepatic lipid accumulation. Increased fatty acid flux from adipose tissue to the liver may lead to the accumulation of lipid species, such as diacylglycerol, which activate atypical protein kinases (protein kinase Cε) to disrupt hepatic insulin signaling. As hepatic insulin resistance is pathway selective (meaning that gluconeogenesis is not repressed but de novo lipogenesis is increased), the disruption of insulin signaling also contributes to hepatic lipid deposition. *DAG*, diacylglycerol; *FA*, fatty acid; *FoxO1*, forkhead box protein O1; *HNF4*, hepatic nuclear factor 4; *PGC-1α*, peroxisome proliferator-activated receptor ɣ coactivator 1; *PKC*, protein kinase C; *SREBP-1*, sterol regulatory element-binding protein-1; *TG*, triglycerides.

HOMA-IR, a parameter that reflects whole-body insulin resistance, was higher after 2 weeks of consumption of liquid fructose compared with liquid glucose (both providing 25% of total energy intake). However, when euglycemic-hyperinsulinemic clamps were performed in a subgroup of participants, no differences were observed between the glucose and fructose groups regarding the suppression of hepatic glucose production. The reasons for the discrepancies between these two studies are not clear, as their duration was similar (2 and 3 weeks), but the study by Johnston et al. points to the possibility of intestinal malabsorption due to the high amount of fructose used (Johnston et al., 2013). More recently, Schwarz et al. showed that in healthy men who received isocaloric diets, one with 25% of their energy requirements as fructose and the other with complex carbohydrates instead of fructose, for 9 days, endogenous glucose production was only increased in the fructose group (Schwarz et al., 2015).

To shed some light on these conflicting results, Lecoultre et al. analyzed several short-term studies (6–7 days) performed in healthy young men who received diets containing different amounts of fructose or glucose (Lecoultre et al., 2013). The results of this analysis showed that

TABLE 1 Clinical studies that examined the effects of fructose or fructose-containing sweeteners on hepatic insulin resistance and related parameters (since 2013)

Author (year)	Type of study	Patients	Intervention	Main results
Aeberli et al. (2013)	Double blind, randomized, crossover	Normal weight males 21–25 years ($n = 9$)	3 weeks, F at 40 g and 80 g/day, G or S at 80 g/day	Hepatic suppression of glucose production during EH clamp lower after F than G
Johnston et al. (2013)	Randomized, parallel, double blind	Centrally overweight males 18–50 years ($n = 32$)	Two intervention periods of 2 weeks, F or G (25% of energy requirements). Initial period isocaloric, 6-week washout, final period hypercaloric	Higher increase in HOMA-IR during isocaloric period after F than G. Hepatic insulin resistance and whole-body glucose disposal unaltered by interventions during EH clamp (subset of $n = 12$)
Lecoultre et al. (2013)	Analysis of data collected in several studies	Healthy males, mean age 22.5 years ($n = 55$)	6–7 days, weight-maintenance diet (control) or overfeed with F (1.5, 3, or 4 g/kg/day) or G 3 g/kg/day	F (3 and 4 g/kg/day) reduced the hepatic insulin sensitivity index. F (4 g/kg/day) and G (3 g/kg/day) increased HGP
Lecoultre et al. (2014)	Randomized, crossover	Healthy males, mean age 23 years ($n = 10$)	6 days, weight-maintenance diet (control) or overfeed with F (4 g/kg/day) ± three types of coffee	F group (without coffee) showed higher rates of HGP compared with the control group
Lowndes et al. (2015)	Randomized, parallel, partially blinded	Men and women (normal weight, overweight, and obese) 20–60 years ($n = 156$)	10 weeks, unsweetened milk, milk sweetened with F or G (9% of weight-maintenance EI), milk sweetened with HFCS or S (18% of weight-maintenance EI)	Fasting glucose and insulin concentrations and HOMA index unaltered by sweeteners
Schwarz et al. (2015)	Randomized, crossover	Healthy males, 18–65 years ($n = 8$)	9 days, two isocaloric diets with 50% of total EI as CH, one of them with F providing 25% of total EI and another with F providing 5% of total EI	HGP during hyperinsulinemia greater with the high-F diet
Lin et al. (2016)	Cross sectional	Adolescents, both genders, 12–16 years ($n = 1454$)		Consumers of >350 mL/day HFCS-containing SSB had higher HOMA-IR values than SSB nondrinkers
Lustig et al. (2016)	Within-subject intervention with repeated measures	Latino and African-American adolescents, both genders, 12–18 years ($n = 43$)	9 days, dietary sugar reduced from 28% to 10% and substituted with starch (isocaloric)	Fasting plasma glucose and insulin and respective AUC during the OGTT significantly reduced

Continued

TABLE 1 Clinical studies that examined the effects of fructose or fructose-containing sweeteners on hepatic insulin resistance and related parameters (since 2013)—Cont'd

Author (year)	Type of study	Patients	Intervention	Main results
ter Horst et al. (2016)	Metaanalysis	46 clinical trials, nondiabetic adults normal weight, overweight, or obese ($n = 1005$)	Short-term F consumption, in isocaloric exchange or in hypercaloric supplementation	F induces hepatic insulin resistance under isocaloric and hypercaloric conditions
Matikainen et al. (2017)	Randomized, parallel, open label	Obese males ($n = 66$), mean age 48 years	12 weeks, F (75 g/day) + ad libitum diet	F did not change responses to OGTT, nor fasting HOMA-IR compared with baseline

Abbreviations: *AUC*, area under the curve; *EH*, euglycemic hyperinsulinemic; *EI*, energy intake; *F*, fructose; *G*, glucose; *HFCS*, high-fructose corn syrup; *HGP*, hepatic glucose production; *HOMA-IR*, homeostasis model assessment of insulin resistance; *n*, number of participants that completed the study; *OGTT*, oral glucose tolerance test; *S*, sucrose; *SSB*, sugar-sweetened beverages.

fructose at the highest concentrations (3 and 4 g/kg/day) reduced the hepatic insulin sensitivity index. Moreover, both fructose (4 g/kg/day) and glucose (3 g/kg/day) increased hepatic glucose production. This dosage of 4 g/kg/day of fructose for 6 days was used in a randomized controlled study performed in healthy men, designed to evaluate the effects of coffee consumption on the adverse metabolic effects induced by fructose (Lecoultre et al., 2014). Regardless of the effects of coffee, it is remarkable that the group receiving fructose showed higher rates of hepatic glucose production than the control group. However, it must be taken into account that these studies, which clearly point to fructose as a causal agent for hepatic insulin resistance (Lecoultre et al., 2013, 2014), were performed using extremely high-fructose doses, which may have increased the total energy intake by around 140% of the typical requirements for weight maintenance.

Other studies have been performed with lower fructose doses, thus assessing the effects of this sugar in a more realistic setting. For example, a randomized prospective study was designed to provide an amount of fructose equivalent to the 50 percentile of fructose consumption in the United States (9% of total caloric intake) (Lowndes et al., 2015). Participants (men and women aged 20–60, normal weight, overweight, and obese) received unsweetened low-fat milk (control) or low-fat milk with added HFCS, sucrose, fructose, or glucose for 10 weeks. None of the added sugars altered fasting glucose or insulin concentrations or the HOMA index, compared with the control group (Lowndes et al., 2015). A possible confounding factor is that the vehicle used in this study was milk and it has been suggested that consumption of milk and other dairy products has a protective effect against several components of the metabolic syndrome, including insulin resistance (Astrup, 2014). Thus, the consumption of sugars in this vehicle may have masked any possible deleterious effects on glucose metabolism.

Children and adolescents seem to be particularly prone to high consumption of added sugars in the form of SSB (Popkin, 2010), a trend that has been related to metabolic alterations, including insulin resistance (Bremer et al., 2010). A cross-sectional study to examine the relationship between SSB consumption, assessed through dietary questionnaires, and

metabolic alterations in adolescents (Lin et al., 2016) showed that the consumption of more than 350 ml/day of beverages containing HFCS is linked to higher indices of insulin resistance (HOMA-IR values). However, due to the study design, a causal relationship could not be established. In contrast, intervention studies in which food consumption is altered in a controlled way can demonstrate causality. Lustig et al. performed a study in children with obesity and metabolic syndrome in which the intervention consisted in reducing the sugar content of their diet from 28% to 10% and substituting it with starch, to maintain the same percentages of fat, protein, and total carbohydrates as in the basal diet self-reported by the participants (Lustig et al., 2016). An oral glucose tolerance test (OGTT), used to predict insulin sensitivity when euglycemic-hyperinsulinemic clamps are difficult to carry out (Stumvoll et al., 2000), was performed at the beginning and at the end of an intervention period of 9 days. The results showed that fasting plasma glucose and insulin, as well as the respective area under the curves (AUC) during the OGTT, were significantly reduced by the intervention, suggesting improved glucose tolerance and insulin sensitivity (Lustig et al., 2016). In contrast, a recent study performed in obese males who received 75 g of fructose/day for 12 weeks, showed that the response to an OGTT was not affected by the intervention (Matikainen et al., 2017).

A limitation of most studies conducted in humans to assess the effects of fructose on metabolic outcomes is the low numbers of participants involved. A recent metaanalysis included 46 clinical trials that assessed the effects of fructose on insulin sensitivity in nondiabetic adults (normal weight, overweight, or obese) (ter Horst et al., 2016). The analysis concluded that short-term fructose consumption, in either isocaloric exchange for other carbohydrates or under conditions of hypercaloric supplementation, induces hepatic insulin resistance, whereas peripheral (muscular) insulin sensitivity remains unaffected. Thus the results of this metaanalysis suggest that fructose negatively affects hepatic insulin sensitivity, at least in nondiabetic adults, and that this effect cannot only be attributed to increased caloric intake.

Clinical studies on fructose consumption and hepatic fat content

Some of the human studies evaluating the effects of high-fructose consumption on hepatic insulin sensitivity, mentioned in the previous section, also assessed its effects on intrahepatic lipids. Most of them found that fructose consumption was associated with an increase in liver fat content (Lecoultre et al., 2013, 2014; Matikainen et al., 2017; Schwarz et al., 2015), and one intervention study in obese children showed that the reduction of dietary sugars reduced intrahepatic lipid content and de novo lipogenesis (DNL) (Lustig et al., 2016). In addition, an intervention study performed in overweight adolescents with >8% hepatic fat who were regular consumers of SSB showed that substituting these SSB with fructose-only or glucose-only sweetened beverages for 4 weeks did not change hepatic steatosis levels (Jin et al., 2014). The authors speculated that the lack of effects on hepatic lipids could be related to the fact that the study was designed to be equicaloric, suggesting that the main cause of improvements in hepatic steatosis when reducing fructose intake is the reduction in caloric intake and therefore in body weight. In fact, in the intervention study performed by Lustig et al., there was a small (1%) but significant reduction in body weight (Lustig et al., 2016). Similarly, a pilot study in

10 obese patients with NAFLD, who were advised to reduce their fructose intake by 50% from baseline for 6 months, showed a decrease in liver fat content, but as this reduction was accompanied by lower fat and total energy consumption, the effect cannot be directly related to reduced fructose intake (Volynets et al., 2013). To be able to better discriminate between sugar-specific effects and those related to calorie consumption, a study (Johnston et al., 2013) was designed to provide high-fructose or high-glucose intake in two separate intervention periods of 2 weeks: the first period was isocaloric (25% of total energy intake was from liquid glucose or fructose, and a controlled diet provided the remaining 75%), and the second period was hypercaloric, as glucose or fructose were provided at the same percentage and the rest of the diet was supplied ad libitum. The results of the study showed that liquid glucose or fructose consumption only increased intrahepatic triglyceride contents under hypercaloric, but not under isocaloric conditions (Johnston et al., 2013).

Key factors that could explain conflicting results in different studies, besides the length of the study, may be the quantity and the form in which fructose is provided, either as a pure substance or as sucrose or HFCS, which are the main sources of fructose in the human diet. Thus, Bravo et al. performed a study in healthy men and women who received low-fat milk sweetened with HFCS or sucrose to provide 8%, 18%, and 30% of total energy requirements, for 10 weeks (Bravo et al., 2013). These doses were chosen because they were within the range of normally consumed sugars in the United States (equivalent to the 25th, 50th, and 90th percentiles for dietary fructose intake in the American population). Hepatic fat content, assessed by computed tomography scans of the liver, was not modified at any of the studied doses, and no difference was found between HFCS and sucrose. Several factors could account for this lack of effect, including the short duration of the study or the technique used to determine intrahepatic fat, which is not as accurate as magnetic resonance spectroscopy. Moreover, sugars were provided in milk, which as mentioned earlier is not the ideal vehicle for this type of study. In addition, the study did not include children or adolescents, the population segment with the highest consumption of fructose, especially in beverages (Marriott et al., 2009; Popkin, 2010). Mager et al. focused on children and youth (ages 7–18) with and without NAFLD and performed an intervention study involving the reduction of fructose intake, glycemic index, and glycemic load, without energy restriction, for 6 months (Mager et al., 2015). Although in this study liver fat content was not measured, the intervention reduced plasma markers of liver dysfunction (ALT and AST) in patients with NAFLD in the absence of changes in body weight or body mass index. Although causality cannot be inferred from observational studies, a cross-sectional study including participants of the Framingham Offspring and Third Generation cohorts showed that SSB intake was associated with fatty liver and ALT levels, after adjusting for potential confounding factors (Ma et al., 2015). In contrast, no association was found between these parameters and the consumption of diet soda, which does not contain added sugars. Thus, the results of this study suggest that added fructose may play a role in the accumulation of hepatic lipids.

All the previously cited studies focused on evaluating the association between fructose consumption and fatty liver. However, NAFLD is a spectrum of disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis (Lewis and Mohanty, 2010). High-fructose intake has also been linked to progression from fatty liver to NASH and hepatic fibrosis (Abdelmalek et al., 2010). Few studies have investigated the effects of dietary fructose on liver fibrosis in humans. A cross-sectional study in a cohort of patients with chronic hepatitis C, a pathology that frequently progresses to liver fibrosis, showed that the severity of

hepatic fibrosis was associated with the consumption of industrial fructose (from processed foods or beverages containing HFCS), but not to the consumption of fructose from fruits (Petta et al., 2013). Although some aspects of this study have been criticized (Kitson et al., 2014), the results stress the role of fructose origin, either natural or industrial, on the metabolic outcomes. Clearly, intervention studies are warranted to better define the role of fructose in the progression from hepatic steatosis to fibrosis.

Molecular insight from experimental data

Several recently published reviews have addressed the metabolic consequences of fructose consumption, focusing mainly on data generated from experimental models (Baena et al., 2014; Geidl-Flueck and Gerber, 2017; Johnson et al., 2013), some of them specifically addressing fructose-related effects on liver metabolism (Herman and Samuel, 2016; Softic et al., 2016). Most of the general information about fructose metabolism referred in this section is extracted from these reviews.

Experimental data, mainly gathered by using rodent models (rats and mice), are consistent in showing that fructose, and to a much lesser extent glucose, is able to induce fat accumulation and insulin signaling impairment in liver tissue. In the following pages, we will try to address and review recently (from 2013 onward) published basic information on two main subjects:

- Key molecular players relaying fructose-related alterations in liver metabolism.
- Experimental design considerations: species used (rats or mice), route of administration (solid chow or liquid supplementation), and length of simple sugar administration. What matters, quantity (calories), type of sugar (fructose or glucose), or both?

Key molecular players relaying fructose-related alterations in liver metabolism

De novo lipogenesis

It has been demonstrated that 26% of liver fat from obese patients with NAFLD derives from DNL, while the same figure for healthy people is less than 5% (Donnelly et al., 2005). Thus DNL is one of the main drivers of NAFLD, and fructose efficiently provides triose phosphates substrates for lipogenesis, being highly lipogenic. This is due to several key molecular switches that are activated by fructose.

- Fructokinase or ketohexokinase (KHK). Fructose is highly efficiently extracted from portal blood, leaving very few molecules for systemic circulation, by liver KHK. This enzyme transforms fructose into fructose-1-phosphate, which in turn is quickly cleaved by aldolase B into lipogenic dihydroxyacetone phosphate and glyceraldehyde. In contrast, glucose is not a good substrate for KHK and is transformed to glucose-6-phosphate by glucokinase (whose activity is tightly regulated) allowing glucose to reach the systemic circulation and be distributed to the whole organism. Lack of liver KHK, specially the A isoform, prevents fructose-derived fat deposition (Ishimoto et al., 2012, 2013). Both KHK and aldolase B are induced by sustained fructose consumption, increasing the

lipogenicity of fructose over time (Baena et al., 2015, 2016b; Erion et al., 2013; Rebollo et al., 2014b; Rodríguez et al., 2016). The transcription factor responsible for this induction is carbohydrate response element–binding protein (ChREBP).

- ChREBP has generated much attention in the last few years, with several excellent published reviews that address its main role as a regulator of the expression of genes related, among others, to DNL, gluconeogenesis and glycolysis (Baraille et al., 2015; Filhoulaud et al., 2013; Geidl-Flueck and Gerber, 2017; Iizuka, 2017a,b). Being highly expressed in metabolically active tissues, such as liver, intestine, and adipose tissue, its transcriptional activity is switched on by allosteric and posttranslational mechanisms—that is, acetylation (Rebollo et al., 2014b)—which are activated by products of glucose and fructose metabolism, independently of the presence of insulin. There are two isoforms of ChREBP, alpha and beta (Herman et al., 2012). ChREBPα is activated by simple carbohydrate metabolism and induces the expression of its target genes; among them is the gene coding for ChREBPβ. Once the level of ChREBPβ protein is increased and reaches the nucleus, its potency as a transcriptional activator of ChREBP-target genes is much greater than that of ChREBPα. While ChREBPα needs a certain degree of carbohydrate metabolism in order to be activated, ChREBPβ is constitutively active. As fructose, in comparison with glucose, is almost exclusively metabolized by the liver, it is a strong inducer of ChREBP expression and activity (Baena et al., 2016a; Rebollo et al., 2014b; Rodríguez et al., 2016; Roglans et al., 2007), intensely activating hepatic DNL. In fact, a reduction in liver ChREBP expression impairs the ability of fructose to induce fatty liver (Erion et al., 2013).
- Sterol response element–binding protein 1c (SREBP-1c). SREBP-1c is, together with ChREBP, a key transcription factor supporting the expression of genes related to DNL. In contrast to ChREBP, its liver expression and activity is upregulated by insulin, and markedly increased in states of insulin resistance and hyperinsulinemia. Sustained, chronic fructose supplementation, but not glucose, increases mouse (Softic et al., 2017) and rat (Sangüesa et al., 2018, 2019) liver SREBP-1c expression. This effect is probably mediated by fructose-related hyperinsulinemia and theoretically should further contribute to fructose-induced DNL.
- Mammalian (mechanistic) target of rapamycin complex 1 (mTORC1). The mTOR system responds to nutritional cues to allow for cell growth and proliferation in situations of a surplus of nutrients and energy. Among many metabolic activities, the mTORC1 complex controls lipid biosynthesis through SREBP-1c activation and fatty acid catabolism by inhibition of lipophagy, thus decreasing the availability of free, oxidizable fatty acids (Shimobayashi and Hall, 2014). In 2014, Sapp et al. demonstrated that fructose treatment of larval zebra fish induced fat liver accumulation by a mechanism related to mTORC1 activation (Sapp et al., 2014); in the same year, we also demonstrated that liquid fructose supplementation activated mTORC1 in rats, inhibiting liver autophagy and promoting liver fat deposition (Baena et al., 2015). Recent results from our laboratory indicate that at the same caloric intake, fructose promotes liver mTORC1 activation more intensely than glucose (Sangüesa et al., 2019), again probably reflecting the high liver extraction ratio of fructose.
- Endoplasmic reticulum stress response (ERSR). The ERSR is a physiological response that enables the endoplasmic reticulum to adapt to the increases in the cellular demand

for the production of properly folded proteins; when unrestricted over time, it can contribute to the pathophysiology of chronic neurodegenerative and metabolic diseases (Wang and Kaufman, 2016). In rodents, liquid fructose supplementation for several periods of time consistently increases the activity of the IRE-1 branch of the ERSR, without significantly affecting the activity of the PERK and ATF-6 branches of the ERSR (Baena et al., 2015, 2016b; Rebollo et al., 2014a; Sangüesa et al., 2019). IRE-1 promotes the formation of the spliced form of the transcription factor X-box binding protein 1 (XBP1). Although XBP1 is considered a master regulator of lipogenesis (Lee et al., 2008), the activation of the IRE-1 system in the liver is reported to prevent hepatic steatosis (Zhang et al., 2011).

Fatty acid β-oxidation activity

The consumption of simple sugars and especially fructose can contribute to the accretion of liver lipids by reducing the activity of the fatty acid β-oxidation system by three nonexclusive and complementary mechanisms: decreased flux of substrates as a consequence of mTORC1-mediated inhibition of autophagy (Baena et al., 2015), malonyl-CoA-mediated allosteric inhibition of the rate-limiting fatty acid oxidative enzyme CPTIα (Wakil and Abu-Elheiga, 2009), and reduced expression of CPTIα (Rebollo et al., 2014b; Roglans et al., 2007).

Liver insulin signaling and gluconeogenesis

Although we have previously shown that liquid fructose, but not glucose, supplementation induces insulin resistance in skeletal muscle and adipose tissue of female rats (Baena et al., 2016a), results obtained in liver tissue are conflicting. Despite consistently reducing the expression of the insulin receptor substrate 2 (IRS2) in the liver, fructose supplementation also consistently increases the expression of the phosphorylated, inactive form of the forkhead box protein O1 (FoxO1). As active FoxO1 is required for gluconeogenic gene transcription (Zhang et al., 2006), the expression of G6Pc and PEPCK is reduced in the livers of fructose-supplemented animals (Baena et al., 2016a,b; Rebollo et al., 2014a; Sangüesa et al., 2019). Moreover, the inactivation of FoxO1 transcriptional activity could be a key factor switching the metabolic flux from gluconeogenesis to lipogenesis in liver tissue (Haeusler et al., 2014).

Experimental design considerations

Species, route of administration, solid chow, or liquid supplementation

Rats, like humans, have a limited ability to convert fructose into glucose in the intestinal wall (Alegret et al., 2011). In studies on mice, higher concentrations of liquid fructose (30%) than those used in rats (10%) are necessary in order to induce fatty liver. Under these conditions, fructose increases the permeability of the gut barrier, allowing the leaking of bacterial endotoxins into systemic circulation, thus confounding the interpretation of the direct effects of fructose on liver metabolism (Bergheim et al., 2008; Spruss et al., 2009). Mice supplemented with a 15% fructose solution for 3 months do not develop neither steatosis nor liver inflammation (Baena et al., 2016b).

Supplementation of fructose to experimental animals can be done in solid form using rodent chow that incorporates a high proportion of fructose (30%–60%, w/w), or in liquid form, by providing ad libitum access to fructose solutions in water ranging from 10% (w/v) in rats to 15%–30% (w/v) in mice. Fructose incorporated into solid chow does not mimic the most usual form of ingestion of fructose in humans (i.e., sweetened beverages) and imposes an extremely high burden of fructose on the liver metabolism, allowing for metabolic changes that are very difficult to reproduce in conditions where fructose supplementation is not as extreme. For example, it has recently been reported that sucrose supplementation in solid form to mice acutely increases the production of fibroblast growth factor 21 (FGF21) by the liver, which acts on the central nervous system to suppress the consumption of simple sugars (Von Holstein-Rathlou et al., 2016), and increases the expression of liver G6Pc (Kim et al., 2016), both effects being mediated by activation of liver ChREBP. Our own experience in female rats supplemented for several periods of time with liquid fructose (10% w/v) indicates that under these experimental conditions, fructose does not modify liver expression and circulating concentrations of FGF21 (unpublished results) and thus does not adequately compensate "liquid" calories by a proportional reduction in the ingestion of "solid" calories and reduces the expression of liver G6Pc, despite increasing the expression and activity of liver ChREBP (Baena et al., 2015, 2016a; Rebollo et al., 2014a,b). Nevertheless, caution should be exerted in directly translating experimental animal-generated data to humans, as Dushay et al. have recently reported that acute ingestion of 75 g of fructose in liquid form by humans transiently increases the serum concentrations of FGF21 (Dushay et al., 2015).

It is undisputed that an excess of ingested calories promotes the development of metabolic diseases. There is, however, a continuous debate over whether, besides the amount of calories ingested, the type of molecule (fructose or glucose) providing them can impact negatively on metabolism. Recent results from our laboratory (Baena et al., 2016a,b; Hutter et al., 2015; Sangüesa et al., 2017) and others (Softic et al., 2017) clearly confirm that for a similar intake of calories, the metabolic response is worse when these calories are provided by liquid fructose than by liquid glucose or a solid western style diet.

Duration of simple sugar administration

The vast majority of experimental supplementation studies in animals are of short duration (up to 2 months) and do not properly mimic human consumption patterns of SSB, which can span decades. In our recent publication (Sangüesa et al., 2019), we supplemented glucose and fructose solutions to female Sprague-Dawley rats over a 7-month period, in order to determine whether chronic fructose supplementation could promote not only fatty liver but also steatohepatitis, with liver manifestations of necrosis and fibrosis. Surprisingly, although 7-month sugar-supplemented rats showed hypertriglyceridemia (in part attributed to decreased expression of the VLDL receptor in hepatic and extrahepatic tissues), they did not show any sign of liver steatosis (Fig. 3) (Sangüesa et al., 2019). We thus argued that the consistent upregulation of the IRE1 branch of the ERSR could be a liver homeostatic response, finally restoring liver lipid metabolism almost to normality. Moreover, it has been recently demonstrated that fructose-driven activation of ChREBP not only promotes the liver lipogenic program but also controls the proapoptotic, PERK branch of the ER stress response,

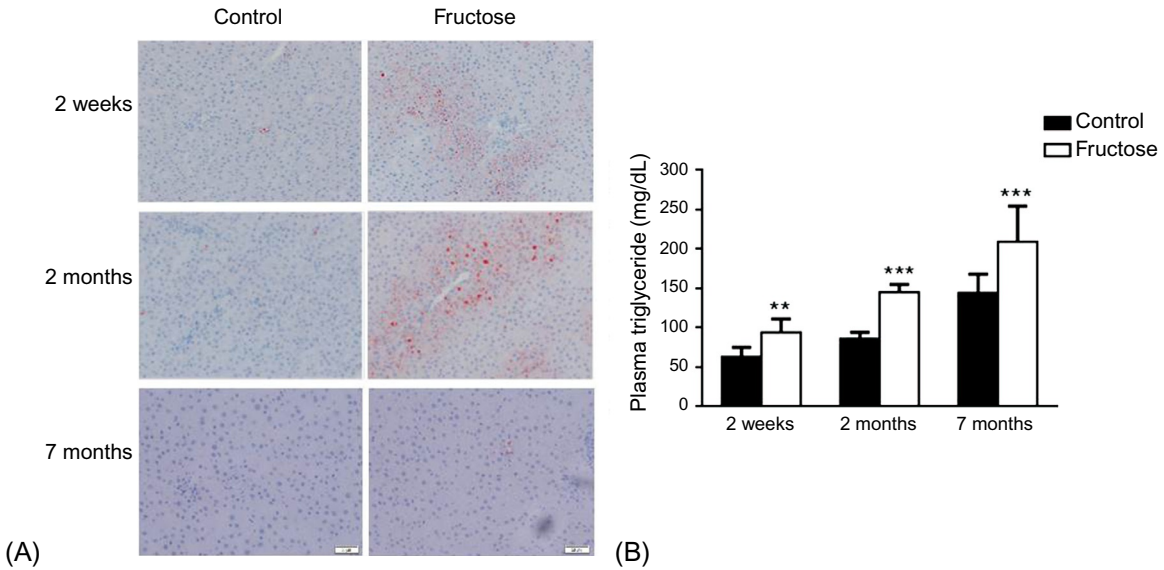

FIG. 3 Effects of liquid fructose supplementation on hepatic lipid accumulation and plasma triglyceride levels in female rats. Representative Oil Red O-stained liver sections (A) and plasma triglyceride levels (B) in female Sprague-Dawley rats control or supplemented with a 10% w/v fructose solution as drinking water for 2 weeks, 2 months, and 7 months. The bar graphs represent the mean ± SD for each group. ** $P < .01$ and ***$P < .001$ versus control values. The results are from our laboratory and were previously published in Rebollo et al. (2014b), Baena et al. (2015), and Sangüesa et al. (2019).

protecting liver tissue from injury and the development of hepatocyte death and fibrosis (Zhang et al., 2017). This mechanism could also explain our results, which showed that even under chronic consumption of fructose as the sole dietary factor, liver injury did not develop. Only when fructose supplementation is combined with other dietary insults provided by, for example, a western-type diet (excess of saturated fatty acids and cholesterol), do signs of liver inflammation and fibrosis clearly develop (Baena et al., 2016b). Nevertheless, caution should be exerted again in generalizing experimental data from rodents. In a very recent publication, Cydylo et al. demonstrated that chronic consumption (7 years) of a high-fructose diet by cynomolgus monkeys sufficed to induce the development of fatty liver and liver fibrosis (Cydylo et al., 2017).

Concluding remarks

Based on the evidence from the clinical studies examined herein, the consumption of high amounts of fructose reduces the insulin-mediated suppression of hepatic glucose production, whereas a reduction in whole-body insulin sensitivity is not always observed (Table 1). This suggests that fructose may specifically impair hepatic insulin sensitivity without affecting glucose uptake by skeletal muscle, the main tissue responsible for glucose disposal. In contrast, studies performed in animal models show insulin resistance in skeletal muscle and reduced hepatic expression of gluconeogenic genes after fructose consumption, suggesting a preserved hepatic response to insulin involving a reduction in hepatic glucose output.

Regarding liver fat content, the results of clinical studies are not conclusive: some of them showed an increase after fructose consumption, but others showed no change (Table 2),

TABLE 2 Clinical studies that examined the effects of fructose or fructose-containing sweeteners on hepatic steatosis and related parameters (since 2013)

Author (year)	Type of study	Patients	Intervention	Main results
Bravo et al. (2013)	Randomized, parallel, partially blinded	Men and women, mean age 42 years ($n = 64$)	10 weeks, low-fat milk sweetened with either HFCS or S at 8%, 18%, or 30% of calories required for weight maintenance	Liver fat content unchanged
Petta et al. (2013)	Cross sectional	Chronic hepatitis C patients ($n = 147$)		Patients with severe liver fibrosis reported a significantly higher intake of total and industrial F, not fruit F. Hepatic steatosis not related to F intake
Volynets et al. (2013)	Pilot study, pre-post, single arm	NAFLD, obese patients ($n = 10$)	6 months, reduction of F intake by 50% from baseline (dietary counseling)	Hepatic fat content significantly reduced

TABLE 2 Clinical studies that examined the effects of fructose or fructose-containing sweeteners on hepatic steatosis and related parameters (since 2013)—Cont'd

Author (year)	Type of study	Patients	Intervention	Main results
Johnston et al. (2013)	Randomized, parallel, double blind	Centrally overweight males 18–50 years ($n = 32$)	Two intervention periods of 2 weeks, F or G (25% of energy requirements). Initial period isocaloric, 6-week washout, final period hypercaloric	During hypercaloric period, liver TG content similarly increased in F and G groups
Lecoultre et al. (2013)	Analysis of data collected in several studies	Healthy males, mean age 22.5 years ($n = 55$)	6–7 days, weight-maintenance diet (control) or overfeed with F (1.5, 3, or 4 g/kg/day) or G 3 g/kg/day	F (3 and 4 g/kg/day) and G (3 g/kg/day) increased intrahepatic lipid content compared with control
Lecoultre et al. (2014)	Randomized, crossover	Healthy males, mean age 23 years (n = 10)	6 days, weight-maintenance diet (control) or overfeed with F (4 g/kg/day) ± three types of coffee	F group (without coffee) showed higher intrahepatic lipid content compared with the control group
Jin et al. (2014)	Randomized, parallel, double blinded	Overweight Hispanic, both genders, 11–18 years. Hepatic fat >8%, regular consumers of SSB ($n = 24$)	4 weeks, usual SSB replaced by only F or only G beverages	Hepatic fat content unchanged
Schwarz et al. (2015)	Randomized, crossover	Healthy males, 18–65 years ($n = 8$)	9 days, two isocaloric diets with 50% of total EI as CH, one of them with F providing 25% of total EI and another with F providing 5% of total EI	Higher DNL and higher liver fat with high-F diet
Ma et al. (2015)	Cross sectional	Framingham Offspring and Third Generation cohorts		SSB consumption Positively, dose-dependently associated with fatty liver and ALT levels
Mager et al. (2015)	Pilot study, pre-post, double arm	Children and adolescents with NAFLD ($n = 9$) and healthy ($n = 13$), 7–18 years	6 months, dietary education to promote low GI (45–55), GL (<80), and F (<7% of total EI) diets	ALT significantly reduced in the NAFLD group at 3 and 6 months
Matikainen et al. (2017)	Randomized, parallel, open label	Obese males ($n = 66$), mean age 48 years	12 weeks, F (75 g/day) + ad libitum diet	Liver weight and fat content significantly increased

Abbreviations: *ALT*, alanine transaminase; *AUC*, area under the curve; *DNL*, de novo lipogenesis; *EH*, euglycemic hyperinsulinemic; *EI*, energy intake; *F*, fructose; *G*, glucose; *GI*, glycemic index; *GL*, glycemic load; *HFCS*, high-fructose corn syrup; *HGP*, hepatic glucose production; *HOMA-IR*, homeostasis model assessment of insulin resistance; *n*, number of participants that completed the study; *NAFLD*, nonalcoholic fatty liver disease; *OGTT*, oral glucose tolerance test; *S*, sucrose; *SSB*, sugar-sweetened beverages; *y*, years.

whereas short-term studies in animal models mostly found that fructose causes hepatic lipid accumulation and hypertriglyceridemia. Our studies performed in female rats showed differential effects of fructose depending on the length of supplementation. Thus, liquid fructose supplementation for 2 weeks to 2 months causes hepatic steatosis and hypertriglyceridemia, whereas after a 7-month supplementation period, hypertriglyceridemia is still observed, but hepatic fat accumulation is absent (Fig. 3). The hepatic steatosis observed in these short-term studies arises from fructose-related activation of the ChREBP-KHK and mTORC1-SREBP-1c axes, together with fatty acid β-oxidation inhibition, but in the context of chronic fructose supplementation, these mechanisms may be compensated by other pathways, including the selective activation of IRE-1, resulting in the absence of hepatic steatosis (Fig. 4).

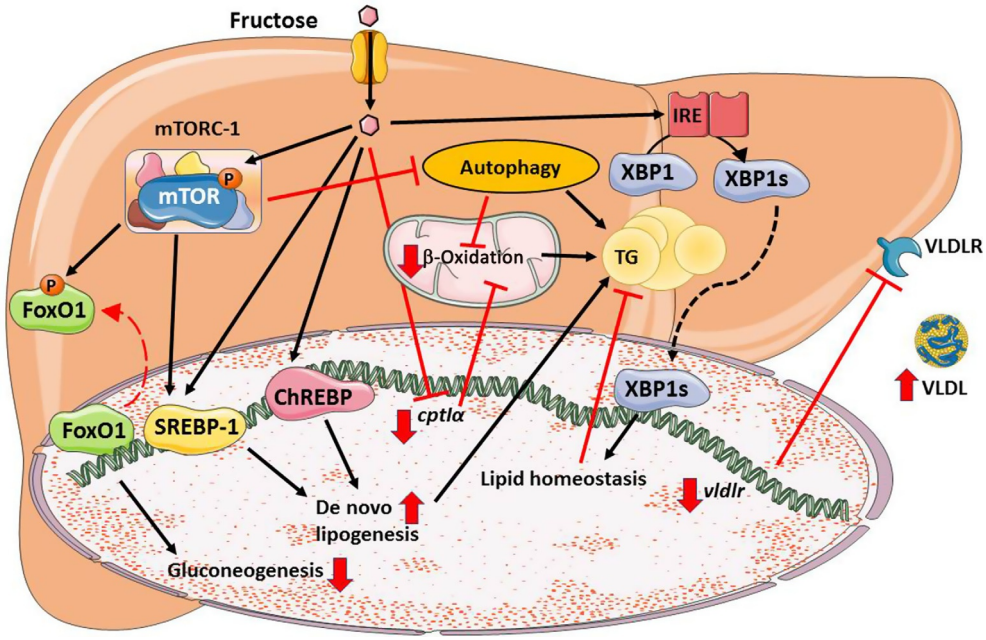

FIG. 4 Fructose affects molecular pathways involved in hepatic lipid deposition. Fructose is a strong inducer of ChREBP and SREBP-1c expression, intensely activating hepatic de novo lipogenesis, which is one of the main drivers of NAFLD. Fructose also activates the mTORC1 complex, which controls gluconeogenesis by increasing the expression of the phosphorylated inactive form of FoxO1, and increases lipid biosynthesis through SREBP-1c activation. Moreover, activated mTORC1 inhibits liver autophagy, decreasing the flux of substrates to the mitochondrial fatty acid β-oxidation system. In addition the rate-limiting fatty acid oxidative enzyme CPTIα is inhibited by fructose. All these mechanisms contribute to liver fat deposition. However, long-term fructose supplementation chronically upregulates the IRE-1 branch of the ERSR and promotes the formation of the spliced form of the transcription factor XBP1. The specific activation of the IRE-1 system in the liver, without significantly affecting the activity of the PERK and ATF-6 branches of the ERSR, is reported to prevent hepatic steatosis. In addition, fructose downregulates the expression of the VLDLR, thus reducing the clearance of VLDL, an effect that contributes to hypertriglyceridemia. *ATF-6*, activating transcription factor 6; *ChREBP*, carbohydrate response element–binding protein; *CPTIα*, carnitine palmitoyltransferase Iα; *ERSR*, endoplasmic reticulum stress response; *FoxO1*, forkhead box protein O1; *IRE-1*, Inositol-requiring protein 1; *mTOR*, mammalian (mechanistic) target of rapamycin; *PERK*, protein kinase RNA-like ER kinase; *SREBP-1c*, sterol regulatory element–binding protein-1c; *VLDL*, very-low-density lipoprotein; *VLDLR*, very-low-density lipoprotein receptor; *XBP1*, X-box binding protein 1; *XBP1s*, spliced form of X-box binding protein 1.

In addition to the duration of sugar supplementation, the type of basal diet to which sugars are added must also be considered as a critical factor. Standard rodent diets are "healthy" diets, essentially of vegetal origin, and the chronic addition of simple sugars in liquid form to these diets, although greatly increasing the total calorie consumption, may not suffice to induce hepatic lipid accumulation. In contrast, real human diets are often rich not only in simple sugars but also in saturated animal fat. Thus, studies in rodents fed standard chow may be useful to unravel how sugars affect molecular pathways under "basal" conditions, but this cannot be considered a real setting regarding human fructose consumption. Rodent-based studies of longer duration, in which sugar supplementation is combined with other typical components of an unhealthy human diet, such as fats of animal origin, will provide a more realistic approximation.

Glossary

De novo lipogenesis (DNL) Metabolic pathway by which the essential components of lipids and fatty acids are synthesized from acetyl-CoA subunits produced during glycolysis of dietary carbohydrates through the sequential action of the enzymes liver-pyruvate kinase (L-PK), ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). The main fatty acid synthesized, palmitate, can be elongated to stearate, and both are converted to unsaturated fatty acids through stearoyl-CoA desaturase 1 (SCD1). Fatty acids are then esterified with glycerol molecules through glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) to render triglycerides.

Endoplasmic reticulum stress response (ERSR) Under conditions of imbalance between protein synthesis and folding the endoplasmic reticulum homeostasis are altered, and this condition is termed endoplasmic reticulum stress. The response of the cell to this alteration aimed at protecting the cell from the stress through restoring homeostasis is the unfolded protein response (UPR), which consists of three branches activated through transmembrane proteins: inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6).

Euglycemic-hyperinsulinemic clamp Gold standard technique for assessing insulin sensitivity in an individual. The procedure consists of establishing a hyperinsulinemic state through the intravenous infusion of high doses of insulin. Under these conditions, endogenous hepatic glucose production is totally suppressed, so hypoglycemia would occur. To avoid this, a glucose solution is infused to keep glucose concentration within the normal range. Under steady-state conditions the rate of exogenous glucose infusion needed to maintain glucose concentration constant at the normal level equals the uptake of glucose by all body tissues and therefore reflects insulin sensitivity.

Gluconeogenesis Metabolic route by which the liver synthesizes glucose from lactate, pyruvate, glycerol, and amino acids (also termed de novo glucose synthesis). The key steps of this pathway are catalyzed by the enzymes phosphoenolpyruvate carboxylase (PEPCK) and glucose-6-phosphatase (G6P), which are under the control of several transcription factors including carbohydrate response element–binding protein (ChREBP), hepatic nuclear factor 4 (HNF4) and its cofactor peroxisome proliferator-activated receptor α coactivator 1 (PGC1α), and forkhead box protein O1 (FoxO1).

HOMA-IR This is an acronym for homeostatic model assessment of insulin resistance. It yields an estimate of β-cell function and insulin sensitivity from the basal (fasting) concentrations of glucose and insulin. The original equation (HOMA1-IR) to assess insulin resistance results from multiplying fasting plasma insulin (in mU/L) and fasting plasma glucose (in mmol/L) and dividing the product by 22.5.

Insulin resistance This condition can be defined as a failure of the cells of the organism, mainly hepatocytes, skeletal muscle cells, and adipocytes, to adequately respond to insulin. Hepatic insulin resistance usually manifests as the inadequate suppression of gluconeogenesis in response to insulin, whereas the insulin-stimulating effect on lipogenesis is maintained or even increased.

Mammalian (mechanistic) target of rapamycin (mTOR) mTOR is a serine/threonine kinase that, together with other proteins, forms two functionally and structurally different complexes termed complex 1 (mTORC1) and

complex 2 (mTORC2). These complexes are energy sensors controlling cellular responses to several nutritional and hormonal stimuli. mTORC1 is activated by amino acids, growth factors, and insulin to enhance anabolic pathways (cellular growth and proliferation, cell cycle progression, synthesis of proteins, and fatty acids) and repress catabolic pathways (autophagy). On the other hand, mTORC2 is activated by growth factors and controls actin cytoskeleton organization and cell proliferation and survival.

Nonalcoholic fatty liver disease (NAFLD) Spectrum of disorders that initially involves the accumulation of triglycerides in hepatocytes (hepatic steatosis). This condition is usually benign, but fatty liver is extremely vulnerable to several proinflammatory and profibrotic mediators, so it can progress to hepatic necroinflammation (nonalcoholic steatohepatitis, NASH) and even further to cirrhosis.

Glucose tolerance test (GTT) This test measures how glucose is cleared from the organism after the administration of a large amount of this sugar. In humans the glucose load is administered orally (OGTT) and dissolved in water, whereas in laboratory animals (rodents), both the oral and intraperitoneal routes are regularly used. After the glucose challenge, blood is extracted at different time intervals, and the concentration of glucose in blood samples is determined. Abnormal blood glucose curves (higher peak levels or levels that return more slowly than normal to basal concentrations) suggest glucose intolerance.

Sugar-sweetened beverages (SSB) Industrially manufactured drinks have been sweetened using high-fructose corn syrup or sucrose (both containing approximately equal amounts of fructose and glucose). Usually, the term SSB includes sodas, colas, fruit punches, lemonade, and other fruit drinks with added sugars.

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CHAPTER

10

Master role of glucose-6-phosphate in cell signaling and consequences of its deregulation in the liver and kidneys

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OUTLINE

SUMMARY POINTS

- This chapter focuses on metabolic adaptions during the fed-to-fasted period to maintain blood glucose.
- In the fed state, glucose is stored under the form of glycogen in the liver and muscles.
- During the postprandial period, glucose is mainly produced from glycogen degradation (glycogenolysis) in the liver, to maintain glycemia.
- Hepatic glycogen metabolism is controlled by the coordinated action of two enzymes, glycogen synthase and glycogen

phosphorylase, both of which are regulated by phosphorylation (regulated by pancreatic insulin or glucagon) and allosteric modulators such as glucose and glucose-6-phosphate.

- During long-term food deprivation, glucose is produced by gluconeogenesis in the liver, kidney, and intestine.
- Gluconeogenesis is the synthesis of glucose from nonsugar carbon compounds. The main precursors are lactate and alanine in the case of liver gluconeogenesis, whereas glutamine is an essential substrate for gluconeogenesis in the kidneys and intestine.
- Glucose homeostasis is balanced by nutrient sensing and hormones that control tissue-specific rates of glucose utilization and production. Insulin induces glucose utilization by tissues and inhibits glucose production, while glucagon activates glucose production.
- This chapter focuses on glucose phosphorylation/dephosphorylation during the fed-to-fasting state cycle in the liver and kidneys.
- Glucose-6-phosphate is the first intermediate of glucose metabolism.
- The postprandial elevation in G6P has a major role in stimulating glycogen storage.
- Glucokinase is the gatekeeper of glucose metabolism in hepatocytes and strongly regulates hepatic glycogen synthesis.
- Glucokinase activity is regulated by an inhibitory protein, GKRP, which sequesters glucokinase in the nucleus during fasting.
- In the kidneys, glucose phosphorylation is mediated by hexokinase.
- Glucose-6-phosphatase is a key enzyme for endogenous glucose production. This enzyme ensures the last common step of both glycogenolysis and gluconeogenesis pathways, that is, the hydrolysis of glucose-6-phosphate into glucose.
- Glucose-6-phosphatase is composed of two subunits (a G6P translocase, G6PT, and a catalytic subunit, G6PC), both localized in the membrane of the endoplasmic reticulum.
- The transcription factor carbohydrate response element-binding protein (ChREBP) is a central metabolic coordinator orchestrating the fate of glucose.
- Insulin-mediated lipogenesis is activated via the regulation of sterol regulatory element-binding protein-1c (SREBP1c), while glucose-mediated lipogenesis is activated via ChREBP.
- This chapter described two metabolic diseases, that is, type 2 diabetes and glycogen storage disease type I, which are characterized by glucose metabolism impairments.
- Type 2 diabetes is characterized by an overproduction of glucose and hyperglycemia, while GSDI is characterized by a lack of endogenous glucose production and hypoglycemia.
- Type 2 diabetes and nonalcoholic fatty liver diseases (NAFLD) are chronic metabolic diseases that are associated with overnutrition.
- GSDI is a rare genetic metabolic disease due to deficiency in G6Pase activity. GSD type Ia patients have mutations in *G6PC*, whereas GSD type Ib patients have mutations in G6P transporter (*SLC37A4).*
- When the storage capacity of glucose in the form of glycogen is exceeded, G6P is transformed into fat via the de novo lipogenesis pathway, leading to steatosis.
- Type 2 diabetes and GSDI patients both develop hepatic steatosis and long-term pathologies, including liver cancer and chronic kidney disease. In these diseases,

hepatic and renal metabolism is characterized by an increased metabolism downstream of G6P.

- Lipid accumulation in the liver and kidneys play a key role in the occurrence of hepatic and renal damage.
- Hepatic steatosis is the major hallmark of NAFLD.
- The transcription factor ChREBP is a major mediator for promoting the conversion of dietary carbohydrates to fat for energy storage.
- In diabetes, NAFLD can progress to liver fibrosis and nonalcoholic steatohepatitis and finally to the development of hepatocellular carcinomas (HCC). However, HCC can also occur from the transformation of hepatocellular adenomas (HCA) developed in fatty liver. In GSDI, most adult patients develop multiple HCA that can transform into HCC with time.
- In diabetes and GSDI, nephropathy can progress to interstitial fibrosis and glomerulosclerosis leading to renal failure.
- A common metabolic feature of CKD and NAFLD is ectopic lipid accumulation, which is suspected to induce organ damage and dysfunction.

Maintaining normoglycemia is a finely tuned, crucial physiological function. Indeed, most mammals, including humans, are incapable of tolerating hypoglycemia for more than a few minutes. Glucose is the major source of energy for most cells and provides precursors for biomolecule synthesis, such as nucleotides. Once a glucose molecule has passed from the bloodstream into a cell, it is rapidly phosphorylated into glucose-6-phosphate (G6P) and enters the glycolysis pathway (Fig. 1). In addition, G6P enters the pentose phosphate pathway (PPP), where it is oxidized by glucose-6-phosphate dehydrogenase to produce ribose-5-phosphate, an intermediate metabolite in nucleotide synthesis required for DNA replication, as well as the production of the biological reductant NADPH. Glucose can also be stored as glycogen in the liver and skeletal muscle. When the storage capacity of glucose under the form of glycogen is exceeded, G6P is transformed into fat via de novo lipogenesis pathway. While hepatic lipid synthesis is a normal physiological process, excessive storage of lipids in the liver leads to the development of steatosis that represents a prepathological state possibly evolving in nonalcoholic fatty liver diseases (NAFLD). Thus G6P is situated at the crossroad of several metabolic pathways and thus plays a key role in cell metabolism. In this review, we will focus on G6P metabolism in the liver and kidneys, the two major glucose-producing organs. We will also analyze the consequences of its deregulation in two metabolic diseases: type 2 diabetes and glycogen storage disease type I (GSDI). Indeed, abnormal plasma concentrations of glucose result in deleterious effects that can alter the normal functioning of the whole organism. Decreased glucose levels are of particular danger for several tissues, especially in neuronal cells or erythrocytes that require glucose ceaselessly under normal physiological conditions. Thus hypoglycemia can lead to impaired brain function, and in more extreme situations, it can lead to death. Conversely, hyperglycemia results in nonenzymatic glycosylation (glycation) and thus loss of function of proteins, glucose-induced oxidative damage, and other adverse effects such as macrovascular and microvascular complications. While type 2 diabetes is characterized by an overproduction of glucose and hyperglycemia, GSDI is due to an absence of glucose production leading to severe hypoglycemia between two meals.

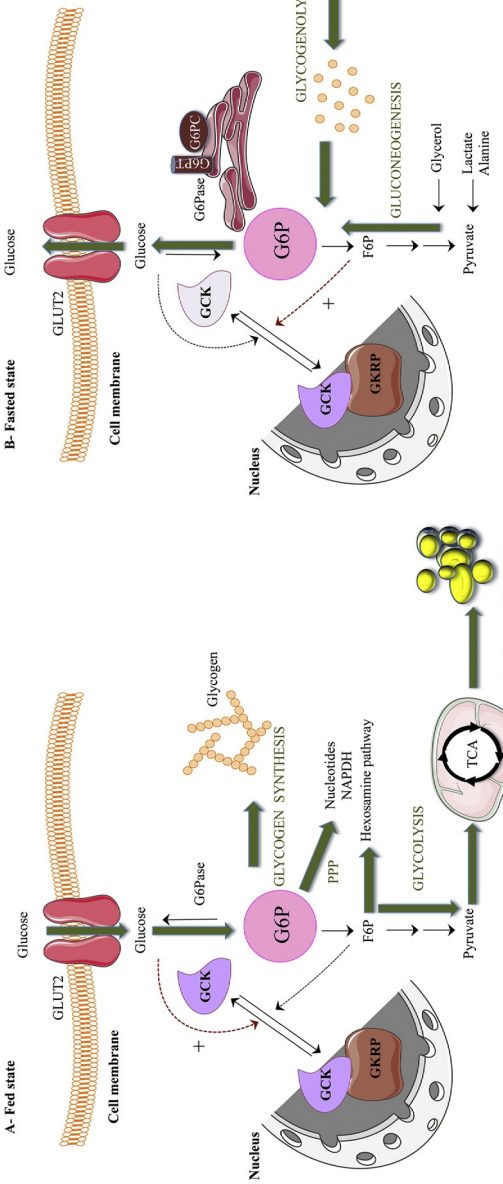

FIG. 1 Schematic glucose metabolism in hepatocytes in the fed state and postabsorptive state. Under fed conditions (A), glucose promotes the dissociation of GCK from GKRP, which leads to the cytoplasmic translocation of GCK. Thus glucose is phosphorylated into G6P, which can enter three metabolic pathways including glycogen synthesis, glycolysis, and PPP. Glucose is then stored under the form of glycogen. G6P is also degraded during glycolysis, providing energy and pyruvate in further decarboxylated to acetyl-CoA, which enters the intramitochondrial tricarboxylic acid cycle (TCA). Alternatively, G6P is degraded in PPP, which provides NADPH and nucleotides. When glucose is in excess, de novo lipogenesis is activated, and G6P is transformed into fat leading to steatosis. (B) Under fasted conditions, glycogenolysis and gluconeogenesis provide G6P as a substrate for glucose production. G6P is then dephosphorylated by G6Pase, which is localized in endoplasmic reticulum. In the liver, glycerol, lactate, and alanine are used as gluconeogenic substrates. Moreover, GCK is sequestered in the nucleus, bound to GKRP, limiting the phosphorylation of glucose. *GLUT2*, glucose transporter 2; *GCK*, glucokinase; *G6Pase*, glucose-6-phosphatase; *G6P*, glucose-6-phosphate; *F6P*, fructose-6-phosphate; *PPP*, pentose phosphate pathway. Illustrations were done with the help of Servier Medical Art.

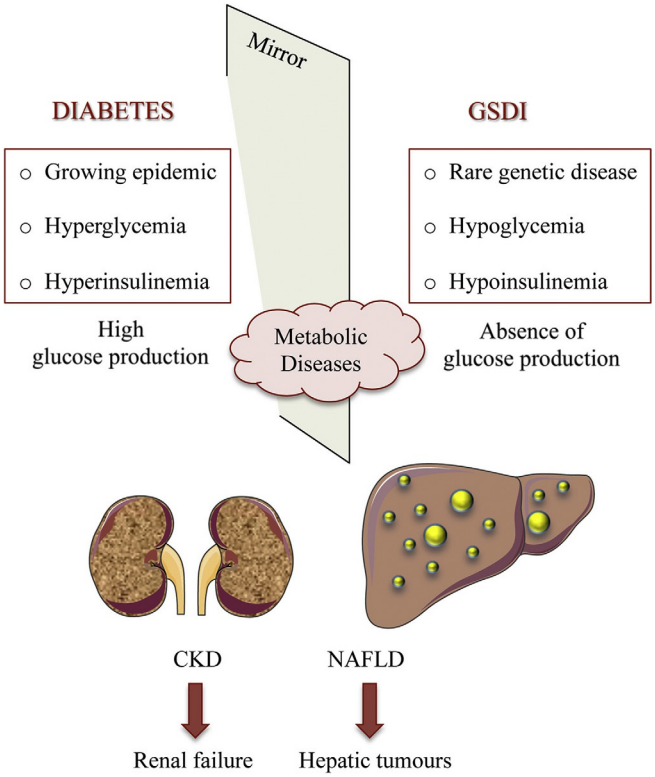

FIG. 2 Development of chronic kidney disease and hepatic tumors in type 2 diabetes and GSDI. Type 2 diabetes and GSDI are two metabolic diseases characterized by glucose metabolism impairments. While type 2 diabetes is characterized by an overproduction of glucose and hyperglycemia, GSDI is due to an absence of glucose production leading to hypoglycemia. Thus these two pathologies could be considered as "mirror" diseases. However, lipids are accumulated in the liver and kidneys of both type 2 diabetes and GSDI patients, which is responsible for the development of NAFLD and CKD. With time, renal failure and hepatic tumors can occur. *CKD*, chronic kidney disease; *NAFLD*, nonalcoholic fatty liver disease. Illustrations were done with the help of Servier Medical Art.

Thus these two pathologies could be considered as "mirror" diseases. However, they share similar hepatic and renal metabolic perturbations. Therefore type 2 diabetic patients and GSDI patients are both prone to hepatic steatosis and tumor development and to chronic kidney disease (CKD) that appear with time (Fig. 2).

Maintenance of blood glucose

The maintenance of blood glucose between 0.7 and 1.2 mg/dl (4–7 mmol/L) is a critical requirement for the body to maintain metabolism and function of several organs including the brain, kidneys, or intestine. While glucose is generally considered as a major source of energy, ketone bodies represent the major energy source of several organs, including the

brain, during fasting periods. Thus, during fasting, the body reduces glucose oxidation to supply PPP for the synthesis of nucleotides, which is essential for cell proliferation (Soty et al., 2017). Thereby, global glucose turnover decreases, and glucose is preserved to supply 3-carbon skeletons to the body during fasting.

During the fed-to-fasted transition, glucose metabolism is finely tuned by a multiorgan cross talk involving hormonal regulation and both central and splanchnic nervous systems (Donovan and Watts, 2014). Insulin and glucagon that are produced by β and α cells, respectively, in the endocrine pancreas, are the main regulators of blood glucose levels. In addition to insulin and glucagon, counterregulatory hormones such as epinephrine are also involved in this tight regulation. The liver and kidneys play a central role in glucose homeostasis by coordinating the storage/utilization and the synthesis of glucose between two meals and fasting in response to hormonal signaling. Moreover, reabsorption of filtered glucose in the renal proximal tubules is also an essential process to prevent excretion of glucose in the urine.

In the fed state the presence of high insulin level favors tissue glucose utilization through the glycolytic pathway and storage of glucose in glycogen in the liver and muscles (around 30%–40% of alimentary glucose). Glycogen is a polymer composed of glucose molecules forming long linear chains linked by α (1–4) bonds and fixed on main ramification chains by α (1–6) branching bonds (Roach et al., 2012). At a molecular level, glucose inhibits glycogen phosphorylase phosphatase, preventing glycogen degradation, whereas G6P allosterically activates glycogen synthase, thus favoring glycogen synthesis (Agius, 2015). Hepatic glycogen represents around 5% of the weight of the liver. A deregulation of its storage and/or metabolism in the liver and/or muscle is responsible for glycogen storage diseases (GSD), which are a family of rare genetic diseases (Hicks et al., 2011; Weinstein et al., 2018). Hypoglycemia is the primary manifestation of the hepatic GSDs (types 0, I, III, VI, IX, and XI). Contrarily to the liver and muscle, glycogen is not accumulated in the normal kidneys. However, when G6P is present in excessive concentrations, this metabolite can be stored as glycogen in the kidneys, as observed in GSDI and Fanconi-Bickel syndrome (due to GLUT 2 mutations), leading to nephromegaly (Berry et al., 1995; Martens et al., 2009). In diabetes, renal glycogen accumulation was reported in hyperglycemic conditions, but further studies are necessary to better understand the tubular formation of glycogen deposits and their relevance in the diabetic kidney (Vallon and Komers, 2011).

In the fasted state the decrease in glycemia reduces intracellular glucose and G6P levels, activating glycogen degradation. Glycogen stores are considered to contribute to endogenous glucose production for about 10–12 h in rodents and 20 h in human subjects (Mutel et al., 2011; Soty et al., 2017). During this postabsorptive period the liver accounts for at least 80% of glucose production, mainly ensured by glycogenolysis. The stimulation of hepatic glycogenolysis during fasting or adrenergic stimulation is dependent on cAMP-PKA signaling. Indeed, both glycogen synthase and glycogen phosphorylase are phosphorylated by PKA, which results in a coordinated inhibition of glycogen synthase and activation of glycogen phosphorylase. Furthermore the increase in glucagon activates the gluconeogenic pathway by inducing the expression of phosphoenolpyruvate carboxykinase-cytosolic form (encoded by *Pck1*) and glucose-6-phosphatase (G6Pase). Gluconeogenesis (GNG) is the de novo synthesis of glucose from noncarbohydrate substrates that takes place only in the liver, kidney, and intestine (Soty et al., 2017). G6Pase catalyzes the hydrolysis of G6P into glucose and inorganic

phosphate, leading to the production of glucose in the bloodstream. This enzyme is expressed exclusively in gluconeogenic organs, but not in the muscles. Therefore G6P is not converted into glucose in the muscle and thus not released in the bloodstream for glucose maintenance, but it is used solely in glycolysis to produce energy for muscle contraction. While the contribution of hepatic glucose production decreases during fasting, the contribution of renal GNG is increased to represent more than 50% of glucose production in fasting rats or humans (Gerich et al., 2001; Owen et al., 1969; Pillot et al., 2009). Moreover, we showed that intestinal GNG takes place to maintain glycemia during prolonged fasts (Croset et al., 2001; Penhoat et al., 2014). It is noteworthy that glucose produced by the gut exerts in the postabsorptive state beneficial metabolic effects. By delivering glucose directly in the portal vein, where glucose is sensed by the nervous system, it induces a gut-brain glucose signal (Soty et al., 2017). This portal glucose signal positively controls different metabolic functions, such as food intake and insulin sensitivity. Thus the regulation of endogenous glucose production is finely regulated in the three gluconeogenic organs, allowing them to coordinately maintain blood glucose. While glucagon is mainly known to modulate hepatic glucose production (Jiang and Zhang, 2003), it has also been shown to modulate the transcription of *G6pc* in the kidneys and intestine, thus modulating renal and intestinal glucose production (Mutel et al., 2011). Recently, we have demonstrated a possible involvement of a cross talk between the kidneys and intestine, via the vitamin D system, and the intestine and liver, via the aforementioned neural gut-brain axis, which might take place in the situation of impaired renal glucose production, such as during CKD (Kaneko et al., 2018).

Glucose phosphorylation and dephosphorylation

In the liver and kidneys, extracellular and intracellular glucose concentrations are in equilibrium due in part to glucose transporter GLUT 2, characterized by a low affinity for glucose (15–20 mM) (Thorens, 1996) (Fig. 1). Once in the cell, glucose is phosphorylated by glucokinase, specifically in the hepatocytes, or by hexokinases (Fig. 1A). Glucokinase (GCK, also named hexokinase IV; encoded by *Gck*) exhibits kinetic properties quite distinct from those of other mammalian hexokinases (Agius, 2016). In vivo the expression and/or activity of GCK is induced in response to an oral glucose and fructose load (Oosterveer et al., 2012; Van Schaftingen et al., 1994). GCK activity is regulated by an inhibitory protein, GKRP, that sequesters GCK in the nucleus during fasting. The interaction of GCK and GKRP is also induced by fructose-6-phosphate (Van Schaftingen et al., 1994). On the contrary, elevated blood glucose (above 5 mM) and fructose-1-phosphate enable dissociation of GCK and translocation in the cytoplasm, where GCK is active (Agius, 2016). It was recently shown that sirtuin 2 (SIRT2) allows glucose-dependent dissociation of GCK from GKRP by deacetylating GKRP in the presence of elevated levels of NAD+ (Watanabe et al., 2018). Consistent with the major role of GCK in control of blood glucose homeostasis, transcription of *Gck* in the liver is induced by insulin through the transcription factor SREBP1c and repressed by glucagon. Unlike other hexokinase, GCK is not inhibited by G6P and has relatively low affinity for glucose (Van Schaftingen et al., 1994). In addition, glucagon is predicted to favor sequestration of GCK in the nucleus and thereby increased hepatic glucose production and impaired hepatic

glucose uptake (Agius, 2016). Thus GCK enables hepatocytes to efficiently trap glucose when blood glucose is increased, playing a central role in glucose homeostasis. In the last decade, GCK activators and allosteric inhibitors of GCK-GKRP interaction have been proposed as a therapy for diabetes, with inconclusive results (Raimondo et al., 2015).

As mentioned earlier, G6P can enter three metabolic pathways including glycogen synthesis, glycolysis, and PPP as illustrated on Fig. 1 (Adeva-Andany et al., 2016). Thus G6P may undergo isomerization into glucose-1-phosphate for glycogen synthesis, isomerization into fructose-6-phosphate, and oxidation into gluconolactone, which initiates PPP. Fructose-6-phosphate may further lead to UDP-*N*-acetylglucosamine production (hexosamine pathway), or it can enter glycolysis to generate pyruvate and acetyl-CoA, via tricarboxylic acid cycle into the mitochondria. PPP is widely known to play a key role in reductive biosynthesis, given that this pathway is the major source of NADPH. While it was proposed that NADPH could be essential for glutathione reduction, it has been recently suggested that PPP is not an efficient pathway to protect the liver from oxidative stress (Jin et al., 2018). In addition, G6P and/or its metabolites (xylulose-5-phosphate or fructose-2,6-biphosphate) may act as signaling molecules to regulate transcription of specific glucose-responsive genes, by recruiting carbohydrate response element-binding protein (ChREBP) and its partner Mlx (Abdul-Wahed et al., 2017; Adeva-Andany et al., 2016). ChREBP, which recognizes conserved carbohydrate response elements (ChoREs) in gene promoters, regulates genes involved in glycolysis (*Lpk*) and fatty acid synthesis (*Fasn, Acaca,* and *Scd1*) (Benhamed et al., 2012; Dentin et al., 2012). Recent data confirmed that the majority of ChREBP target genes are involved in metabolic processes in the liver and white adipose tissue, as well as in insulin signaling and tumorigenesis (Poungvarin et al., 2015). The various hormonal and nutritional regulations of ChREBP expression and activity during fasting and upon carbohydrate feeding have been detailed in a recent review (Abdul-Wahed et al., 2017). Thus ChREBP is considered as a glucose sensor and a general integrator of metabolic signals according to nutritional states.

As soon as the intestinal absorption of glucose is completed (postprandial period), hepatic G6P is mainly derived from glycogen breakdown, while GNG becomes the major source of G6P after prolonged fasting. In its phosphorylated form, glucose cannot be exported in the circulation, and thus it is retained in the cell. In glucose-producing organs, G6P is then dephosphorylated into glucose by G6Pase (Fig. 1B). G6P is first translocated from the cytosol into the endoplasmic reticulum (ER) by the G6Pase transporter (G6PT encoded by *SLC37A4*), and subsequently dephosphorylated by the catalytic subunit of G6Pase (G6PC encoded by *G6PC1*). Both subunits are deeply embedded in the ER membrane, and the catalytic site of G6PC faces the ER lumen. Glucose is finally released from the cytosol into the bloodstream through GLUT2. Indeed, GLUT2 deficiency leads to Fanconi-Bickel disease (GSD type XI) characterized by ketotic hypoglycemia during periods of fasting and hepatomegaly secondary to glycogen accumulation (Thorens, 2015). However, liver-GLUT2 deficiency in mice does not result in major perturbations in hepatic glucose production, which has suggested that glucose produced in the ER lumen could be released from a vesicle trafficking to the plasmatic membrane (Burcelin et al., 2000; Guillam et al., 1998). It is noteworthy that glucose release from the ER lumen by a pathway that would compartmentalize glucose away from the cytosol, where it could be rephosphorylated by GCK, could represent an interesting adaptation during fasting.

Deregulation of glucose homeostasis in diabetes and glycogen storage disease type I

Hepatic and renal metabolic disruption in type 2 diabetes and GSDI

Type 2 diabetes is currently considered as an epidemic metabolic strongly linked to the development of obesity, which can be generated from a decrease in physical activity and an increase in caloric consumption in combination with genetic predisposition to insulin resistance (Chen et al., 2012). Indeed, type 2 diabetes is characterized by insulin resistance of the tissues capable of capturing glucose under the action of insulin and/or the lack of insulin production due to the decline in β-cell function. The lack of appropriate insulin signaling, associated with endogenous glucose overproduction, leads to hyperglycemia (Lin and Accili, 2011). Thus the liver and kidney are chronically exposed to hyperglycemia. In the liver, it was shown that glucose uptake is impaired in obese diabetic mice, due to a decreased activity of SIRT2, blocking the dissociation of GCK and GKRP (Watanabe et al., 2018). This has also been associated with low hepatic *GCK* mRNA expression in diabetic patients (Haeusler et al., 2015). Indeed, high glucose represses the expression of the *Gck* gene and concomitantly induces *G6pc* gene expression (Arden et al., 2011). Thus glucose production is activated in diabetes, consequence an overall increase in GNG and of G6P production (Haeusler et al., 2015). This leads to aberrant glucose sensing that results in the accumulation of triglycerides (TG) in the liver and the development of steatosis via the concomitant activation of de novo lipogenesis and suppression of lipid oxidation. Thus diabetes, characterized by glucose metabolism dysfunction, is tightly linked to liver disease, more precisely to nonalcoholic fatty liver disease (NAFLD) (Bhatt and Smith, 2015; Hazlehurst et al., 2016). Indeed, up to 70% of diabetic patients may present NAFLD (Hazlehurst et al., 2016; Williamson et al., 2011). Moreover, hypertriglyceridemia is a common lipid abnormality in persons with type 2 diabetes. In the kidneys, similar metabolic perturbations (increased de novo lipogenesis, decreased lipid oxidation, activation of hexosamine pathway…) are observed, leading to the development of chronic kidney disease (CKD) (Vallon and Komers, 2011), which will be described in the succeeding text.

GSDI (also known as the von Gierke's disease) is a rare genetic disease, with an incidence of 1/100,000 live births, due to a deficiency in G6Pase. Mutations in the gene encoding G6PC are responsible for GSD Type Ia and mutations in G6PT are responsible for GSD Type Ib (Bruni et al., 1999; Chevalier-Porst et al., 1996; Kishnani et al., 2014). Consequently, patients with GSDI cannot produce glucose during short fasts and may suffer from severe hypoglycemia episodes (Kishnani et al., 2014). The deficiency in G6Pase leads to the accumulation of G6P in the liver and kidneys, leading to a complete metabolic reprograming, characterized in part by increased glycolysis and lipogenesis (Gjorgjieva et al., 2016a). Furthermore, we have recently demonstrated a strong reduction in hepatic GCK flux in GSDIa mice and an activation of glycogen turnover (Hijmans et al., 2017). GSDI patients show hepatomegaly and severe steatosis caused by strong glycogen and lipid accumulation, respectively. This is associated with lactic acidosis, hyperuricemia, hypercholesterolemia, and hypertriglyceridemia(Bandsma et al., 2014; Kishnani et al., 2014). Furthermore, in these patients, de novo lipogenesis and cholesterol synthesis were found to be increased 40-fold and 7-fold, respectively (Bandsma et al., 2008). The production of VLDL was unchanged compared with control values, but conversion of VLDL into intermediate density lipoproteins was relatively delayed in GSDI patients.

Lipid vesicles are present in abundance in GSDI livers, mainly in the periportal zone, which corresponds to the location of the highest expression of G6Pase in the liver (Rajas et al., 2007). Associated with glycogen accumulation, lipids contribute further to the development of hepatomegaly. Recently, our results highlighted the crucial role that lipids also play in the development of CKD in GSDIa, independently of the occurrence of NAFLD (Monteillet et al., 2018). While hepatic manifestations of GSDI have been long known, CKD was acknowledged as a complication of GSDI later on, in the 1980s, when renal histology of GSDI patients revealed glomerulosclerosis, interstitial fibrosis, and tubular atrophy (Kishnani et al., 2014). Indeed, G6Pase deficiency results in glycogen and lipid accumulation in renal proximal tubules of the kidney cortex. It has been reported that most adult GSDI patients over 25 years of age develop first signs of CKD, which can progress to renal failure with aging (Kishnani et al., 2014). Many patients also present nephrocalcinosis (due to hypercalciuria and hypocitraturia) that enhance the likelihood of urine calcium precipitation. Because of hyperuricemia, uric acid nephrolithiasis and gout nephropathy can develop. Unfortunately the loss in renal function, glomerulosclerosis, and interstitial fibrosis can further take place, and these defects can lead to renal failure at later stage.

Thus type 2 diabetes and GSDI share similar hepatic and renal metabolic dysfunctions leading to hepatic steatosis and CKD (Rajas et al., 2013).

Deregulation of glucose homeostasis and hepatic steatosis

When the storage capacity of glucose in the form of glycogen in the liver is exceeded, glucose is stored under the form of lipids. As previously mentioned, high G6P concentrations activate glycolysis, leading to the production of pyruvate. Pyruvate may then be transported in the mitochondria, where it is converted into acetyl-CoA, a precursor for the synthesis of fatty acids and cholesterol. Fatty acids can then be elongated, desaturated, esterified, and used to synthetize TG. Triglycerides are stored in lipid vesicles in the cytosol, transported to the adipose tissue via the export of very-low-density lipoproteins (VLDL), or metabolized through the β-oxidation pathway (Postic and Girard, 2008). At a molecular level, lipid accumulation in the liver can be due to excessive lipid synthesis or import of fatty acids, a decrease in lipid degradation, or problems in the export of VLDL (Postic and Girard, 2008). In insulin-resistant states, hyperglycemia and hyperinsulinemia are responsible for enhanced lipogenesis partly through the activation of ChREBP and SREBP1c. Indeed, inhibition of ChREBP expression in obese diabetic ob/ob mice leads to reversal of hepatic steatosis (Dentin et al., 2006; Iizuka et al., 2006). In GSDI, lipid synthesis is activated by ChREBP but independent of liver X receptor (LXR) and SREBP1c (Grefhorst et al., 2010). The lack of SREBP1c activation could be due to low insulin signaling in GSDI (Tong et al., 2009). Besides lipogenesis, ChREBP is known to potentiate glycolysis and nucleotide biosynthesis, and it can have an inhibitory role on oxidative phosphorylation (OXPHOS) (Tong et al., 2009). Thus ChREBP could be a metabolic switch orchestrating the metabolic reprograming in GSDI and diabetic cells.

Contrarily to what is generally believed, there is no clear link between hepatic insulin resistance and fat storage in the liver. For example, despite severe hepatic steatosis, GSDIa mice are protected against diabetes induced by high-fat/high-sucrose diet (Abdul-Wahed et al., 2014). More likely the consequences of hepatic steatosis are dependent on the specific nature

of generated lipid species and/or their subcellular localization. The fractional contribution of hepatic de novo lipogenesis is also dependent on dietary carbohydrate intake. For example, fructose increases lipogenesis more strongly than glucose, through a significant activation of ChREBP (Kim et al., 2016). Moreover, this induces the expression of the hepatokine FGF21, which appears to exert a protective effect on the liver against steatosis (Fisher et al., 2017).

NAFLD encompasses a large spectrum of disease seriousness ranging from simple steatosis to inflammation, fibrosis, and potentially cirrhosis. Simple hepatic steatosis is thought to be a benign reversible condition characterized by the accumulation of TG, in the absence of indicators of liver disease. However, NAFLD is a fertile ground for the development of hepatic tumors. In a subset of obese/diabetic individuals, NAFLD may transition to nonalcoholic steatohepatitis (NASH) characterized by the development of inflammation and fibrosis. However, hepatocarcinogenesis is also observed in nonfibrotic, noncirrhotic NAFLD livers (Baffy et al., 2012; Calzadilla Bertot and Adams, 2016). An uncertain fraction of tumors in nonfibrotic livers likely arise through transformation of HCA (Baffy et al., 2012; Stoot et al., 2010) (Fig. 3). Interestingly the same HCA to HCC transformation has been recently described in GSDI livers (Gjorgjieva et al., 2018). Despite the important levels of accumulated glycogen and lipids in the liver, as well as the important metabolic imbalance, GSDI patients present low-grade hepatic inflammation (Kishnani et al., 2014). Hepatic transaminase (AST/ALT) levels are also usually normal, especially in patients with optimal metabolic control and in patients not bearing hepatic tumors (Kim et al., 2008). The absence of oxidative stress and inflammatory responses in the case of GSDI might be the reason as to why hepatic fibrosis is not associated with GSDI (Kishnani et al., 2014). Indeed, GSDI patients and related animal models do not present fibrosis in the liver, contrarily to other types of glycogen storage diseases. Consequently, these patients do not develop cirrhosis. The comparison of HCC occurrence in NAFLD and GSDI livers argues for a dominant role of metabolic reprograming in the molecular induction of tumor development. Recently, we showed that GSDIa hepatocytes exhibited the main characteristics of cancer cell metabolism, with a Warburg-like metabolic

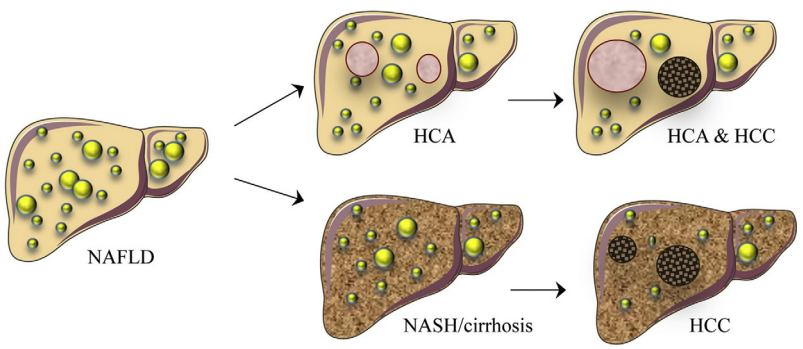

FIG. 3 Schematic steps of the development of hepatocellular adenomas and carcinomas. Hepatic steatosis is the major hallmark of NAFLD-like pathologies, which can lead to the development of hepatic tumors. In most cases, NAFLD progresses to nonalcoholic steatohepatitis (NASH) due to inflammation and fibrosis and ultimately to HCC. However, the development of HCC is more and more observed after the occurrence of HCA in the absence of inflammation and fibrosis. *HCA*, hepatocellular adenomas; *HCC*, hepatocellular carcinomas. Illustrations were done with the help of Servier Medical Art.

reprograming that predisposes GSDIa livers to tumor development (Gjorgjieva et al., 2018). Indeed, we observed a hyperactivation of the glycolysis pathway characterized by an overexpression of the M2 isoform of pyruvate kinase in the tumors and an increase in lactate production. Moreover, OXPHOS analyses revealed a decrease in mitochondrial respiration with a reduction of pyruvate oxidation. This offers advantages to proliferating cells, providing substrates for cellular membrane biogenesis and amino acids and nucleotide synthesis for cell division. Altogether, these metabolic perturbations were associated with a decrease in cellular defenses, including decreased autophagy, chronic ER stress and loss of tumor suppressors such as p53, HNF1α, and PTEN (Gjorgjieva et al., 2016a, 2018). Interestingly, as observed in GSDIa, a decrease in autophagy levels has also been reported in other NAFLD (Koga et al., 2010; Mao et al., 2016; Yang et al., 2010). Furthermore, lipid accumulation and defective autophagy induce ER stress in NAFLD conditions (Yang et al., 2010). Interestingly, there is growing evidence indicating that ChREBP plays a central role in hepatocarcinogenesis. Indeed the suppression of ChREBP in hepatoma and colorectal cancer cells led to a metabolic switch from aerobic glycolysis to mitochondrial respiration coupled with reduced lipogenesis and nucleotide synthesis and decreased proliferative and tumorigenic potential in vivo (Tong et al., 2009). It was also shown that advanced glycation end products (AGEs) that are elevated in diabetic patients stimulate both the expression and activity of ChREBP and promote cancer cell proliferation (Chen et al., 2014). In GSDIa the overexpression of ChREBP has been linked to glucose and lipid metabolism reprograming (Abdul-Wahed et al., 2014; Gjorgjieva et al., 2018). In this context, enhanced ChREBP could partly account for increased proliferation of hepatocytes by favoring cancer cell metabolism. Further investigation is required to unravel the exact role of ChREBP in hepatocarcinogenesis in the context of NAFLD.

In conclusion, both diabetic and GSDI patients develop severe hepatic steatosis and are prone to the development of hepatic tumors. It is noteworthy that tumor occurrence is higher in GSDI patients than in diabetes. Interestingly, molecular mechanisms involved in hepatocarcinogenesis in this steatotic context seem different from HCC induced by some viral infections and alcoholic liver diseases. By comparing hepatocarcinogenesis in diabetes and GSDI, we can propose a dominant role of hepatic metabolic reprograming in this process. Thus a rational therapeutic approach for the treatment of NAFLD, before the development of hepatic tumors, could be to increase hepatic energy expenditure and thereby increase hepatic fat oxidation. Recently, it was shown that T3-glucagon conjugate or glucagon-like peptide 1 agonist-gastric inhibitory peptide-glucagon triconjugate improved glycemia and NAFLD in diabetes, without the harmful effects of each hormone (Finan et al., 2015, 2016). Liver-targeted mitochondrial uncouplers, which can dissipate the inner mitochondrial membrane proton gradient in the liver, are also a promising class of agent for the treatment of NAFLD in the context of diabetes (Perry et al., 2015). Interestingly, lipid-lowering treatments that increased fatty acid oxidation by using pharmacological activators of PPARα have recently shown to prevent hepatic damages in both diabetes and GSDI (Monteillet et al., 2018; Musso et al., 2016; Waskowicz et al., 2018).

Deregulation of glucose homeostasis and chronic kidney disease

The molecular mechanism leading to CKD in GSDI patients is thought to be very similar to the one observed in diabetic patients (Mundy and Lee, 2002; Rajas et al., 2013; Yiu et al., 2008).

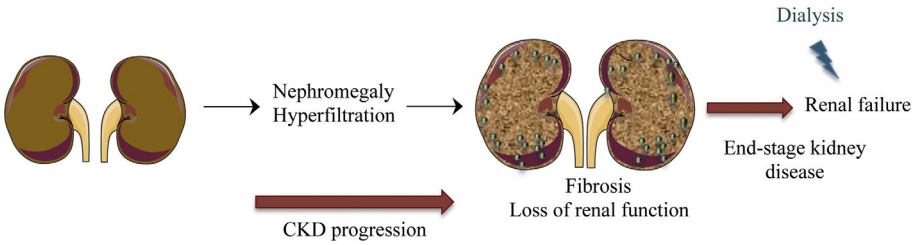

FIG. 4 CKD progression until the end-stage kidney disease. After the first alterations of kidneys, hyperfiltration is often observed before the progressive lack of renal function (decreased glomerular filtration rate). In type 2 diabetes and GSDI, the accumulation of lipids in the renal cortex induces interstitial fibrosis and glomerulosclerosis, responsible of CKD. Then the progression of CKD into renal failure requires dialysis and then kidney transplantation. Illustrations were done with the help of Servier Medical Art.

Indeed the increased flux downstream of G6P leads to the accumulation of ectopic lipids in the kidneys (Fig. 4). This results in the activation of the renin-angiotensin system (RAS) observed in both diabetic and GSDI CKD (Mundy and Lee, 2002; Yiu et al., 2008). Lipids are precursors for the de novo synthesis of glycerolipids, including diacylglycerol (DAG). DAG is a potent activator of protein kinase C (PKC) and acts through binding to a specific domain of the regulatory subunit of the enzyme. The activation of PKC leads to the activation of RAS. In RAS, angiotensinogen (Agt) is the precursor of angiotensin II (Ang II) that is produced from two sequential enzymatic reactions: the conversion of Agt in angiotensin I by renin and the conversion of Ang I in Ang II by Ang-converting enzyme (ACE). Ang II stimulates the proliferation of mesangial cells, glomerular endothelial cells, and fibroblasts and acts as a profibrogenic factor. Many profibrotic effects of RAS are mediated by transforming growth factor-β1 (TGF-β1). Indeed, renal fibrosis development is induced by TGF-β1, which activates the expression of the extracellular matrix genes (Lan, 2011; Meng et al., 2015). In addition, TGF-β1 is a potent inducer of epithelial-to-mesenchymal transition that characterizes the phenotype of renal interstitial fibrosis. Thus several treatments inhibiting the RAS/TGF-β1 axis, such as ACE inhibitors, are used to retard progression of CKD in diabetic and GSDI patients (Gjorgjieva et al., 2016b; Martens et al., 2009; Patney et al., 2018). Interestingly, as lipids are a major contributor to the development of CKD, lipid-lowering drugs, such as fenofibrate via the activation of PPARα, could represent a suitable therapeutic strategy to prevent nephropathy. Some studies already evoked renoprotective effects of fenofibrate in diabetes and GSDI (Lewis and Wanner, 2012; Monteillet et al., 2018; Ting et al., 2012). Unfortunately, while lipid-lowering effects of fibrate were corrective of both pathologies featuring the liver and kidneys of GSDIa mice, this strategy did not insure normal glycemia (Monteillet et al., 2018).

To conclude, by comparing hepatic and renal diseases in diabetes and GSDI, we emphasize the striking similarities of molecular metabolism dysfunction in these two diseases. A stimulated metabolism downstream of G6P is a common causal feature leading to the accumulation of ectopic lipids in the liver and kidneys. In the liver, hepatic steatosis results in HCA/HCC development with time. In the kidneys, renal lipids are responsible for kidney injuries, finally responsible for the loss of renal filtration function. It is a noteworthy observation that, while diabetes and GSDI are characterized by abnormal glucose metabolism, lipid-lowering drugs might represent a potential therapeutic approach to prevent both NAFLD and CKD development in both diabetes and GSDI.

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CHAPTER

11

Glucose transporter 1 and prognosis in cancer

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OUTLINE

SUMMARY POINTS

- This chapter focuses on the association between glucose transporter 1 (GLUT1) and prognosis in cancer.
- Altered glucose metabolism in cancer cells is known as the Warburg effect.
- The Warburg effect is characterized by the increased uptake of glucose and the conversion of glucose to lactate under adequate oxygen tension.
- The 14 GLUTs identified display different affinities for sugars and vary in terms their distribution in organs.

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- Several studies have been published linking other GLUT family members to tumorigenesis, especially GLUT3, but GLUT1 has been the main focus of investigation.
- High expression of GLUT1 has been reported to be associated with worse prognosis for various cancers.
- GLUT1 overexpression may be associated with chemoradiotherapy resistance in various cancers.
- Small molecules targeting GLUT1 exert antiproliferative effects in vitro and in vivo.
- GLUT1 may be a significant biomarker for predicting cancer prognosis and may also be a therapeutic target for new cancer therapies.

Key facts of GLUT1

- In 1926 Otto Warburg discovered that the conversion of glucose to lactic acid in the presence of adequate oxygen was a specific metabolic abnormality of cancer cells.
- GLUT1 has a high affinity for glucose (K_m: 3 mmol/L) and can also transport galactose, mannose, glucosamine, and DHA.
- GLUT1 expression in normal tissue is restricted to erythrocytes and endothelial cells at the blood-brain barrier and placenta.
- GLUT1 expressions were remarkably upregulated in tumor lesions compared with normal lesions.
- GLUT1 overexpression has been reported to be associated with worse prognosis and maybe be associated with chemoradiotherapy resistance in various cancers.
- The significance of GLUT1 expression in cancer has highlighted its potential use as a biomarker.
- The functional and therapeutic importance of inhibiting GLUT1 is increasingly being recognized, and glycolysis may become a target of anticancer strategies.

Introduction

In 1926 Otto Warburg discovered that anaerobic glycolysis in the presence of adequate oxygen was a cancer-specific metabolic alteration (Warburg, 1956). Warburg hypothesized that anaerobic glycolysis in cancer cells results from a defect in mitochondrial metabolism. The role of dysfunctional glucose metabolism in cancer, defined as the "Warburg effect," is now firmly established. Cancer cells increase their consumption of glucose (Younes et al., 1996) via nonoxidative ATP production during which glucose is converted to lactate in the presence of adequate oxygen (Warburg, 1956). Thirty-six ATP molecules per glucose molecule are produced by the tricarboxylic acid (TCA) cycle in mitochondria, whereas only two ATP molecules per glucose molecule are generated by glycolytic metabolism of glucose to pyruvate in the cytoplasm. The high level of glycolysis, representative of the Warburg effect, is exploited in clinical practice to perform metabolic imaging.

For example, ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) is a noninvasive imaging technique that measures tumor metabolic activity based on alterations of glucose metabolism (Plathow and Weber, 2008). Many oncogenes regulate glucose metabolism, including phosphoinositide 3-kinase (PI3K) (Plas and Thompson, 2005), Akt (Elstrom et al., 2004), AMP-activated protein kinase (AMPK), p53 (Vousden and Ryan, 2009; Stambolic et al., 2001), c-Myc, and hypoxia-inducible factor (HIF). Particularly, c-Myc and HIF1A induce a transcriptional program for glycolysis that targets glucose transporters (GLUTs) and the genes encoding glycolytic enzymes (hexokinase 2 [HK2], pyruvate kinase type M2 [PKM2], pyruvate dehydrogenase kinase isozyme 1 [PDK1], and lactate dehydrogenase A [LDHA]). The 14 known GLUTs have different affinities for sugars and vary in terms of their distribution among organs. An association between overexpression of GLUT1 and undesirable prognosis for various cancers has been reported. Recent metabolic and genetic analyses have revealed the molecular mechanisms by which GLUT1 contributes to the Warburg effect and tumorigenesis. Small molecule inhibitors targeting GLUT1 have been identified and are currently being investigated in preclinical studies. In this chapter the significant molecular insights into the clinical impact of GLUT1 expression will be discussed.

GLUT1

GLUT1 (human SLC2A1: 1p34.2) has 492 amino acid residues and possesses 12 transmembrane domains, an exoplasmic N-linked glycosylation site, and cytosolic N and C termini as well as a large endofacial loop (Mueckler et al., 1985). GLUT1 in normal tissue is expressed in erythrocytes and endothelial cells at the placenta and blood-brain barrier (Pardridge et al., 1990). The sequence similarity analyses against GLUT1 have led to the identification of glucose transporter-like proteins. Together with GLUT1, these proteins constitute the solute carrier 2A family (SLC2A, protein symbol GLUT). The 14 GLUTs are divided into three classes according to their sequence similarity: Class 1 (GLUTs 1–4 and 14), Class 2 (GLUTs 5, 7, 9, and 11), and Class 3 (GLUTs 6, 8, 10, 12, and 13/HMIT) (Joost and Thorens, 2001). These GLUTs play an important role in embryonic development, and different GLUTs have discrete affinities for glucose, fructose, or other sugars, such as mannose (Table 1). The affinity of GLUT1 for glucose is high (K_m: 3 mmol/L), and GLUT1 can also transport mannose, galactose, glucosamine, and DHA (Mueckler and Thorens, 2013). The human erythrocyte cytoplasm carries about one-third of the glucose in the blood, and the expression level of GLUT1 in the erythrocytes is very high. At the resting state the central nervous system oxidizes the circulating glucose after crossing the blood-brain barrier via GLUT1. Subsequently, glucose is transported into the parenchymal cells of the brain through GLUT1 (astrocytes) and GLUT3 (neurons). During exercise the skeletal muscle utilizes most of the circulating glucose in the endothelium via GLUT1 and subsequently via GLUT4. Evaluation of the GLUT families using the Gene Expression Omnibus data set revealed that GLUT1 mRNA levels were substantially upregulated in the tumor tissue compared with those in the normal tissue in esophageal squamous cell carcinoma (ESCC) (GDS 3838), colorectal cancer (CRC) (GDS 4382), and pancreatic cancer (GDS 4336). Some studies have linked other GLUT family members to the progression of cancer, particularly GLUT3 (Younes et al., 1997; Ayala et al., 2010; Fonteyne et al., 2009); however, the major focus of investigation has been GLUT1. GLUT1 expression in tissue microarray slides of 1955 samples has

TABLE 1 Affinities for sugars and site of expression of GLUT1

	Glucose	Galactose	Glucosamine	Fructose	Others	Site of expression
GLUT1 (SLC2A1)	3	17	2.5		Mannose 20 DHA 1.1	Erythrocytes, endothelial cells, placenta
GLUT2 (SLC2A2)	17	92	0.8	76	Mannose 125	Kidney, intestine, liver, pancreas
GLUT3 (SLC2A3)	1.4	8.5			Xylose DHA Mannose	Brain, testis
GLUT4 (SLC2A4)	5		3.9		DHA 0.98	Muscle, fat, heart
GLUT5 (SLC2A5)				6		Kidney, intestine, testis
GLUT6 (SLC2A6)	+					Spleen, brain, leukocytes
GLUT7 (SLC2A7)	0.3			0.3		Intestine, colon, testis, prostate
GLUT8 (SLC2A8)	2	+		+		Testis, brain, adrenal, liver, spleen, fat
GLUT9 (SLC2A9)	0.61			0.42	Urate 0.9	Liver, kidney, placenta, pancreas
GLUT10 (SLC2A10)		+			2-d-D-Glu 0.3	Heart, lung, brain, liver, muscle
GLUT11 (SLC2A11)	0.16			0.16		Heart, kidney, muscle, placenta, pancreas
GLUT12 (SLC2A12)	+					Muscle, fat, heart, intestine, placenta, prostate
HMIT (SLC2A13)					Myoinositol 0.1	Brain, adipose tissue
GLUT14 (SLC2A14)						Testis

been evaluated using immunohistochemistry (IHC). GLUT1 positivity was detected in 47% of prostate adenocarcinomas, 42% of uterine cervix SCC, 36% of head and neck SCC, 29% of thyroid cancers, 18.6% of glioblastomas, 10% of gastric cancers, 9.4% of retinoblastomas, and 5% of breast adenocarcinomas (Carvalho et al., 2011).

The association between GLUT1 expression and prognosis in cancer

Five metaanalyses (oral SCC [OSCC], colorectal cancer, extrahepatic biliary tract, pancreatic cancer, and nonsmall cell lung cancer) (Table 2) and nine other studies (ESCC, gastric

TABLE 2 Impact of GLUT1 on prognosis in metaanalysis

Organ	Studies *N*	Patients *N*	Expression correlated with	Prognosis			Reference
				Total	HR (95%CI)	Subgroup	
OSCC	13	1301	T stages, tumor grade, size, LN meta. Distant meta	OS: poor	1.88 (1.51–2.33)		Medicine 2016 (Li et al., 2016)
CRC	14	2077	Poorly differentiated Higher in stage III + IV	DFS: NS OS: NS	1.71 (0.78–3.72) 1.28 (0.86–1.91)	Rectal cancer OS: poor	Oncotarget 2017 (Yang et al., 2017)
Extrahepatic biliary tract	17	2371	NA	OS: poor	2.09 (1.52–2.89)		Biomark Med. 2015 (Jones et al., 2015)
PC	8	538	Size, LN meta	OS: poor	1.79 (1.19–2.70)		Oncotarget 2017 (Sharen et al., 2017)
NSCLC	10	1665	T stages, tumor grade, size, LN meta. Sex	DFS: poor OS: poor	1.73 (1.35–2.23) 2.21 (1.42–3.42)		Oncotarget 2017 (Tan et al., 2017)

OSCC, oral squamous cell carcinoma; *CRC,* colorectal cancer; *PC,* pancreatic cancer; *NSCLC,* nonsmall cell lung cancer; *LN,* lymph node; *meta.,* metastasis; *NA,* not available; *OS,* overall survival; *DFS,* disease-free survival; *NS,* not significant; *HR,* hazard ratio; *CI,* confidence interval.

cancer, hepatocellular carcinoma, and gallbladder carcinoma) (Table 3) have reported the association between GLUT1 expression and prognosis.

OSCC

Thirteen studies covering 1301 OSCC subjects were evaluated in a metaanalysis (Li et al., 2016). These analyses demonstrated that GLUT1 positivity was correlated with advanced tumor stage ($n = 7$, odds ratio [OR] $= 2.99$, 95% confidence interval [CI]: 2.01–4.46, $P < .001$), size of tumor ($n = 5$, OR $= 3.36$, 95% CI: 2.04–5.51, $P < .001$), presence of lymph node (LN) metastasis ($n = 5$, OR $= 3.15$, 95% CI: 1.89–5.25, $P < .001$), distant metastasis ($n = 2$, OR $= 3.06$, 95% CI: 1.19–7.9, $P = .02$), and higher tumor grade ($n = 5$, OR $= 3.34$, 95% CI: 1.12–9.94, $P = .031$). Overexpression of GLUT1 was also associated with shorter overall survival (OS) ($n = 8$, hazard ratio [HR] $= 1.88$, 95% CI: 1.51–2.33, $P < .001$).

Analyses of datasets from The Cancer Genome Atlas confirmed that patients with head and neck squamous cell carcinoma with a favorable metabolic and immune gene signature (low GLUT1, high CD8A, and high COX5B) had better short-term outcomes and longer survival compared with patients with an unfavorable metabolic and immune gene signature (Krupar et al., 2018).

TABLE 3 Impact of GLUT1 on prognosis and correlation with clinicopathological features

						Prognosis		
Organ	**Condition**	**Total *N***	**%**	**Cutoffs**	**Expression correlated with:**	**Univariate**	**Multivariate**	**Reference**
ESCC	No preoperative treatment	145	43	>50%	pT3, v+ Microvessel density (MVD)	DFS: poor CSS: poor	DFS: NS CCS: NS	Ann. Surg. Oncol. 2014 (Sawayama et al., 2014)
ESCC	Curative operation	63	48	>30%	No correlation	OS: poor	OS: NS	Dis Esophagus 2005 (Tohma et al., 2005)
ESCC	T1b patients	96	71	Score 4–6	N+	DFS: poor CSS: poor	NS	Ann. Diagn. Pathol. 2010 (Ogane et al., 2010)
GC		617	30	>1%	pap > por or tub, T-stage N+, ly+, v+, H+, stage	OS: poor	OS: poor	Cancer 2001 (Kawamura et al., 2001)
GC		152	24	>30%	T2–T4, N+, diffuse type	DFS: NS OS: NS	DFS: NS OS: NS	Int. J. Oncol. 2013 (Hur et al., 2013)
GC		193	43		Age > 65, T2–T4, N+, stage, intestinal type	OS: poor	OS: NS	Int. J. Med. Sci. 2013 (Jung et al., 2013b)
HCC		63	37	Scoring ≧Score1	SUV, TNR, Ki67LI	DFS: poor OS: poor	NA	J. Hepatol. 2011 (Kitamura et al., 2011)
GB		56	34	>50%	Perinecrotic areas	OS: poor	NA	Pathol. Oncol. Res. 2011 (Legan et al., 2011)
GB		71	52		Histological tumor type tumor stage	OS: poor	NA	Hepatogastroenterology, 2002 (Kim et al., 2002)

ESCC, esophageal squamous cell carcinoma; *GC*, gastric cancer; *HCC*, hepatocellular carcinoma; *GB*, gallbladder cancer; *OS*, overall survival; *DFS*, disease-free survival; *NA*, not available; *NS*, not significant.

Esophageal cancer

Functional genomic analysis based on gene set enrichment analysis revealed GLUT1 as a prognostic marker in esophageal cancer. Of 141 esophageal cancer patients, 114 (80.9%) displayed GLUT1 positivity, which was associated with shorter OS (HR = 2.08, 95% CI: 1.1–3.94, $P = .024$) in multivariate analysis. GLUT1 positivity was also observed in 182 (69.5%) of the 262 patients with shorter OS in the validation set (Blayney et al., 2018). In another study, GLUT1 positivity was observed in 41 (28.2%) of the 145 patients and was correlated with advanced tumor stage (OR = 2.984, 95% CI: 1.208–7.371, $P = .018$) and vascular invasion (OR = 2.771, 95% CI: 1.118–6.871, $P = .028$). In univariate Cox hazard analysis, high

expression of GLUT1 was a significant indicator of both shorter relapse-free survival (HR = 2.021, 95% CI: 1.100–3.712, $P = .023$) and shorter esophageal cancer-specific survival (HR = 2.223, 95% CI: 1.121–4.411, $P = .022$) (Sawayama et al., 2014).

Gastric cancer

Several studies have reported the association between GLUT1 expression and the prognosis of gastric cancer patients. Fifty tubular adenomas and 617 gastric cancers of the stomach were evaluated for GLUT1 expression by IHC. GLUT1 was not detected in adenomas, but was detected in 182 (29.5%) of 617 gastric cancers. Overexpression of GLUT1 was correlated with depth of invasion ($P = .0001$), the presence of LN metastasis ($P = .0001$), venous invasion ($P = .0001$), lymphatic invasion ($P = .0001$), and liver metastasis ($P = .0001$). GLUT1 positivity was not significantly correlated with peritoneal dissemination ($P = .0833$). Positive expression of GLUT1 was significantly associated with shorter survival GLUT1 ($P = .0001$) (Kawamura et al., 2001). Furthermore, GLUT1 positivity was significantly associated with LN metastasis ($P = .008$), advanced tumor stage ($P = .044$), and advanced stage ($P = .009$). OS was shorter in patients with GLUT1 expression than in those without GLUT1 expression in univariate analysis ($P = .042$). However, in multivariate analysis, GLUT1 was not an independent prognostic factor. By contrast, other studies found no association between GLUT1 and the prognosis of gastric cancer. Schlößer et al. evaluated the expression of GLUT1, 3, 6, and 10 by IHC in gastric cancer specimens from 150 patients who underwent total gastrectomy, and only GLUT3 expression was identified to be associated with gastric cancer survival (Schlosser et al., 2017).

Colorectal cancer

Fourteen studies covering 2077 colorectal cancer patients were evaluated in a metaanalysis (Yang et al., 2017). GLUT1 positivity was not significantly correlated with disease-free survival (DFS) (HR = 1.71, 95% CI: 0.78–3.72, $P = .179$) or OS (HR = 1.28, 95% CI: 0.86–1.91, $P = .22$). However, GLUT1 positivity in rectal cancer was identified as a significant biomarker for shorter DFS (HR = 2.47, 95% CI: 1.21–5.05, $P = .013$) in a subgroup analysis. GLUT1 positivity was also significantly associated with female sex ($n = 4$, OR = 2.92, 95% CI: 2.16–3.95, $P < .001$), advanced tumor stage ($n = 6$, OR = 1.73, 95% CI: 1.17–2.58, $P = .007$), LN metastasis ($n = 8$, OR = 2.14, 95% CI: 1.66–2.75, $P < .001$), higher Dukes' stage ($n = 5$, OR = 2.92, 95% CI: 2.16–3.95, $P < .001$), and liver metastasis ($n = 3$, OR = 1.82, 95% CI: 1.06–3.12, $P = .03$).

Extrahepatic biliary tract cancer

Thirty-seven studies, involving a total of 2371 extrahepatic biliary tract cancer patients, were evaluated in a metaanalysis (Jones et al., 2015), and nine biomarkers were associated with OS were identified. These were VEGF (HR = 2.32, 95% CI: 1.57–3.44), COX-2 (HR = 1.94, 95% CI: 1.01–3.71), GLUT1 (HR = 2.09, 95% CI: 1.52–2.89), cyclin D1 (HR = 1.96, 95% CI: 1.02–3.76), p16 (HR = 0.68, 95% CI: 0.47–0.98), p27 (HR = 0.48, 95%

CI: 0.3–0.78), E-cadherin (HR = 0.47, 95% CI: 0.35–0.63), fascin (HR = 2.19, 95% CI: 1.35–3.55), and Ki-67 (HR = 1.69, 95% CI: 1.02–2.79).

Pancreatic cancer

A total of eight studies covering 538 patients were evaluated in a metaanalysis (Sharen et al., 2017). GLUT1 positivity was associated with shorter OS (HR = 1.79, 95% CI: 1.19–2.70, $P = .005$). Overexpression of GLUT1 was correlated with tumor size (over 2 cm; OR = 2.16, 95% CI: 1.2–3.9, $P = .01$) and the presence of LN metastasis (OR = 3.29, 95% CI: 1.38–7.84, $P = .007$). However, GLUT1 positivity was not significantly associated with age, sex, TNM stage, vascular invasion, or histological grade.

Nonsmall cell lung cancer

Ten studies covering a total 1665 patients were evaluated in a metaanalysis (Tan et al., 2017). Overexpression of GLUT1 was correlated with shorter DFS (HR = 1.73, 95% CI: 1.35–2.23, $P < .001$) and OS (HR = 2.21, 95% CI: 1.42–3.42, $P < .001$). Furthermore, overexpression of GLUT1 was associated with gender (OR = 2.29, 95% CI: 1.17–4.49, $P = .015$), depth of invasion (OR = 2.46, 95% CI: 1.79–3.38, $P < .001$), tumor size (OR = 2.77, 95% CI: 1.73–4.44, $P < .001$), and histological grade (OR = 6.99, 95% CI: 4.71–10.38, $P < .001$).

Regulators of GLUT1

c-Myc (Dang et al., 2009) and HIF1A (Griffiths et al., 2005) are key transcription factors that regulate GLUT1 expression. The expression of approximately 15% of all human genes is affected by the MYC protein; therefore the deregulation of MYC may result in alterations in metabolic and other physiological pathways that contribute to tumorigenesis (Lutz et al., 2002). The c-Myc oncogene is upregulated in various cancers, thus causing cancer-specific metabolic alteration (Dang et al., 2009). The regulatory signals that increase c-Myc expression are absent in normal cells, and MYC mRNA and protein levels are therefore low. Gene expression regulated by MYC is often activated or deregulated in human cancer, and c-Myc appears to be at the crossroads of several crucial tumorigenesis processes and pathways. Deregulation of MYC due to mutations (Tuupanen et al., 2012), gene amplification (Calcagno et al., 2008), epigenetic modifications (Amente et al., 2011), and chromosomal translocation or insertion (Liu et al., 2013) has been demonstrated in various cancers. MYC expression, as detected by IHC, has also been investigated in a number of cancers, although to a lesser extent than HIF1A expression. Overexpression and promoter hypomethylation of c-Myc may be associated with gastric carcinogenesis, where deregulation of c-Myc mainly correlates with shorter survival (de Souza et al., 2013). c-Myc-positive IHC is correlated with shorter survival in pancreatic cancer (He et al., 2014); however, c-Myc positivity is not correlated with prognosis in CRC patients (Baba et al., 2010; Rasheed et al., 2009).

The transcriptional activity of HIF is increased following hypoxia to activate an adaptive response. HIF regulates the expression of genes responsible for glucose metabolism, angiogenesis, and cell survival (Lu et al., 2002). Oxygen-independent and oxygen-dependent pathways both affect HIF expression levels. HIF is composed of two regulatory subunits, HIF1A and endothelial PAS domain protein 1 (EPAS1; HIF-2A), and both of these proteins are overexpressed in various cancers (Zhong et al., 1999; Talks et al., 2000). HIF1A expression is a potential biomarker that could be used to predict the clinical outcome of various cancers. Overexpression of HIF1A is correlated with shorter OS in ESCC (Matsuyama et al., 2005; Ogane et al., 2010), gastric cancer (Zhang et al., 2013; Lin et al., 2014), CRC (Baba et al., 2010), and hepatocellular carcinoma (HCC) (Zheng et al., 2013). High HIF1A expression may be correlated with unfavorable outcome of adjuvant chemotherapy with 5-FU-based regimens in gastric cancer patients (Nakamura et al., 2009, 2010) (Table 4). HIF-2A is correlated with shorter OS in gastric cancer patients (Griffiths et al., 2008) but not CRC patients (Baba et al., 2010; Rasheed et al., 2009) (Table 5).

GLUT1 expression is one of the most consistently upregulated proteins in tumors with BRAF or KRAS mutations (Yun et al., 2009). The expression of GLUT1 in CRC tissue was positively correlated with the presence of KRAS/BRAF mutations and the accumulation of FDG (Kawada et al., 2012). EGFR and MAPK upregulation were associated with increased phosphorylation of PKM2 at Ser37. This provides a link back to glucose metabolism, because nuclear PKM2 induces c-Myc, which in turn leads to increased GLUT1 expression (Yang et al., 2012).

GLUT1 expression and chemoradiation therapy

Anticancer treatments, such as chemotherapeutic drugs and radiation, induce intracellular oxidative stress (ROS) in targeted cells. The fixation of radiation-induced DNA damage required intracellular ROS (Sattler and Mueller-Klieser, 2009). Therefore intracellular ROA accumulation may enhance or induce resistance to radiation and may cause chemotherapy resistance (Hirschhaeuser et al., 2011). In rectal cancer, overexpression of GLUT1 was correlated with tumor regression grade, and GLUT1 positivity may be a predictor of the response to chemoradiotherapy (Korkeila et al., 2011; Brophy et al., 2009). The mRNA levels of 20 genes (PCNA, MKI67, CDKN1A [p21Cip1], CDK2, CHEK1, PDRG1, LGR5, PROM1 [CD133], CD44, SOX2, POU5F1 [OCT4], LKB1, VEGF, EGFR, HGF, MET, HIF1, GLUT1, BAX, and BCL2) were examined following microdissection of 52 specimens of locally advanced rectal cancer patients who were treated with preoperative chemoradiation therapy. Elevated expression of PDRG1 and GLUT1 was associated with a weak pathological response according to three kinds of tumor regression grading systems, JSCCR, Dworak, and Rödel (Saigusa et al., 2012).

Furthermore, overexpression of GLUT1 was associated with 5-FU resistance in colon cancer cells (Liu et al., 2014). Inhibiting carbohydrate metabolism lead to reduced ATP production; elevated $[Ca^{2+}]_i$ and cleaved caspase-3 levels; increased apoptosis; and a remarkable sensitization to 5-FU, a genotoxic agent, in colorectal cancer cells. Patients with advanced CRC who had high expression of the transient receptor potential channel C5 (TRPC5)/GLUT1 displayed a poorer outcome after chemotherapy; the significant correlation between

TABLE 4 Impact of HIF1A on prognosis in metaanalysis

Organ	Studies *N*	Patients *N*	Expression correlated with	Prognosis			Reference
				Total	HR (95% CI)	Subgroup	
ESCC	12	1261	Depth of invasion, N+, Stage, VEGF	OS: poor	0.32 (0.01–0.89)	Poor CRT outcomes	Pathol Oncol Res. 2013 (Sun et al., 2013)
GC	12	1555		DFS: NS OS: poor	1.67 (0.99–2.82) 1.34 (1.13–1.58)		Asian Pac. J. Cancer Prev. 2013 (Zhang et al., 2013)
GC	9	1103	Differentiation, T-stage N+, ly+, v+, stage	OS: poor	0.36 (0.31–0.64)		World J. Gastroenterol. 2014 (Lin et al., 2014)
GC	10	1333	T-stage, N+, v+, distant metastasis, stage	OS: poor	1.51 (1.32–1.73)		PLoS ONE 2014 (Chen et al., 2014)
HCC	7	953	Tumor grade, N+, v+	DFS: poor OS: poor	2.14 (1.39–3.29) 1.65 (1.38–1.97)		PLoS ONE 2013 (Zheng et al., 2013)
PC	8	557	T-stage, N+	OS: poor	1.88 (1.39–2.56)		Pancreatology 2014 (Ye et al., 2014)
NSCLC	13	1420		OS: poor	1.60 (1.14–2.25)		Gene 2014 (Wang et al., 2014)
Glioma	24	1422	WHO grade, microvascular density	3y-OS: poor	0.48 (0.35–0.66)		Int. J. Clin. Exp. Med. 2015 (Liu and Cao, 2015)

ESCC, esophageal squamous cell carcinoma; *GC,* gastric cancer; *HCC,* hepatocellular carcinoma; *PC,* pancreatic cancer; *OS,* overall survival; *DFS,* disease-free survival; *NS,* not significant.

chemoresistance and high TRPC5 expression was dependent on the GLUT1 expression level (Wang et al., 2018). Another study demonstrated that gefitinib-resistant NSCLC cells increased GLUT1 expression levels and glucose uptake. Pharmacological and genetic inhibition of GLUT1 blocked the proliferation of NSCLC cells with primary and acquired resistance to gefitinib. Therefore inhibiting GLUT1 activity and thus carbohydrate metabolism may be a mechanism-based approach to target EGFR inhibitor-resistant NSCLC, irrespective of EGFR mutational status (Suzuki et al., 2018).

There is also evidence that GLUT1 inhibition may synergize with radiation in breast cancer. The combination of WZB117, a GLUT1 inhibitor, with radiation has synergistic inhibitory effects on breast cancer cells (Zhao et al., 2016). Adriamycin-resistant MCF-7 cells are also partially resensitized to the drug by inhibition of GLUT1, probably via increasing AMPK activation, inactivating the mTOR pathway, and increasing the translocation of BAX to mitochondria (Chen et al., 2017).

TABLE 5 Impact of HIF1A, HIF2A, and MYC on prognosis

Organ	Total N	%	Cutoffs	Expression correlated with	Prognosis		Reference
					Univariate	Multivariate	
HIF-1A							
CRC	731	19	>50%	COX-2, CIMP-high LINE1 hypomethylation	CSS: poor OS: poor	CSS: poor OS: poor	Am. J. Pathol. 2010 (Baba et al., 2010)
RC	90	54		N+, v+, stage	DFS: poor CSS: poor	OS: poor	Br. J. Cancer 2009 (Rasheed et al., 2009)
RC	92	55	Scaling system	pT4, N+, v+ (T3,4/N+/−)	DFS: poor OS: poor	DFS: poor OS: poor	Int. J. Colorectal Dis. 2006 (Theodoropoulos et al., 2006)
HIF-2A							
GC	80	38	>Score 0	Diffuse type	DFS: poor OS: poor	CSS: NS OS: NS	Br. J. Cancer 2008 (Griffiths et al., 2008)
CRC	731	19	>50%	Low tumor grade, male, BMI < 30	CSS: NS OS: NS	CSS: NS OS: NS	Am. J. Pathol. 2010 (Baba et al., 2010)
RC	90	64		No correlation	DFS: NS CSS: NS		Br. J. Cancer 2009 (Rasheed et al., 2009)
MYC							
GC	125	77	>10%	Intestinal type, late-onset Deeper tumor extension, M+	NA	NA	PLoS ONE 2013 (de Souza et al., 2013)
PC	70	52	Score 5–9	Perineural invasion, stage	OS: poor	OS: poor	Int. J. Clin. Exp. Pathol. 2014 (He et al., 2014)
CRC	731	19	>50%	Low tumor grade Male, BMI < 30	CSS: NS OS: NS	CSS: NS OS: NS	Am. J. Pathol. 2010 (Baba et al., 2010)
RC	90	64		No correlation	DFS: NS CSS: NS		Br. J. Cancer 2009 (Rasheed et al., 2009)

CRC, colorectal cancer; *RC,* rectal cancer; *GC,* gastric cancer; *PC,* pancreatic cancer; *CSS,* cancer-specific survival; *OS,* overall survival; *DFS,* disease-free survival; *NS,* not significant; *NA,* not available; *HR,* hazard ratio; *CI,* confidence interval.

Inhibitors of GLUT1

Phloretin, a natural GLUT inhibitor found in apples and pears, exerts antiproliferative effects in colon cancer and HCC cell lines (Cao et al., 2007; Wu et al., 2009). Several small molecule GLUT1 inhibitors have been reported. In a nude mouse study, a daily intraperitoneal injection

TABLE 6 Antiproliferative effect of GLUT1 inhibitors.

Inhibitor	Cancer type (cell lines)	Dose in vitro	Dose in vivo	Combination or drug resistance	Reference
Phloretin	CRC (SW620)	50 μM		Daunorubicin	Cancer Chemother. Pharmacol. 2007 (Cao et al., 2007)
STF-31	Renal carcinoma (RCC4)	1 μM	11.6 mg/kg		Sci. Transl. Med. 2011 (Chan et al., 2011)
WZB117	Lung cancer (A549)	10 μM	10 mg/kg/day		Mol. Cancer Ther. 2012 (Liu et al., 2012)
Resveratrol	Lewis lung carcinoma	50 μM	100 mg/kg	Rapamycin	J. Nucl. Med. 2013 (Jung et al., 2013a)
Salicylketoximes	Lung cancer	30 μM			ChemMedChem 2015 (Granchi et al., 2015)
BAY-876			0.3 mg/kg		ChemMedChem 2016 (Siebeneicher et al., 2016)

CRC, colorectal cancer.

of 10 mg/kg WZB117 for 10 weeks resulted in a >70% reduction in the volume of human lung cancer xenografts. However, this beneficial effect was accompanied by a body weight loss of ~1–2 g, aberrant lymphocyte and platelet counts, and hyperglycemia (Liu et al., 2012). Other small molecule GLUT1 inhibitors described in the literature are resveratrol (Jung et al., 2013a), salicylketoxime, and STF-31 (Granchi et al., 2015). Other highly potent GLUT1 inhibitors have been identified using an ultrahigh-throughput screen of a library of ~3 million compounds. These include phenylalanine amides (Kapoor et al., 2016) and BAY-876 (Siebeneicher et al., 2016), each of which has been demonstrated to be efficacious (Table 6).

Conclusions and future perspectives

The significance of GLUT1 expression in cancer has highlighted its potential use as a biomarker. The therapeutic importance of inhibiting GLUT1 for treating cancer cells has also been recognized, and inhibiting glycolysis has become an important anticancer strategy. The expression of GLUT1 is correlated with an undesirable prognosis in various cancers, and overexpression of GLUT1 may be associated with chemoradiotherapy resistance in several cancers. Thus novel small molecules that inhibit GLUT1 also inhibit proliferation and may decrease chemoradiotherapy resistance in gastrointestinal, lung, and breast cancers.

Future investigations should continue to evaluate whether inhibitors of metabolite transporters and glycolytic enzymes, such as GLUT1, are effective oncotherapeutics. These inhibitors should also be evaluated for their adverse effects, feasibility, and tolerance for use in clinical practice. Investigating imaging techniques that monitor glucose metabolism and predict drug response will be required to refine patient selection strategies for testing these metabolic inhibitors.

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CHAPTER

12

ChREBP and cancer

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OUTLINE

SUMMARY POINTS

- This chapter focuses on the relationship between ChREBP and cancer.
- ChREBP is a transcription factor that regulates de novo lipogenesis and the pentose phosphate pathway.
- Recent reports indicate that ChREBP is an oncoprotein for prostate cancer and hepatocellular carcinoma and tumor suppressor for leukemia.
- During epithelial-mesenchymal transition program, ChREBP levels are suppressed by TGF signals.
- In primary tumors and metastatic tumors, ChREBP levels are high.

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Introduction

ChREBP is a glucose-activated transcription factor that belongs to the basic helix-loop helix/leucine-zipper family of transcription factors (Uyeda and Repa, 2006; Abdul-Wahed et al., 2017; Iizuka, 2017). ChREBP heterodimerizes with Max-like protein (Mlx), and the ChREBP/Mlx heterodimer binds to the carbohydrate response element (ChoRE) in promoters of ChREBP target genes (Cairo et al., 2001; Ishii et al., 2004). ChREBP is abundantly expressed in the liver, kidney, adipose tissues, pancreatic β cells, muscle, and intestine (Yamashita et al., 2001; Iizuka et al., 2004). ChREBP functions in de novo lipogenesis, glycolysis, pentose phosphate shunt, and gluconeogenesis through regulating gene expression (Uyeda and Repa, 2006; Abdul-Wahed et al., 2017; Iizuka, 2017). Recent studies have established a relationship between ChREBP and tumor progression. During tumor progression, ChREBP expression levels and metabolism are altered. In this chapter, I discuss the biological and structural characteristics of ChREBP and the relationship between ChREBP and several cancers. Finally, I review the use of several antidiabetic and antihyperlipidemic drugs that modulate ChREBP activities for cancer therapy.

ChREBP, a glucose-activated transcription factor

Glucose stimulation is known to induce glycolytic and lipogenic genes such as the liver-/red blood cell-type pyruvate kinase (*Pklr*) and fatty acid synthase (*Fasn*). The promoters of these genes contain the ChoRE (Ma et al., 2006; Jeong et al., 2011; Poungvarin et al., 2015). ChREBP-α was cloned as the transcription factor that binds to the ChoRE in the promoter of *Pklr* (Yamashita et al., 2001). ChREBP-α is localized in the cytosol, and upon glucose stimulation, ChREBP-α translocates from the cytosol to the nucleus to activate gene transcription (Kawaguchi et al., 2001). ChREBP-α contains a low-glucose inhibitory domain (LID) and a glucose response conserved element (GRACE) (Li et al., 2006). Under high-glucose conditions, GRACE is activated by glucose and promotes ChREBP transactivation activity, while under low-glucose conditions, LID can inhibit the ChREBP activity conferred by GRACE (Li et al., 2006). ChREBP-β was later cloned as another isoform of ChREBP-α (Herman et al., 2012). Notably, ChREBP-β contains only GRACE and lacks LID (Herman et al., 2012), and therefore ChREBP-β is constitutively active under any glucose conditions (Herman et al., 2012). Thus ChREBP-β is more efficient and has potent transcription activities compared with ChREBP-α. Moreover, *Chrebp-β* mRNA expression is induced by ChREBP-α, while ChREBP-β suppresses *Chrebp-α* mRNA expression (Jing et al., 2016). Therefore this feedback loop system seems to be important to reach the glucose threshold for ChREBP-mediated gene expression (Iizuka, 2017) (Fig. 1).

ChREBP is also regulated by several mechanisms: nucleo-cytosol shuttling via dephosphorylation/phosphorylation by metabolites (xylulose-5-phosphate (Xu-5-P), glucose-6-phosphate (G-6-P)), conformational changes induced by metabolites, posttranscriptional modifications, and protein degradation (Table 1) (Uyeda and Repa, 2006; Abdul-Wahed et al., 2017; Iizuka, 2017) (Fig. 1). Xu-5-P also activates the nuclear translocation and DNA binding of ChREBP through dephosphorylation by protein phosphatase 2A (Kabashima et al., 2003;

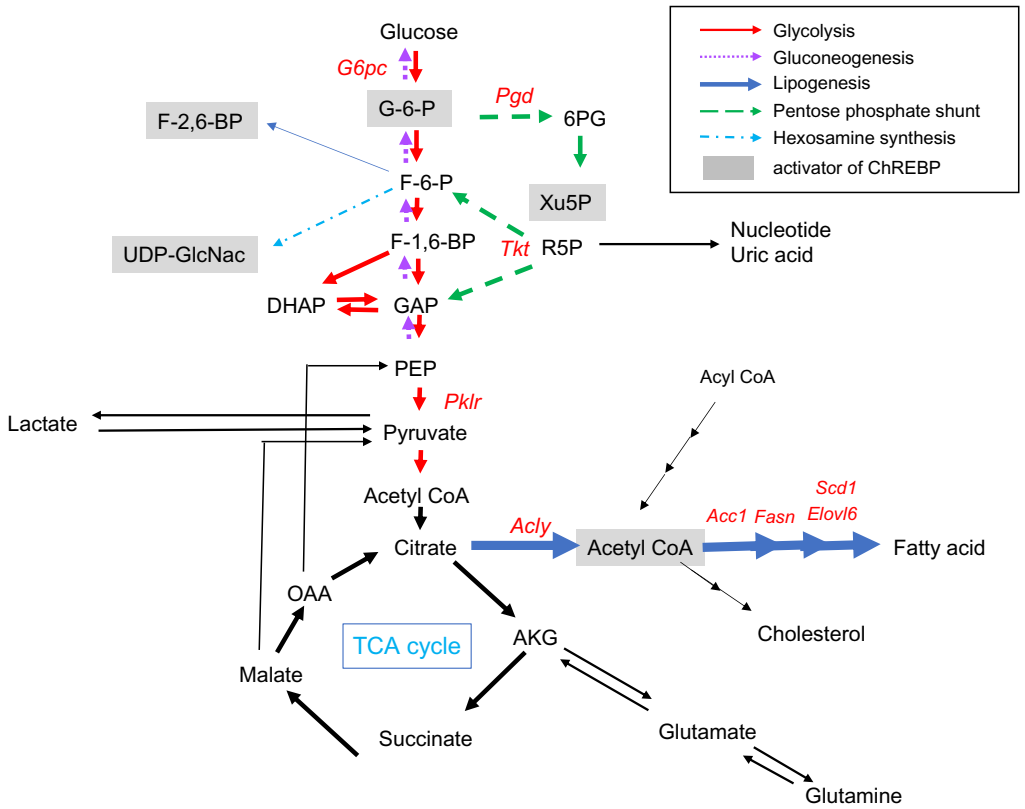

FIG. 1 ChREBP activators in metabolic pathways. Glucose-6-phosphate (G-6-P) is a metabolite in the glycolytic and gluconeogenic pathways. Xylulose-5-phosphate (Xu5P) is a metabolite in the pentose phosphate pathway. Fructose 2,6-bisphosphate (F-2,6-BP) is a metabolite that affects phosphofructokinase and fructose 1,6-bisphosphatase (F-1,6-BP) to regulate glycolysis and gluconeogenesis. Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is an end product in the hexosamine biosynthesis pathway. These metabolites work as ChREBP activators. Genes shown in *red* (*gray color in print version*) are ChREBP target genes.

Iizuka et al., 2013). G-6-P was postulated to induce allosteric modification of ChREBP (Li et al., 2010; Dentin et al., 2012; McFerrin and Atchley, 2012). ChREBP activity is also affected by posttranslational modifications, such as acetylation (Bricambert et al., 2010) and O-linked β-*N*-acetylglucosamine (O-GlcNAc) modification (Ido-Kitamura et al., 2012).

ChREBP activity is negatively regulated by its nuclear export into the cytosol. During starvation, plasma levels of free fatty acids increase, so intracellular levels of adenosine monophosphate (AMP) also increase (Kawaguchi et al., 2002; Nakagawa et al., 2013; Sato et al., 2016). Increased AMP leads to activation of AMP-dependent protein kinase (AMPK). AMPK can suppress ChREBP transcription activity by inhibiting its DNA-binding activity (Kawaguchi et al., 2002). Interestingly, AMP itself can also inhibit nuclear localization of ChREBP by stabilizing cytosol localization of ChREBP (Kawaguchi et al., 2002; Nakagawa et al., 2013; Sato et al., 2016). During fasting, glucagon also increases hepatic cAMP levels, and thereby, protein kinase A (PKA) suppresses hepatic ChREBP activity through

TABLE 1 Key facts of metastasis

- Metastasis is a characteristic of cancer that distinguishes metastatic cancers from benign tumors
- Metastasis is the spread of cancer cells from the place where they first formed to another part of the body
- Some cancer cells can penetrate the walls of lymphatic or blood vessels and circulate through the bloodstream to other sites and tissues in the body
- Epithelial-mesenchymal transition in metastasis is a process that provides tumor cells with invasive, migratory, and stem cell properties, allowing them to disseminate and propagate at distant sites
- Metastatic tumors have similar characteristics as the primary tumor
- Metastasis is a key element in cancer staging systems such as the TNM staging system

Key facts of metastasis, including explanations of metastasis and epithelial-mesenchymal transition, and the importance of evaluating metastasis.

phosphorylation (Yamashita et al., 2001; Kawaguchi et al., 2001). These observations indicate that ChREBP transcription activity is negatively regulated by AMP and cAMP. Because ChREBP is increased in proliferating tumor cells (Tong et al., 2009), AMPK and PKA might be an important therapeutic target for cancer therapy (Table 2).

ChREBP target genes in the liver include genes related to glucose uptake (*Glut5* and *Glut2*) and glycolysis (*Pklr*), fructose metabolism (*Khk*), gluconeogenesis (*G6pc* and *Fbp1*), the pentose phosphate shunt (*Pgd1* and *Tkt*), lipogenesis (*Fasn*, *Acc1*, *Acyl*, *Scd-1*, and *Elovl6*), hormone and hormone receptors (*Fgf-21*, *Gcgr*, and *Adipor2*), and transcription factors (*Bhlhb2*, *Klf-10*) (Iizuka et al., 2004; Iizuka et al., 2006; Poungvarin et al., 2015; Noordeen et al., 2010; Ma et al., 2006; Iizuka, 2017). *Chrebp-β* is also a target gene for ChREBP-α

TABLE 2 ChREBP transcriptional activities are regulated by several nutritional and hormonal factors

ACTIVATION	
Metabolites	Mechanism
Glucose-6-phosphate	Allosteric activation
Xylulose-5-phosphate	Dephosphorylation by protein phosphatase 2A
Acetyl CoA	Acetylation
UDP-GlcNac	UDP-GlcNacylation
INACTIVATION	
Hormones and metabolites	Mechanism
Glucagon Epinephrine	Phosphorylation by cAMP-dependent protein kinase
Free fatty acids	Phosphorylation by AMP-dependent protein kinase
Ketone bodies AMP	Allosteric activation of the interaction ChREBP-14-3-3 through direct binding (stabilizing cytosolic localization of ChREBP)

(Herman et al., 2012). Interestingly, ChREBP also downregulates several target genes (Ma et al., 2006; Jeong et al., 2011; Poungvarin et al., 2015). For example, the tumor suppressor gene p53 is downregulated by ChREBP (Tong et al., 2009). Given that pentose phosphate shunt and de novo lipogenesis are needed for tumor cell proliferation (Tong et al., 2009), ChREBP also plays important roles in cancer development.

Cancer and ChREBP

Prostate cancer and ChREBP

Prostate cancer is one of the most commonly diagnosed cancers worldwide. Many patients with well-differentiated prostate tumors are cured by androgen deprivation therapy, radiation therapy, or surgical therapy; however, patients with poorly differentiated tumors show poor prognosis because these tumors are castration insensitive and can metastasize to bone (Litwin and Tan, 2017). During the initiation and progression of prostate cancer, intracellular glucose levels and lipid metabolism are greatly altered. In normal prostate, epithelial cells secrete citrate into prostate fluids through aerobic glycolysis and inhibition of *m*-aconitase by zinc (Fig. 2A) (Elia et al., 2016). In the development of early-stage prostate cancer (hormone-sensitive prostate cancer), decreased zinc transporter causes a reduction of intracellular zinc concentrations and thus aconitase is not inhibited (Fig. 2B) (Elia et al., 2016). Consequently, cancer cells switch their metabolism to generate energy through the TCA cycle. However, glucose uptake is low, and lactate produced by cancer-associated fibroblasts can fuel cancer growth (reverse Warburg effect) (Elia et al., 2016). In the development of late-stage prostate cancer, glycolysis is increased, and lactate production is increased with increased LDH-A and MCT1 expression (Warburg effect) (Fig. 2C) (Elia et al., 2016). Lipogenesis, sterol synthesis, and cholesterol synthesis are also upregulated. Thus, during prostate cancer progression, reverse Warburg metabolism is switched into a mixed Warburg metabolism (Elia et al., 2016) (Fig. 2B and C).

Glycolysis is a rate-limiting reaction that is regulated by phosphofructokinase, which is activated by fructose-2,6-phosphate (F2,6P). The levels of F2,6P are regulated by the PFK2 family member, PFKFB4. Increased PFKFB4 lowers F2,6P and hence decreases PFK1 activity, shunting G-6-P into the pentose phosphate pathway (PPP) for cell proliferation. In metastatic prostate cancer, PFKFB4 mRNA expression is increased compared with primary prostate cancer, and silencing of PFKFB4 prevented tumor growth in a xenograft model (Ros et al., 2012). These findings suggested that PFKFB4 has an important role in the development of metastatic prostate cancer. Considering that p53 downregulates PFKFB4 expression by binding to its promoter and mediating transcriptional repression via histone deacetylases (Ros et al., 2017), ChREBP activation might indirectly cause an increase *Pfkfb4* expression through its suppression of p53.

Lipogenesis is also upregulated in prostate cancer (lipogenic phenotype). Genes related to lipogenesis (*Acly*, *Acc1*, and *Fasn*), unsaturated fatty acid synthesis (*Scd1*), and elongation of very-long-chain fatty acids (*Elovl7*) are upregulated in prostate cancers (Elia et al., 2016), and the expression levels of these genes have been correlated with prostate cancer progression (Swinnen et al., 2004; Zhang et al., 2014). Akt and mTORC1 are required for

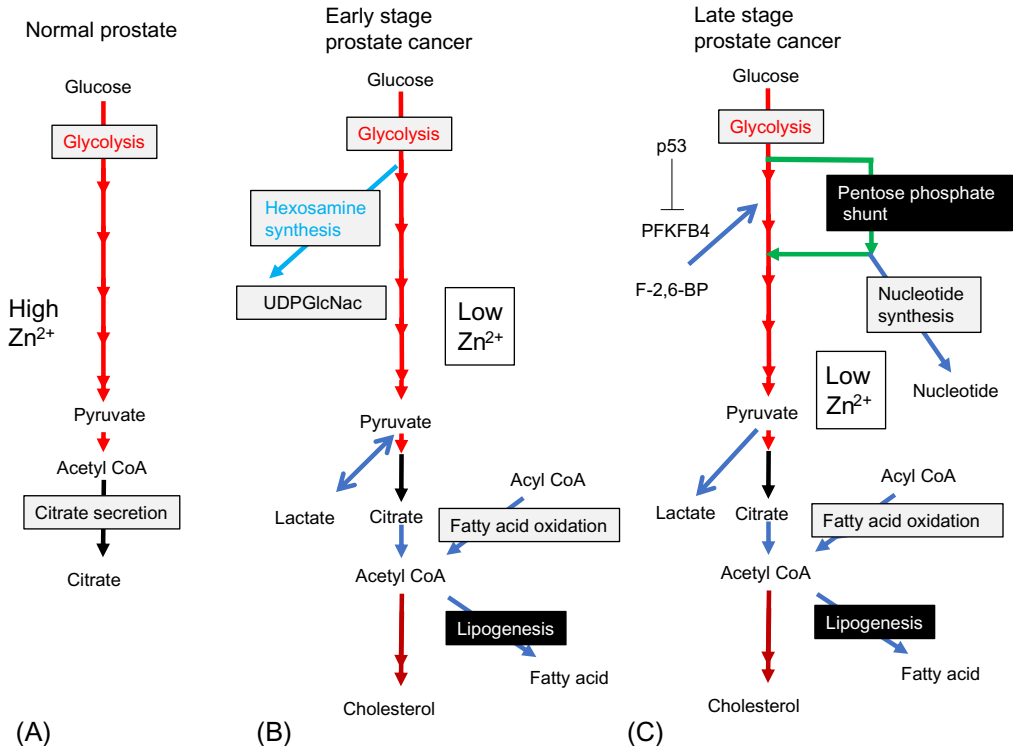

FIG. 2 Metabolic comparison between normal prostate, early-stage and late-stage prostate cancer. Metabolic pathways in (A) normal prostate, (B) early prostate cancer, and (C) late prostate cancer. (A) In normal prostate the TCA cycle enzyme *m*-aconitase is blocked by high intracellular zinc levels. Aerobic glycolysis is thus increased, and citrate is excreted into prostate fluids. (B, C) In early- and late-stage prostate cancer, intracellular Zn^{2+} levels are decreased and enable the utilization of citrate through TCA cycle. Flux in hexosamine biosynthetic pathway is increased in early-stage prostate cancer and then decreased in late-stage prostate cancer. Lipogenesis and pentose phosphate pathways are increased along with ChREBP levels during tumor progression.

SREBP1-mediated lipogenic gene induction (Zhang et al., 2014). Akt also activates lipogenic enzyme activities through direct phosphorylation of these enzymes (Elia et al., 2016; Swinnen et al., 2004). Prostate cancers show constitutive activation of the PTEN/PI3K/Akt/mTORC1 pathway; mutation, altered expression, and copy number alterations of genes in this pathway have been detected in 42% of primary prostate tumors and 100% of metastatic tumors (Swinnen et al., 2004). A previous study showed that Akt facilitates the activation of NRF2, and NRF2 then induces genes involved in the PPP, which produces Xu-5-P, an activator of ChREBP (Bitting and Armstrong, 2013). These findings suggest that ChREBP also might have roles in prostate cancer progression through the PTEN/PI3K/Akt/mTORC1 pathway.

FFA uptake and peroxisomal branched chain fatty acid β-oxidation are also increased in prostate cancers with a low rate of glucose utilization (Zha et al., 2005). ChREBP gene deletion caused a decrease fatty acid β-oxidation (Iizuka et al., 2006). Regarding cholesterol synthesis

the mevalonate pathway is also upregulated in prostate cancers (Tan et al., 2016). ChREBP gene deletion did not affect cholesterol synthesis or SREBP2 levels, indicating that ChREBP might not contribute to this pathway (Iizuka et al., 2004).

Recent studies have reported a relationship between ChREBP and prostate cancer (Wang et al., 2014; Kaushik et al., 2016). In both normal and cancer prostate cells, there is an intramolecular interaction between ChREBP and the androgen receptor (AR) (Wang et al., 2014). ChREBP is required for the optimal transcriptional activity of AR in promoting prostate-specific antigen gene induction (Wang et al., 2014). Moreover, mRNA levels of hexosamine biosynthesis pathway genes (GNPNAT1 and UAP1) and hexosamine biosynthesis pathway activities were significantly downregulated in castration-resistant prostate cancer compared with localized prostate cancer (Kaushik et al., 2016). Knockdown of GNPAT1, a hexosamine biosynthesis pathway gene, in castration-resistant prostate cancer-like cells resulted in increased cell proliferation and aggressiveness. In castration-resistant prostate cancer cells expressing AR splice variant-7 (22Rv1 cells), knockdown of hexosamine pathway genes (GNPNAT1 or GFAT) caused an increase in the ChREBP mRNA and protein levels as well as ChREBP activities (Kaushik et al., 2016). Another study showed that increased binding of Sp1 to the ChREBP promoter causes *Chrebp* expression in advanced prostate cancers (Kaushik et al., 2016). These findings suggested that increased ChREBP expression might partly contribute to prostate cancer progression.

Liver cancer and ChREBP

Hepatocellular carcinoma (HCC) cells show increased glycolysis and diminished gluconeogenesis compared with normal liver cells (Fig. 3A and B) (Elia et al., 2016). In addition, the activities of fetal liver phenotype enzymes (glucose transporter 1, hexokinase 2, glucose-6-dehydrogenase, pyruvate kinase M2, and alpha fetoprotein) are increased in HCC cells, while the activities of adult liver phenotype enzymes (glucokinase and liver type pyruvate kinase) are decreased (Elia et al., 2016). Regarding lipid metabolism the expressions of lipogenic genes (*Fasn*, *Acc1*, *Acly*, and *Hmgcr*) and transcription factor genes (*Lxr*, *Srebp1c*, and *Chrebp*) are increased in liver cancer (Fig. 3A and B). Moreover, mRNA expression of *Scd1*, an important gene in the synthesis of unsaturated fatty acids, is correlated with HCC progression and suppression of *Scd1* reduced proliferation of HCC cells (Calvisi et al., 2011; Ma et al., 2017).

In normal hepatocytes, glutaminolysis, the conversion from glutamine into glutamate, is promoted by liver type glutaminase 2 (Fig. 3A) (Elia et al., 2016). However, in HCC cells, glutaminolysis is diminished, and glutamine synthesis is increased (Elia et al., 2016). Glutamine synthetase expression is highly correlated with HCC progression (Fig. 3B). At present the effects of ChREBP on glutamine/glutamate metabolism are not yet reported. As glutamine/glutamate metabolism is important for not only mitochondrial energy metabolism but also lipogenesis, further investigation will be needed.

Previous studies have established a relationship between ChREBP and the tumor suppressor protein p53. Transient transfection of HepG2 cells with ChREBP siRNAs led to a decline of cell proliferation compared with the siRNA control (Tong et al., 2009). The mechanism underlying the regulation of proliferation is thought to be due to the relationship between ChREBP

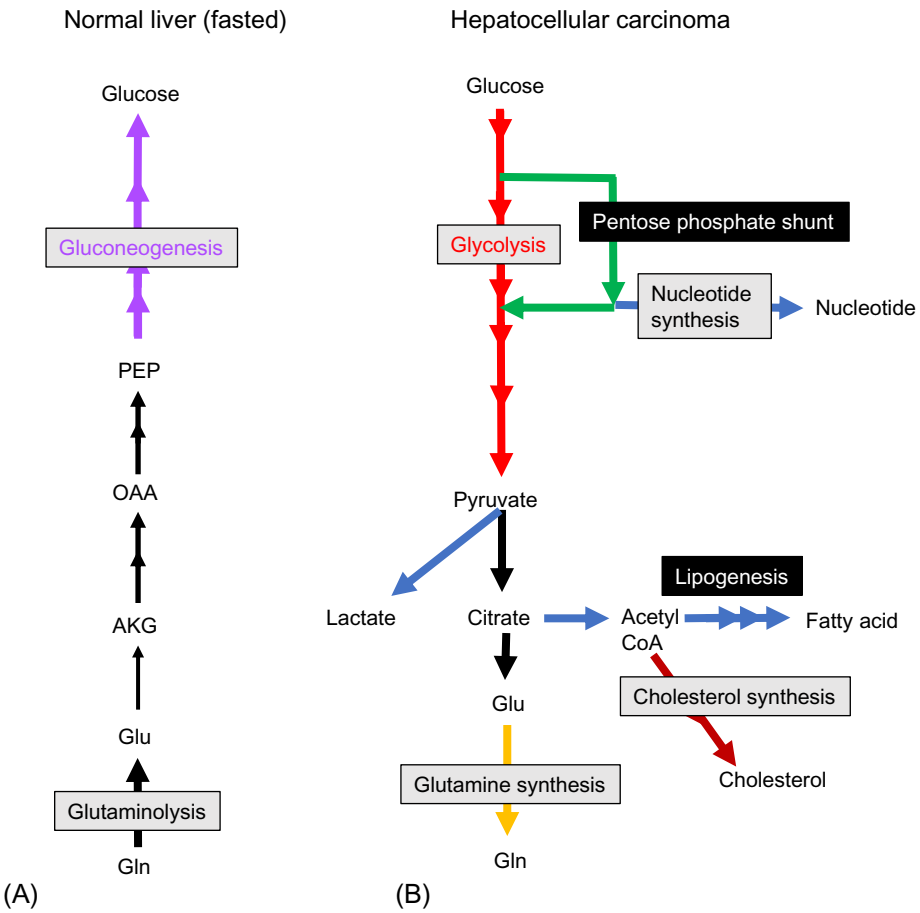

FIG. 3 Metabolic comparison between the normal liver and hepatocellular carcinoma. Metabolic pathways in the (A) normal liver and (B) hepatocellular carcinoma (HCC). Compared with normal liver cells, gluconeogenic flux is decreased in HCC. In contrast, glycolysis, pentose phosphate shunt, de novo lipogenesis, and cholesterol synthesis are increased. Unlike other tumor cells, glutamine synthesis, rather than glutaminolysis, is increased in HCC. Lipogenesis and pentose phosphate pathway are increased along with ChREBP levels during tumor progression.

and p53. In HCT116 colorectal cancer cells, suppression of ChREBP resulted in diminished aerobic glycolysis, de novo lipogenesis, and nucleotide biosynthesis, but stimulated mitochondrial respiration, suggesting a metabolic switch from aerobic glycolysis to oxidative phosphorylation by p53 activation (Tong et al., 2009). p53 might partly mediate the metabolic phenotype induced by ChREBP suppression in cancer cells (Tong et al., 2009).

A recent study showed that ChREBP protein and mRNA are increased in human HCC with poor outcome compared with HCC with better outcome and normal liver (Ribback et al., 2018). Hepatic Myr-Akt1 overexpression causes hepatocarcinogenesis (Calvisi et al., 2011). In Myr-Akt1-overexpressing mice, ChREBP is upregulated in preneoplastic and neoplastic liver lesions (Calvisi et al., 2011; Ribback et al., 2018). Moreover, gene deletion of *Chrebp*

caused delay or impairment of hepatocarcinogenesis driven by Akt or Akt/c-Met overexpression in mice (Calvisi et al., 2011; Ribback et al., 2018). In contrast, ChREBP gene deletion did not protect against hepatocarcinogenesis induced by coactivation of Akt and Ras/MAPK signaling (Ribback et al., 2018). These results suggested that the effects of *Chrebp* silencing on HCC suppression might be dependent on the Akt cascade, but not on the Ras/MAPK/ERK cascade (Calvisi et al., 2011). Furthermore, concomitant suppression of ChREBP and the MAPK cascade suppressed tumor growth in an Akt-/Ras-transfected mouse HCC cell line (Ribback et al., 2018). Therefore both ChREBP suppression and RAS/MAPK/ERK inhibition might be therapeutic targets for HCC.

Breast cancer and ChREBP

Breast cancer subtypes show different metabolic phenotypes (Elia et al., 2016); triple negative breast cancer (TNBC) lacks estrogen receptor, progesterone receptor and HER2, and estrogen receptor-positive breast cancers are positive for estrogen receptor. TNBC shows a higher death incidence rate compared with estrogen-positive breast cancer owing to the lack of specific therapeutic approaches and their prevalence for distant metastasis (Elia et al., 2016). Regarding the differences of these subtypes, TNBC display the classical Warburg effect, while estrogen receptor-positive breast cancers show the reverse Warburg effect. While TNBCs show increased glutaminolysis, estrogen receptor-positive breast cancers show increased glutamine synthesis. TNBC shows decreased lipogenesis and increased uptake of exogenous fatty acid and cholesterol, while estrogen receptor–positive breast cancer shows upregulated de novo fatty acid synthesis compared with TNBC (Elia et al., 2016).

A previous study demonstrated no relationship between breast cancer risk and ChREBP polymorphism (Campa et al., 2009). Two different breast progression arrays revealed that the intensity of ChREBP protein immunostaining correlated with malignant progression; however, *ChREBP* mRNA expression data mined from the Curtis breast series showed a clear and significant decrease of *ChREBP* mRNA with malignant progression (Airley et al., 2014). Consistent with the latter findings, *ChREBP* mRNA levels were positively correlated with 5-year disease-specific survival (Airley et al., 2014). The mechanism underlying how decreased ChREBP expression promotes breast cancer progression remains unclear and may be dependent on differences in cell types. As estrogen receptor–positive breast cancer shows increased de novo lipogenesis, ChREBP might have some roles in the progression of estrogen receptor-positive breast cancer rather than TNBC. Further investigation will be needed to clarify the role of ChREBP in breast cancer progression.

Leukemia and ChREBP

As described earlier, ChREBP functions as an oncoprotein in HCC and prostate cancer. However, some studies demonstrated that ChREBP functions as a tumor suppressor in some leukemias (Zeng et al., 2016). In a murine acute myeloid leukemia model, the deletion of ChREBP resulted in blockage of the differentiation of leukemia-initiating cells and significantly reduced survival (Zeng et al., 2016). The authors concluded that ChREBP promoted the differentiation of leukemia-initiating cells to inhibit leukemogenesis through the

TXNIP/RUNX1 pathway (Zeng et al., 2016). However, ChREBP was not required for the normal repopulation abilities of hematopoietic stem cells (Zeng et al., 2016). *Chrebp* expression levels in both bone marrow cells and leukemia cells of mice were very low (Zeng et al., 2016). Further investigation will be needed to clarify the role of ChREBP in leukemogenesis.

The contributions of ChREBP to metastatic progression

As described earlier, abnormal ChREBP activation promotes metastatic progression of prostate cancer and HCC. Metastatic progression of cancer is characterized by altered lipid metabolism and is responsible for cancer-related mortalities (Nath and Chan, 2016). Recent studies using the multicancer translation of The Cancer Genome Atlas (TCGA) pan-cancer datasets identified genetic alterations in metabolic genes associated with the metastatic progression of cancer. Copy number alterations in genes in the electron transport chain (*SCO2*), fatty acid uptake (*CAV1* and *CD36*), and lipogenesis (*PPARA*, *PPARD*, and *MLXIPL* also termed as *ChREBP*) were elevated in metastatic tumors (Nath and Chan, 2016). Moreover, increased copy number or expression levels of certain genes (*CAV1*, *CD36*, *ChREBP*, *CPT1c*, and *CYP2E1*) were associated with worse survival. These findings indicate that ChREBP has important roles in the metastatic progression of cancers.

Epithelial-to-mesenchymal transition (EMT) is an important process in tumor metastasis (Sounni et al., 2014; Tsai and Yang, 2013). During cancer progression the activation of EMT increases tumor cell invasiveness promotes tumor cell intravasation and ensures tumor cell survival in the peripheral system. CAV1 and CD36 were strongly associated with an EMT program across multiple cancers; however, ChREBP expression was not associated with EMT (Nath and Chan, 2016). In fact, some studies reported that downregulation of ChREBP through a TGFβ/Snail-dependent mechanism is a first step in EMT during nonsmall-cell lung carcinoma metastasis (Jiang et al., 2015). These findings suggested that suppression of ChREBP might contribute to EMT activation in metastasis. Moreover, colonization at distant tissues requires ATP production to promote tumor cell proliferation. Cancer cells colonizing a distant organ have increased energy needs that are fueled in an environment-dependent manner (Elia et al., 2018). In contrast, colonization at distant tissues and macrometastasis requires EMT revision. Cancer cells within an established secondary tumor (macrometastasis) have a similar cellular phenotype as cancer cells within a primary tumor (Elia et al., 2018). Breast cancer-derived lymph node metastasis display increased transketolase, which produces Xu-5-P in the PPP (Elia et al., 2018). Moreover, the increased activity of the PPP can enable epigenetic changes that allow pancreatic cancer cells to proliferate as liver and lung metastases (Elia et al., 2018). These findings are compatible with the evidence that *Chrebp* mRNA levels are increased in metastatic cancers (Nath and Chan, 2016). Therefore altered ChREBP activities might modulate cancer progression (Fig. 4).

Antidiabetes and antilipid drugs show ChREBP inhibitor activities

Atrovastatin

Statins are inhibitors against HMG CoA reductase (HMGCR) that function to lower plasma cholesterol levels. Inhibition of HMGCR activity results in decreased levels of mevalonate and its downstream products that affect critical cell functions such as membrane integrity, cell

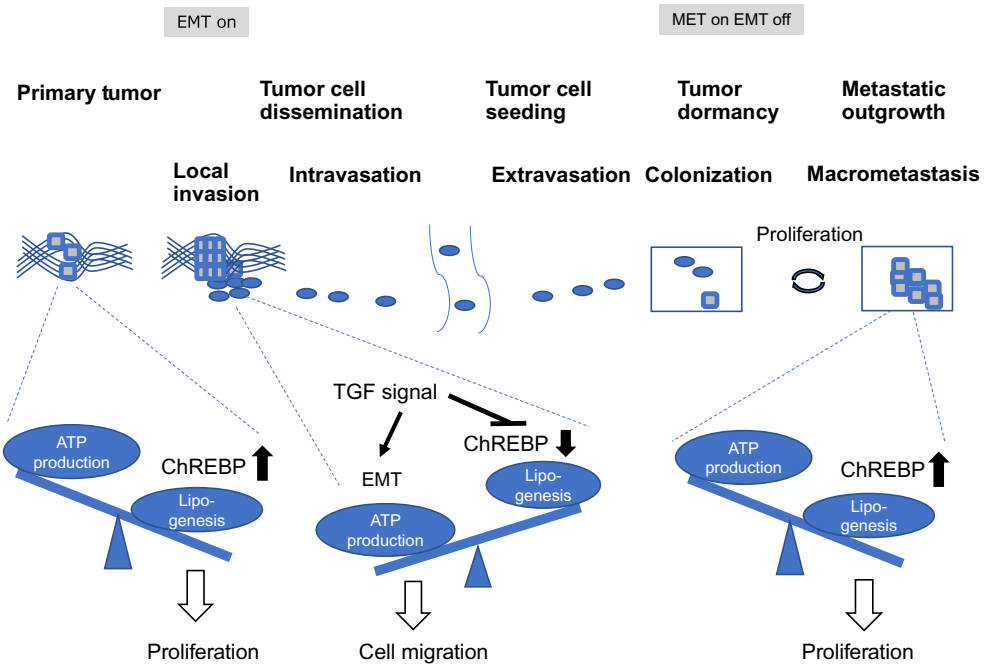

FIG. 4 Altered ChREBP expression at different stages of carcinogenesis. At the primary tumor stage, *Chrebp* expression is increased because of cell proliferation. At the stage of tumor dissemination, epithelial tumor cells lose their polarity and are converted into a mesenchymal phenotype (epithelial-mesenchymal transition, EMT). *Chrebp* expression is suppressed by TGFβ because of increased ATP demand for cell migration. At the metastatic colonization stage, the reverse process of EMT (mesenchymal-epithelial transition, MET) occurs, and cells are redifferentiated. At the metastatic outgrowth stage, *Chrebp* expression is increased, and tumor cells proliferate again.

signaling, protein synthesis, and cell cycle progression (Hindler et al., 2006). In addition, various antitumor effects of statin have been described, including inhibition of tumor growth, induction of apoptosis, repression of metastases, and antiangiogenic effects (high dose only) (Yi et al., 2017). In human, statins are reported to reduce the risk of HCC, pancreatic cancer, prostate cancer, gastric cancer, colorectal cancer, and breast cancer (Hindler et al., 2006). Moreover, atorvastatin treatment prevents fructose-induced ChREBP translocation and an increase in ChREBP DNA-binding activity (Rodríguez-Calvo et al., 2009). Atorvastatin also increased levels of phospho-PKA protein, increased PKA activity, and activated PKA, as well as inhibited the nuclear localization and DNA-binding activity of ChREBP (Rodríguez-Calvo et al., 2009). In an in vitro study, atorvastatin exposure to HepG2 cells enhanced levels of phospho-AMPK protein (AMPK activation) (Rodríguez-Calvo et al., 2009). Together, this indicates that ChREBP also might have some role in the prevention of cancer progression by statins.

Metformin

Metformin is a first-line drug given via the oral route for type 2 diabetes mellitus (T2DM) patients (Heckman-Stoddard et al., 2017). Multiple metaanalyses of case-control and cohort studies have reported a decreased in overall cancer incidence of approximately 10%–40%

with metformin use, along with a decrease in mortality by a similar range (Heckman-Stoddard et al., 2017). Metformin has been also associated with a decreased risk of breast, colon, liver, pancreas, prostate, endometrium, and lung cancer across metaanalyses (Heckman-Stoddard et al., 2017).

Metformin activity occurs mainly through direct action, via AMP kinase, and indirect action, via suppression of both the PI3K/Akt/mTOR and the Ras/Raf/MAPK pathways in insulin/IGF-1 signaling (Zi et al., 2018). Both AMPK activation and suppression of the insulin/IGF-1 pathway inhibit the mTOR pathway and thereby reduce cell proliferation and induce apoptosis. Moreover, in melanoma and breast cancer cells, metformin inhibits the expression of EMT markers (such as Snail) via activating AMPK (Xiao et al., 2016; Cerezo et al., 2013). Metformin was associated with marked reductions in cancer-specific mortality for colon, lung, and early-stage prostate cancer and improvements in overall survival for breast, colon, gynecological, liver, lung, prostate, and pancreatic cancer (Heckman-Stoddard et al., 2017). These findings suggest that metformin might play roles in the protection against tumor metastasis through the suppression of cell proliferation. Therefore metformin has cancer-preventing effects at different stages of cancer progression.

Metformin also inhibits ChREBP transcription activities via AMPK (Li et al., 2015; Sato et al., 2016). Metformin prevents the nuclear entry of ChREBP from the cytosol and inhibits the binding capacity of ChREBP to the promoter of thioredoxin interacting protein in endothelial cells (Li et al., 2015). Similarly, metformin suppresses ChREBP transcription activities in rat primary hepatocytes (Sato et al., 2016). These effects were mediated through AMPK activation. Although whether there is a relationship between metformin and cancer prevention remain unclear, metformin might be beneficial for preventing proliferative cancer cells through suppression of ChREBP activation resulting in suppression of de novo lipogenesis.

Conclusion

Increased ChREBP expression is associated with the progression of many types of cancer except acute myeloid leukemia. As ChREBP regulates lipogenic gene expression, ChREBP might contribute to tumor cell proliferation via upregulation of de novo lipogenesis and nucleotide synthesis. Statin and metformin regulate both pathways including ChREBP suppression through AMPK activation and thus may be desirable drugs for preventing cancer progression in patients with T2DM and hypercholesterolemia.

Glossary

Carbohydrate response element-binding protein (ChREBP) ChREBP is a glucose-activated transcription factor that regulates the pentose phosphate shunt and lipogenesis; ChREBP function is related to cell proliferation.

Hepatocellular carcinoma Hepatocellular carcinoma is a primary liver cancer that occurs in patients with chronic viral infection (hepatitis B or C) and nonalcoholic steatohepatitis.

Prostate cancer Prostate cancer is one of the most common cancers among males over the age of 50. Prostate-specific antigen testing is used as a screening test to detect prostate cancer.

Epithelial-mesenchymal transition Epithelial-to-mesenchymal transition is a cellular process loosely defined as a loss of cell polarity and tight junction and a gain of mesenchymal traits of mobility.

Pentose phosphate pathway The pentose phosphate pathway is a metabolic pathway that supplies NADPH and ribose 5-phosphate necessary for de novo lipogenesis and nucleotide synthesis, respectively.

De novo lipogenesis De novo lipogenesis is a pathway that converts excess carbohydrate into fatty acids, which are esterified to storage triacylglyceride. Glucose and insulin induce lipogenic gene expression through ChREBP and SREBP1c activation, respectively.

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CHAPTER

13

Metabolic syndrome: Protective potentials of dietary phenolic acids

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OUTLINE

SUMMARY POINTS

- This chapter focuses on the protective potentials of phenolic acids against metabolic syndrome.
- Metabolic syndrome is a metabolic disorders characterized by obesity, hyperglycemia, dyslipidemia, insulin resistance, and hypertension.
- Dietary phenolic acids form part of the daily diet intake, and they are responsible for the physiological and pharmacological values of functional foods.
- Phenolic acids enhance glucose uptake, inhibit FOXO-1, inhibit gluconeogenesis, and stimulate the activity of glycogen synthase through PI3K and Akt pathways.
- Phenolic acids lower dyslipidemia in metabolic syndrome by enhancing AMPK, preventing the translocation of sterol-binding protein-1c and transcription of

acetyl-CoA carboxylase and fatty acid synthase.

- The conversion of angiotensin I to angiotensin II, which is associated with hypertension and myocardial infarction, is also prevented by phenolic acids through the inhibition of angiotensin-converting enzyme.
- Oxidative stress resulting from the overproduction of reactive oxygen species generated in metabolic syndrome leads to peroxidation of membrane lipids, protein oxidation, DNA oxidation, and glycation. Phenolic acids enhanced the reactive oxygen species-detoxifying system and protects against lipid peroxidation, protein oxidation, and DNA fragmentation.
- Phenolic acid prevents inflammation in metabolic syndrome by inhibiting the release and translocation of NFκB into the nucleus. Consequently the release of proinflammatory factors is prevented.

Introduction

Metabolic syndrome is a cluster of metabolic disorders including hyperglycemia, insulin resistance, obesity, hypertension, and oxidative stress (Eckel et al., 2010). For any individual to be considered as having metabolic syndrome, such individual must present a condition with hyperglycemia, insulin resistance and obesity, or hypertension and oxidative stress (Kelliny et al., 2008). The etiologies of metabolic syndrome are largely due to genetic predisposition, physical inactivity, low socioeconomic status, smoking, heavy alcohol consumption, age, obesity, and deficiency of type 1 muscle fibers (Aydin et al., 2014). Several models have been documented for metabolic syndrome in rodents and extensively reviewed (Oron-Herman et al., 2008). In addition, the pathophysiology and mechanism of induction by these models are reported in literatures (Aydin et al., 2014; Das, 2014; Lim et al., 2010).

Currently the management and treatment of metabolic syndrome are directed at the components of metabolic syndrome (Ajiboye et al., 2016a, b). Furthermore, the emphasis is placed on regulation of diet, abstinence from smoking, moderate exercise, and drugs (Han and Lean, 2010). Demand for health-promoting foods has led to increasing investigations into bioactive compounds (phenolic acids, polyphenols, and micro- and macronutrients) that may have medicinal properties (Oloyede et al., 2013).

Phenolic acids are ubiquitous dietary phytochemicals present in fruits, nuts, and vegetables (Vinayagam et al., 2015). They are classified as benzoic or cinnamic derivatives depending on the phenolic backbone (Vinayagam et al., 2015). Experimental evidence has shown that phenolic acids improve the indices of diabetes by regulating carbohydrate and hepatic glucose metabolism (Latha and Daisy, 2011; Srinivasan et al., 2007). While there are varieties of phenolic acids in medicinal foods and fruits, caffeic acid, ferulic acid, gallic acid, and protocatechuic acid are the most common (Vinayagam et al., 2015). These compounds have also been reported to improve hyperglycemia, insulin resistance, dyslipidemia, and oxidative stress in fructose-induced metabolic syndrome (Ibitoye and Ajiboye, 2018). Other pharmacological activities of these compounds include antioxidant, antibacterial, hepatoprotective, and antiinflammatory activities (Ajiboye et al., 2018; Ajiboye et al., 2017a; Ibitoye and Ajiboye, 2019). The detailed pharmacological activities have been

extensively reviewed (Han et al., 2007; Rice-Evans et al., 1996; Vinayagam et al., 2015). In this chapter, we provide the potentials and mechanism of actions of phenolic acids in the treatment and management of metabolic syndrome.

Influence of phenolic acids on the components of metabolic syndrome

Hyperglycemia

Elevated blood glucose termed hyperglycemia is a component of metabolic syndrome (Eckel et al., 2010) and has been demonstrated in experimental models and clinical findings (Ajiboye et al., 2016a, b, 2017b). Fructose increases the synthesis of forkhead box protein O1 (FOXO1) (Dong et al., 2008) leading to the increase production of glucose through gluconeogenesis (Fig. 1; Lim et al., 2010). Studies have demonstrated that phenolic acids including caffeic acid, ferulic acid, gallic acid, and protocatechuic acid lowered blood glucose in diabetic and metabolic syndrome models (Ibitoye and Ajiboye, 2018). Caffeic acid improves glucose uptake in insulin-resistant mouse hepatocyte by increasing the expression of glycogen synthase kinase and phosphorylation of glycogen synthase at Ser641 in insulin-resistant mouse hepatocytes (Fig. 1) (Huang and Shen, 2012). Furthermore, it inhibited the activity of phosphoeneol carboxyl kinase and glucose 6-phosphatase, leading to the reduction of glucose via gluconeogenesis (Huang and Shen, 2012). Ferulic acid also lowers gluconeogenesis by inhibiting the interaction of FoxO1 with genes associated with gluconeogenesis (Fig. 1) (Narasimhan et al., 2015).

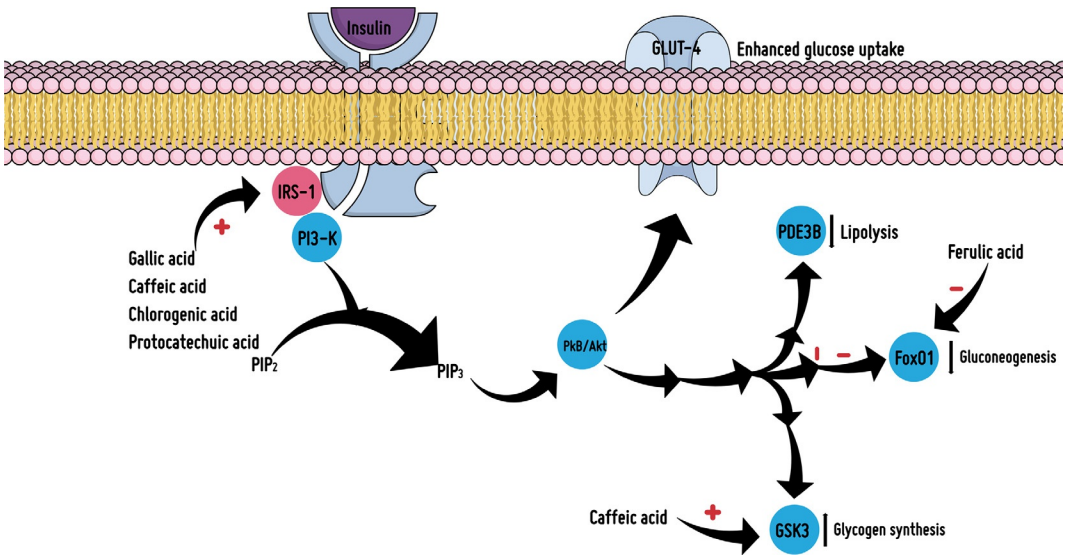

FIG. 1 Phenolic acids stimulate glucose uptake and glycogen synthesis while inhibiting gluconeogenesis via the PI3k pathway.

Interestingly, another study reported that caffeic acid lowers blood glucose by increasing the expression of insulin signaling-related proteins: (i) insulin receptor, (ii) phosphatidylinositol 3-kinase, (iii) Akt/protein kinase B, and (iv) insulin-degrading enzymes (Fig. 1). Indeed, these enhanced glucose uptake in neuro-2a mouse neuroblastoma (Chang et al., 2015). Gallic acid improves blood glucose by enhancing the level of peroxisome proliferator-activated receptor-γ expression in the adipose tissue (liver and muscle) of rats (Gandhi et al., 2014). It also improved insulin-dependent glucose transport in adipose tissue through translocation and activation of glucose transporter-4 (GLUT4) in PI3k/phosphorylated protein kinase B (p-Akt)-dependent pathway (Gandhi et al., 2014). In another related study, gallic acid activated AMP-activated protein kinase (AMPK) in the liver, muscle, and adipose tissue (Doan et al., 2015). It also activates peroxisome proliferator-activated receptor-γ coactivator. This activation stimulates AMPK and could result to increased insulin sensitivity and modulate body weight (Doan et al., 2015). Furthermore, gallic activates Sirt1, which could result to the increase in phosphoenolcarboxykinase and glucose 6-phosphate and inhibit gluconeogenesis (Doan et al., 2015).

Insulin resistance

The contribution of insulin resistance as one of the underlying factors to metabolic syndrome has been extensively reviewed and documented (Asrih and Jornayvaz, 2015). Indeed, studies have reported the development of insulin resistance in fructose overload (Huang et al., 2016; Prakash et al., 2014; Rabie et al., 2015; Shawky et al., 2014; Zhao et al., 2017). This event leads to increase glucose production, decrease glucose uptake, and hyperinsulinemia (Huang et al., 2016; Prakash et al., 2014; Rabie et al., 2015; Shawky et al., 2014; Zhao et al., 2017). Enhanced insulin sensitivity has been demonstrated in high fructose diet-induced metabolic syndrome rats treated with phenolic acids (Ibitoye and Ajiboye, 2018). Interestingly, gallic acids, caffeic acid, and chlorogenic acid improve insulin sensitivity by increasing the expression of insulin-signaling proteins. Insulin receptor substrate-1 (IRS), phosphatidylinositol 3-kinase, Akt/protein kinase B, and glucose transporter are particularly increased by these phenolic acid (Fig. 1) (Eid et al., 2012, 2017; Goto et al., 2012; Huang et al., 2009, 2016; Mubarak et al., 2013). The activation AMPK could further stimulate insulin sensitivity (Doan et al., 2015).

Recently, protocatechuic acid was shown to activate key components of insulin-signaling pathway: IRS-1, PI3K, and Akt (Fig. 1) (Scazzocchio et al., 2015). These insulin-signaling proteins were reported to be equally responsible for the restoration of insulin responsiveness of obese individuals (Ormazabal et al., 2018).

Dyslipidemia

Experimental studies have demonstrated the development of dyslipidemia in metabolic syndrome (Ajiboye et al., 2016a, b, 2017b). Fatty acids accumulate in high-fructose overload and are packaged as triglycerides into heavily fat-laden very low-density lipoproteins (VLDLs), causing dyslipidemia as they are cleared with low efficiency (Lim et al., 2010). Ibitoye and Ajiboye (2018) reported that phenolic acids (caffeic acid, ferulic acid, and gallic

acid) reversed high fructose diet–induced dyslipidemia in rats. Indeed, gallic acid lowered free fatty acids, triglycerides, and VLDL while elevating the level of HDL in high fructose-induced metabolic syndrome rats (Huang et al., 2016).

Gallic acid regulates dyslipidemia by upregulating AMP-activated protein kinase (AMPK) activity through the activation of peroxisome proliferator-activated receptor-γ coactivator1α (PGC1α; Fig. 2) (Doan et al., 2015). Similarly, caffeic acid and protocatechuic acid activate AMPK (Lee et al., 2007; Talagavadi et al., 2016; Tsuda et al., 2012), which could lead to the inhibition of sterol regulatory element-binding protein-1c (SREBP-1c) translocation into the nucleus and subsequent inhibition of lipogenesis and fatty acid synthesis (Fig. 2). Indeed, caffeic acid regulates the expression of gene (fatty acid synthase) responsible for lipogenesis in high-fat diet-induced metabolic syndrome (Liao et al., 2013). Interestingly, Herranz-López et al. (2017) reported that the phenolic acids of *Hibiscus sabdariffa* inhibits sterol regulatory element-binding protein-1c (SREBP-1c). This leads to downregulation in the expression of acetyl-CoA carboxylase and fatty acid synthase (Fig. 2), an action that lowers fatty acid synthesis and stimulates lipolysis (Herranz-López et al., 2017).

Furthermore the combined treatment of caffeic acid and ferulic acid induced the low-density lipoprotein receptor, SREBP, and LXRa mRNA in the liver of obese rats. In a related study, 5-caffeoylquinic acid, a derivative of caffeic acid, modulates the expression of

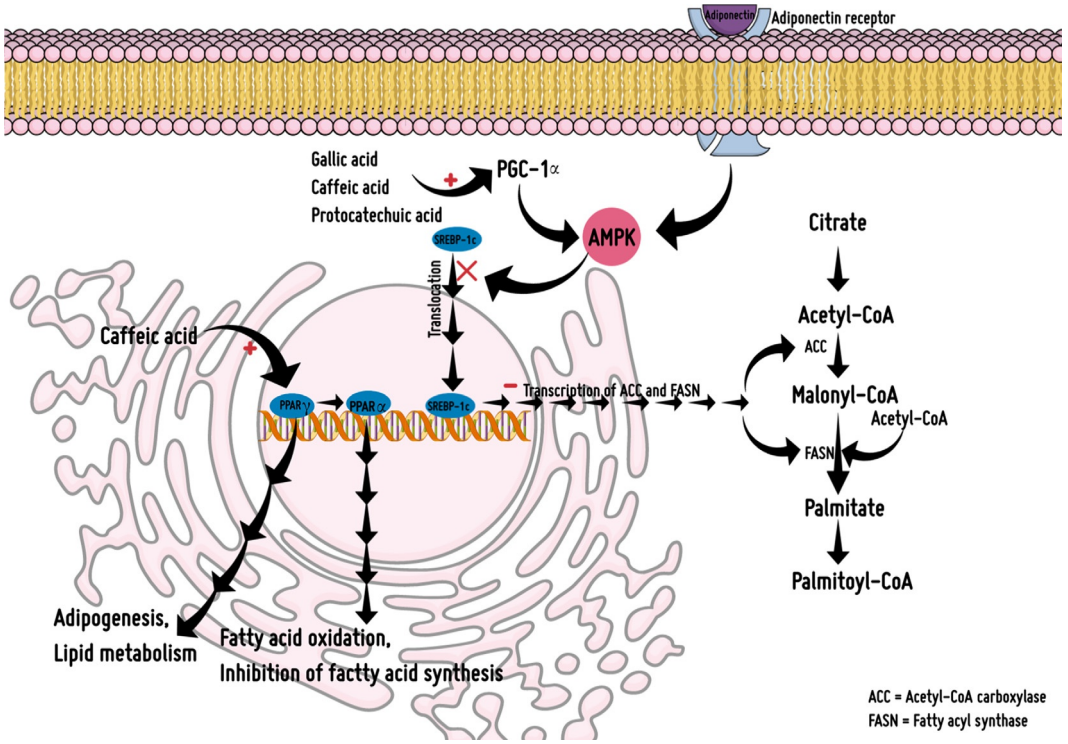

FIG. 2 Phenolic acids improve dyslipidemia by stimulating AMPK, which prevents the translocation of sterol regulatory element-binding protein-1c and the transcription of enzymes of fatty acid synthesis.

peroxisome proliferator-activated receptor α (PPAR α) and liver X receptor α (LXRα) (Huang et al., 2015). Strikingly, these phenolic acids improve lipid homeostasis by modulating the peroxisome proliferator-activated receptors expression leading to the activation of AMPK and downregulation of SREBP and fatty acid synthesis (Fig. 2).

Hypertension

Phenolic acids have the potential to prevent the development of hypertension possibly by preventing the loss of plasma nitric oxide, as well as cardioprotective effects by reducing angiotensin-converting enzyme activity and oxidative stress in the heart in rats. Interestingly, ferulic acid has been reported to suppress nitric oxide through the inhibition of endothelial nitric oxide synthase in metabolic syndrome rats (Senaphan et al., 2015). Similarly, protocatechuic acid regulates blood pressure by nitric oxide production (Semaming et al., 2015). Phenolic acids also serve as signaling molecule that promotes relaxation of the blood vessels, which will cause hypertension. These phenolic acids have been reported to inhibit angiotensin-converting enzyme, leading to the prevention of hypertension (Al Shukor et al., 2013). In support of this, chlorogenic acid significantly ($P \leq .05$) reduced both systolic and diastolic blood pressure. The inhibition of angiotensin-converting enzyme prevents the release of angiotensin II, myocardial infarction, and hypertension in metabolic syndrome (Fig. 3). Furthermore the inhibition of this enzyme stimulates the release of nitric oxide, which tones the heart.

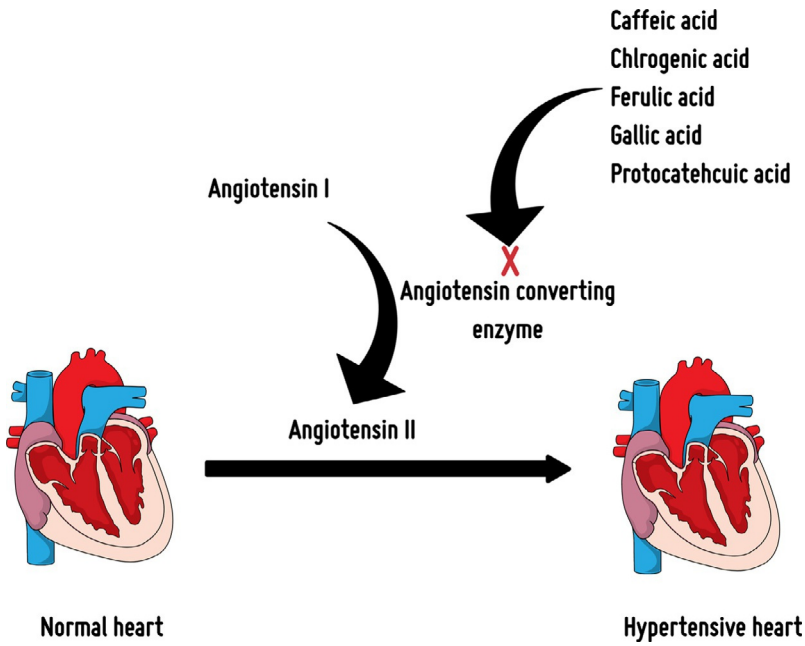

FIG. 3 Phenolic acid prevents the accumulation of angiotensin II by inhibiting the activity of angiotensin-converting enzymes leading to the release of nitric oxide that tones the heart muscle.

Oxidative stress

Oxidative stress, although not a primary event, has been demonstrated in metabolic syndrome (Ajiboye et al., 2016a, b, 2017b). It results from the imbalance in the cellular antioxidants and free radicals including reactive oxygen species produced, leading to damage of cellular macromolecules (Ajiboye, 2015). Redox imbalance occurs in fructose-induced metabolic syndrome when vicious cycles (TNF, reactive oxygen species, peroxynitrite, and products of lipid peroxidation) block the flow of electron in the respiratory chains. In addition, superoxide anion radical generated during the fructose metabolism (fructosylation of protein) contributes to reactive oxygen species generation. The antioxidant activities of phenolic acids have been reported in different animal models. We recently demonstrated the antioxidant properties of phenolic acids (caffeic, ferulic, gallic, and protocatechuic acids) in high-fructose-induced metabolic syndrome (Ibitoye and Ajiboye, 2018). This property could be due to enhanced expression of nuclear erythroid related factor-2 (Ajiboye et al., 2014) by these compounds (Song et al., 2016; Varì et al., 2011). In all the elevated glucose could lead to the generation of reactive oxygen species through (i) glycation of membrane protein, (ii) inhibition of glutathione reductase, (iii) NADH oxidase, and (iv) increased mitochondrial respiration (Fig. 4). Caffeic, chlorogenic, ferulic, gallic, and protocatechuic acids could halt the process of lipid peroxidation, protein oxidation, and DNA oxidation by scavenging these reactive species and also inducing the ROS-detoxifying system (Fig. 4).

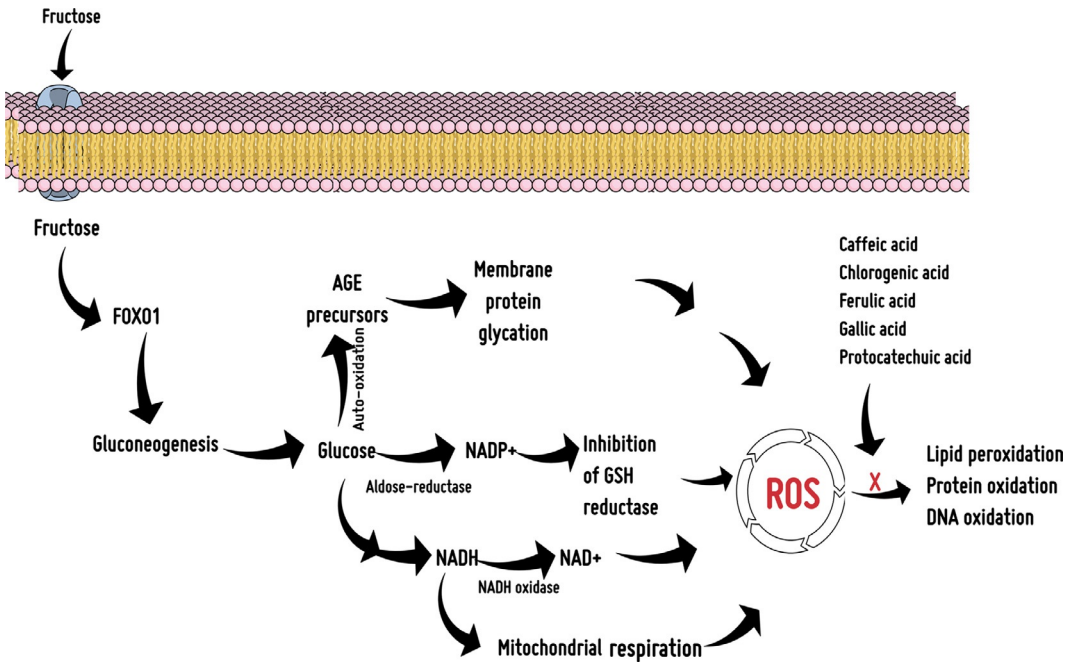

FIG. 4 Phenolic acids prevent oxidative stress in metabolic syndrome by stimulating the detoxification of reactive oxygen species leading to the prevention of lipid peroxidation, protein oxidation, and DNA oxidation.

Inflammation

Inflammation is a second hit event and central mechanism underlying insulin resistance and metabolic syndrome (Welty et al., 2016) making it an important target in the therapeutic management of this disorder (Ajiboye et al., 2016a, b). Phenolic acids are important regulator of inflammation. Interestingly, caffeic, ferulic, gallic, and protocatechuic acids have been reported to reversed metabolic syndrome-induced alteration tumor necrosis factor-α (TNF-α), interleukins-6, and interleukins-8 (Ibitoye and Ajiboye, 2018; Senaphan et al., 2015; Wang et al., 2018). Caffeic and chlorogenic acids mediate their antiinflammatory activity by downregulation of inflammatory transcription factor, nuclear factor-κB (NF-κB). This ultimately results in the inhibition of monocyte chemoattractant protein-1 (MCP-1), intercellular cell adhesion molecules (ICAM), and cyclooxygenase-2. This mechanism is possibly responsible for the reversal of metabolic syndrome-mediated increase in inflammatory cytokines. In all, phenolic acids halt the release and translocation of NFκB into the nucleus (Fig. 5). This event prevents metabolic syndrome-mediated increase in the proinflammatory cytokines (Fig. 5).

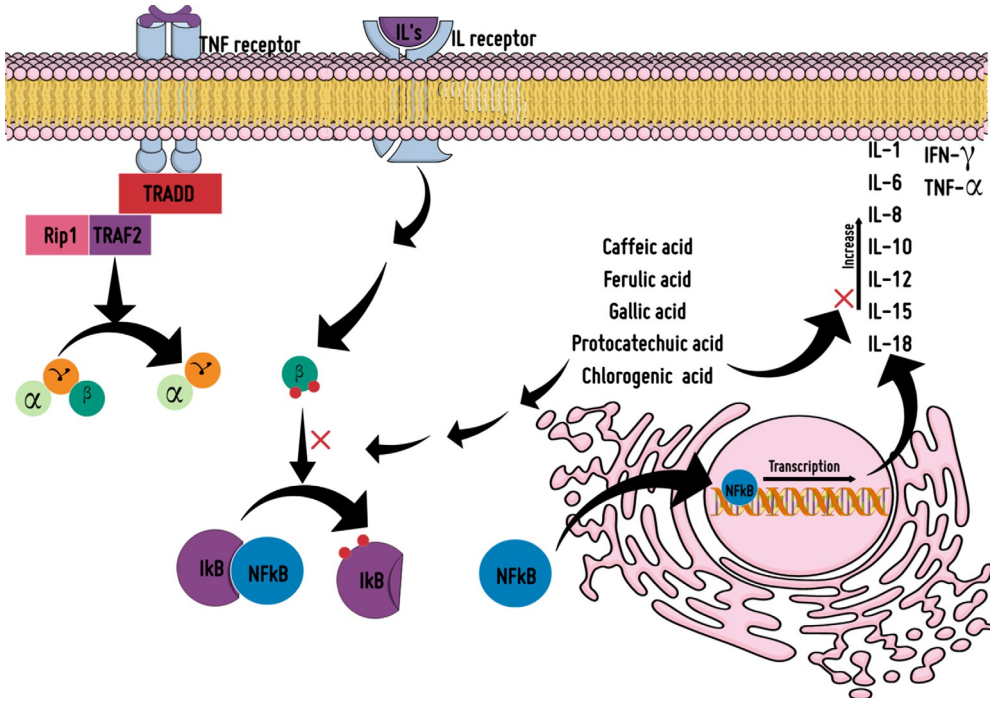

FIG. 5 Phenolic acids protect metabolic syndrome-induced inflammation by stopping the translocation of NFκB and preventing the increase in the inflammatory cytokines.

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CHAPTER

14

Ischemia-reperfusion injury and the roles of excess fructose: Effects on the heart and other organs

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OUTLINE

Key facts

- Excessive consumption of dietary carbohydrates is implicated in the pathogenesis of IRI.
- Excess fructose can reduce high-density lipoproteins and increase the circulation of triglycerides that may worsen myocardial IRI.
- By increasing the production of reactive oxygen species, fructose can reduce coronary blood and damage cardiac functional proteins.
- High-fructose diets may induce diabetic nephropathy to increase renal IRI.

- Excess fructose may worsen cerebral IRI via a proinflammatory mechanism comprising tumor necrosis factor-alpha and complement-protein expression.
- Excess fructose can induce diabetes, and it is likely that via this mechanism, fructose may worsen lung IRI.

Introduction

Obstruction of blood flow (often caused by atherosclerosis) is known as ischemia and affects the heart (Hausenloy and Yellon, 2016), kidney (Malek and Nematbakhsh, 2015), brain (Bai and Lyden, 2015), and lungs (Kirchner et al., 2015; Parambil et al., 2005). After percutaneous intervention is applied to restore blood flow to these organs (reperfusion), a large area of the organ exposed to ischemia is salvaged, but a smaller percentage undergoes further damage (referred to as ischemia-reperfusion injury or IRI) (Kalogeris et al., 2012). Long-term effects of IRI include death if IRI occurs in the heart (Hausenloy and Yellon, 2013), hypertension if it occurs in the kidney (Greite et al., 2018), and multiorgan damage if it occurs in the brain and lungs (Weyker et al., 2012).

Research has focused on understanding the pathophysiology of IRI (Carden and Granger, 2000), to gain knowledge that can aid in the design of modalities to protect organs and increase their longevity (Hausenloy and Yellon, 2013). This approach is also geared toward identifying specific factors that may contribute to IRI. Evidence suggests that contributing factors include lifestyle (Ravingerova et al., 2012), genetic predisposition (Burne et al., 2000; Yu et al., 2018), and other diseases (Fu et al., 2011; Lejay et al., 2016). Excessive consumption of dietary carbohydrates is also implicated (D'Annunzio et al., 2012; Maarman et al., 2012) as a contributing factor that renders organs more susceptible to IRI (Verma et al., 2002). Accordingly, recent work has shown that excess fructose, in particular, has a causal role in IRI (Maarman et al., 2017). However, the exact role of fructose in IRI is not fully understood and considering that it forms a considerable part of the human diet (Pereira et al., 2017), it is necessary to delineate its contribution to IRI. Therefore, the aim of this chapter is to review and discuss literature on fructose and IRI and to delineate the instrumental roles of fructose in IRI and stimulate novel hypotheses. Possible mechanisms employed by fructose are highlighted to provide an overview of the current literature.

Roles of fructose in IRI

Experimental evidence: A brief overview

Fructose is a simple carbohydrate that initially became well known as a replacement sweetener for patients with diabetes (Sievenpiper, 2017). This was mostly because fructose was considered a "natural" sweetener present in fruits, vegetables, and sugar cane (Rippe et al., 2017) and was shown to have a lower glycemic index compared with glucose (Sievenpiper, 2017). However, over the last decade, it was highlighted that the excessive consumption of fructose (Rippe et al., 2017) has pathologic consequences. Subsequently, others proposed that smaller

"catalytic doses" (Rippe et al., 2017; Sievenpiper, 2017) of fructose may have beneficial metabolic effects; however, this was recently refuted (Braunstein et al., 2018; Noronha et al., 2018).

Irrespective, there is overwhelming experimental evidence that excessive fructose consumption has pathologic effects (Madlala et al., 2016) on various organs (Hamden et al., 2018; Harrell et al., 2018; Mastrocola et al., 2018; Mellor et al., 2010; Shortliffe et al., 2015). The main hypothesis is that the pathologic effects of excess fructose are mediated by the increased production of reactive oxygen species (Maarman et al., 2017; Madlala et al., 2016; Mellor et al., 2010) and cytokines (Harrell et al., 2018; Pektas et al., 2016; Zhang et al., 2017). It is appreciable that via these factors, excess fructose can alter a number of pathologic processes that may contribute to or exacerbate IRI. However, the actions and underlying mechanisms of fructose might vary dependent on the IRI of the various organs (the heart, kidney, brain, and lungs).

Myocardial IRI and excess fructose

Myocardial infarction is a leading cause of death worldwide (Hausenloy and Yellon, 2013). In spite of percutaneous coronary intervention or thrombolytic therapy, the remnant of myocardial infarction is myocardial IRI (Hausenloy and Yellon, 2013). Myocardial infarction can be caused by pharmacological substances (Kameczura et al., 2013), thrombotic coronary occlusion (Frangogiannis, 2015; Heusch and Gersh, 2017; Montecucco et al., 2016), lifestyle factors (Akesson et al., 2014; Andres et al., 2011), or diet (Akesson et al., 2014; Iqbal et al., 2008). In addition, there is substantial experimental evidence that dietary factors also effect the outcomes of myocardial IRI (Donner et al., 2015; du Toit et al., 2008; Andreadou et al., 2017; Maarman et al., 2012).

A study by Mastrocola et al. (2016) exposed mice to a diet high in fructose (25% energy from fructose, for 12 weeks). Hearts from these mice displayed elevated hypertrophy and inflammatory markers (Mastrocola et al., 2016). In addition, an ex vivo IR protocol (30-min global no-flow, normothermic ischemia followed by 60-min reperfusion) led to greater infarct size and lactic dehydrogenase release in fructose-fed mice (Mastrocola et al., 2016). These findings were corroborated by another study during which rats were administered a high-fructose diet (60% energy from fructose, for 10 weeks) (Prakash et al., 2011). With an in vivo approach, myocardial IR was induced with left anterior descending coronary artery occlusion, followed by 180 min of reperfusion. The hearts of these rats displayed increased myocardial IRI, reflected by the greater myocardial infarcts, increased myocardial oxidative stress, and cardiac injury markers (Prakash et al., 2011). In concert, these studies demonstrate that excess fructose exacerbates myocardial IRI.

The underlying mechanisms for the adverse myocardial IRI are not fully understood. However, previous literature (Maarman et al., 2017) suggests that excess fructose may worsen myocardial IRI by deteriorating serum lipid profiles, increasing reactive oxygen species, and hampering cardiac function during IR episodes (Fig. 1). Excess fructose can reduce high-density lipoproteins and increase the circulation of triglycerides (Gruzdeva et al., 2013; Hwang et al., 1987; Reaven, 2012) that may worsen myocardial IRI. Furthermore, by increasing the production of reactive oxygen species, it can reduce coronary blood and damage cardiac functional proteins (Maarman et al., 2017; Miller et al., 1999) (Fig. 1).

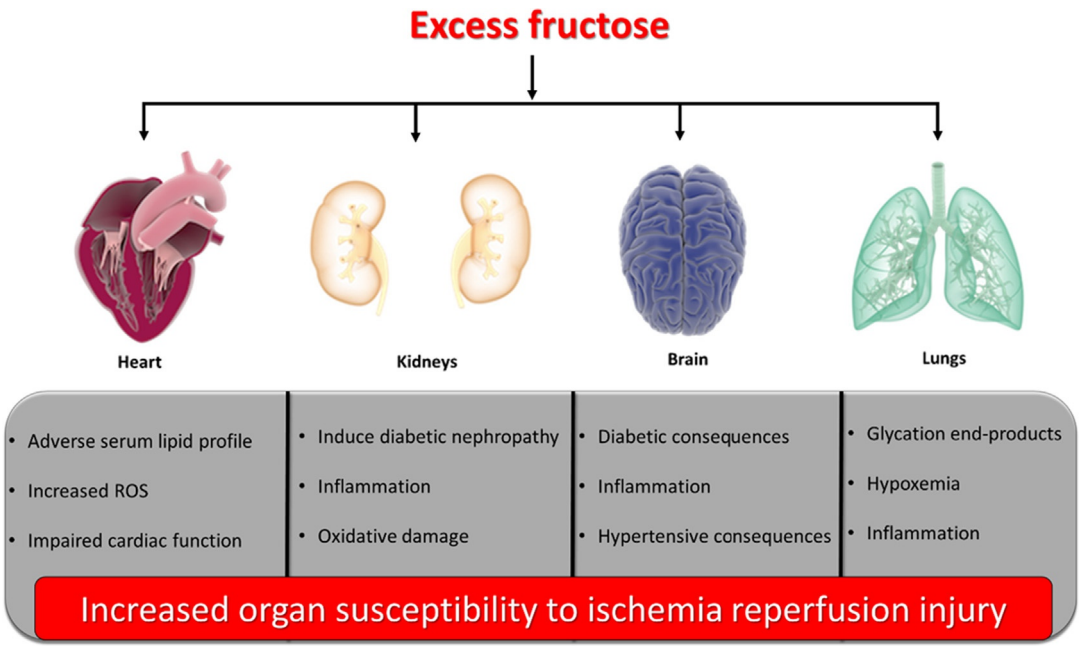

FIG. 1 This figure provides an overview of the literature discussed in this chapter and depicts how excess fructose adversely affects various organs. Via these mechanisms, excess fructose my increase organ susceptibility to ischemia reperfusion injury. *ROS*: reactive oxygen species.

Renal IRI and excess fructose

Renal infarction refers to renal parenchymal damage that is caused by restricted renal blood supply during kidney failure (Caravaca-Fontan et al., 2016; Kwon et al., 2016). Its prevalence is widely underdiagnosed and underreported (Kwon et al., 2016) and believed to occur mostly as an acute, medical emergency (Seetho et al., 2009). Upon restoration of blood flow and oxygenation, significant renal IRI is induced (Malek and Nematbakhsh, 2015). Few experimental studies have shown a link between renal IRI and dietary components (Jongbloed et al., 2017; Melin et al., 2003; Sharyo et al., 2008; Yang et al., 2017).

Rats whose diet was supplemented with fructose (1% energy from fructose, for 5 weeks) displayed significant renal injury/damage (Yang et al., 2014). In this study, the fructose-induced renal injury was reflected by excessive renal interstitial collagen deposition (Yang et al., 2014). Data demonstrated that fructose caused these renal defects by altering proinflammatory cytokines (interleukin-6 and tumor necrosis factor-alpha) and growth factors (plasminogen activator inhibitor and transforming growth factor-beta1) (Yang et al., 2014). Diets high in fructose also cause diabetes in rats (Lozano et al., 2016), and recent evidence shows that diabetes worsens renal IRI in rats (Ozbilgin et al., 2016). The mechanisms for diabetes-induced worsening of renal IRI include diabetic nephropathy (Maisonneuve et al., 2000). Unfortunately, there is a lack of studies investigating the impact of excess fructose on renal IRI. Therefore, future studies could test the hypothesis that a high-fructose diet induces

diabetic nephropathy to increase renal IRI (Fig. 1). However, it is plausible that excess fructose could increase renal injury and susceptibility to IRI, via diabetic nephropathy and inflammation (Fig. 1).

Cerebral IRI and excess fructose

Cerebral infarction is classified as either ischemic or hemorrhagic and refers to the cessation of cerebral blood flow (Saenger and Christenson, 2010). Statistics show that the global burden of cerebral infarction (absolute number of people affected by it) has increased significantly (Feigin et al., 2017; Thrift et al., 2016). It is caused by intracranial thrombosis or extracranial embolism or weakening and rupture of cerebral vessels (Saenger and Christenson, 2010). Upon restoration of blood flow due to recanalization, the brain displays moderate to severe IRI (Al-Mufti et al., 2018; Nour et al., 2013; Xu et al., 2018).

Literature suggests that there is a correlation between cerebral infarction/IRI and diet (Lakkur and Judd, 2015; Medeiros et al., 2012). Moreover, there is evidence that excess dietary carbohydrates can aggravate cerebral IRI in experimental models (Dong et al., 2015; Liu et al., 2015). A recent study investigated the impact of a high-fructose diet (55% energy from fructose) on cerebral measurements after induction of middle cerebral artery occlusion (90-min ischemia) (Harrell et al., 2018). Rats exposed to the fructose diet displayed a trend toward an increase in cerebral legions (though not statistically significant), as well as elevated inflammatory markers (Harrell et al., 2018). These findings suggest that excess fructose may worsen cerebral IRI via a proinflammatory mechanism comprising tumor necrosis factor-alpha and complement-protein expression (Fig. 1), while other research suggests that excess fructose could exacerbate cerebral IRI by inducing hypertension (Jiang et al., 2018; Klein and Kiat, 2015) or diabetes (Liu et al., 2015) (Fig. 1).

Lung IRI and excess fructose

Lung infarction has been described as the interruption of blood flow to the lung (Kirchner et al., 2015). It occurs in approximately 30% of patients with pulmonary thromboembolism (Miniati et al., 2015), malignancy, nonthrombotic embolism, and inflammatory or infiltrative lung diseases (Parambil et al., 2005). Restoration of blood supply to the ischemic lung area results in lung IRI (Weyker et al., 2012). The mechanisms of lung IRI include structural damage brought on by inflammatory and oxidative processes (Laubach and Sharma, 2016).

Although the causes of lung IRI are not generally related to diet, there is evidence that diet-related consequences can aggravate lung IRI (LaPar et al., 2012; Williams and Denlinger, 2017). This is partly mediated by advanced glycation end products, hypoxemia, and inflammation (LaPar et al., 2012; Williams and Denlinger, 2017). Currently, there is no available study on the effects of excess fructose on lung IRI, but considering that excess fructose can induce diabetes (Lozano et al., 2016), it is likely that via this mechanism, fructose may worsen lung IRI. Furthermore, there is evidence that diabetes makes the lung prone to injury (Honiden and Gong, 2009; Zheng et al., 2017) (Fig. 1). Therefore, future studies could investigate whether excess fructose-induced diabetes can increase the susceptibility of the lung to IRI.

Conclusions

The evidence reviewed in this chapter supports the hypothesis that excess fructose may contribute to IRI in a variety of organs. This effect of excess fructose appears to be underlined by its ability to induce diabetes, hypertension, oxidative stress, and inflammation.

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Conflict of Interest

The author has no conflict of interest to declare.

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CHAPTER

15

Linking pathways and processes: Retinoic acid and glucose

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OUTLINE

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SUMMARY POINTS

- This chapter focuses on the effect of all-trans-retinoic acid (*at*RA) on glucose and lipid metabolism.
- RA is synthesized from retinol by retinol dehydrogenase, and retinal dehydrogenases and cellular retinoid-binding proteins facilitate the retinoid metabolism.
- The effects of RA are mainly mediated through its nuclear receptor, retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimer.
- Carbohydrate response element-binding protein (ChREBP), sterol response element-binding protein (SREBP), and liver X receptor (LXR) are nutrient-sensing transcription factors (TFs).
- Hepatocyte nuclear factor (HNF)4α, peroxisome proliferator-activated receptor (PPAR)γ, and LXR are TFs regulating ChREBP, SREBP, and LXR expressions.
- RA regulation of HNF4α expression thereby affects expression of downstream TFs: PPAR, LXR, SREBP, and ChREBP.
- RA modulates small heterodimer partner (SHP) expression that affects several transcriptional activities linked to metabolism.
- RA is an inhibitor of the antioxidant response element-binding TF, Nrf2, continuous activation of which delays the onset of diabetes.
- CRABP2 is a substrate for Sirt1, glucose-dependent NAD^+-dependent protein deacetylase, in embryonic stem cells.
- Genetic modulation of RA synthesis affects energy metabolism.
- Polyol pathway enzyme, aldose reductase, is the regulator of epigenetic control of RAR-mediated transcription.

Introduction

Vitamin A (retinoid) is essential for multiple physiological processes, including vision, immune functions, reproduction, embryonic development, and cellular growth and differentiation (Blomhoff and Blomhoff, 2006). Vitamin A deficiency causes hepatic glycogen depletion, resulting from reduction of gluconeogenesis due to inadequate expression of phosphoenolpyruvate carboxykinase (*Pck1*), a key gluconeogenic enzyme (Shin and McGrane, 1997). Thus vitamin A also plays a role in the regulation of energy metabolism. Many of these pleiotropic activities of vitamin A are mediated by the activation of specific nuclear receptors, retinoic acid receptors (RARs), and retinoid X receptors (RXRs), which modulate transcription of numerous target genes. However, the mechanism of vitamin A action is not limited by RAR/RXR receptor. Indeed, retinoic acid (RA), an active metabolite of vitamin A, induced *Pck1* expression is mediated through p38 mitogen-activated protein kinase (MAPK)-stimulated activating transcription factor (ATF)-2 (Lee et al., 2002).

It is well known that RA suppresses adipogenesis. However, we showed the effect of RA on lipid accumulation changed depending on the extracellular glucose concentration in 3T3-L1 cell culture (Abd Eldaim et al., 2017). That is, the cells treated with RA in normal glucose (5.5 mM) suppressed lipid accumulation, whereas in high glucose (25 mM) the cells treated with RA enhanced lipid accumulation. The changes in lipid accumulation by RA were

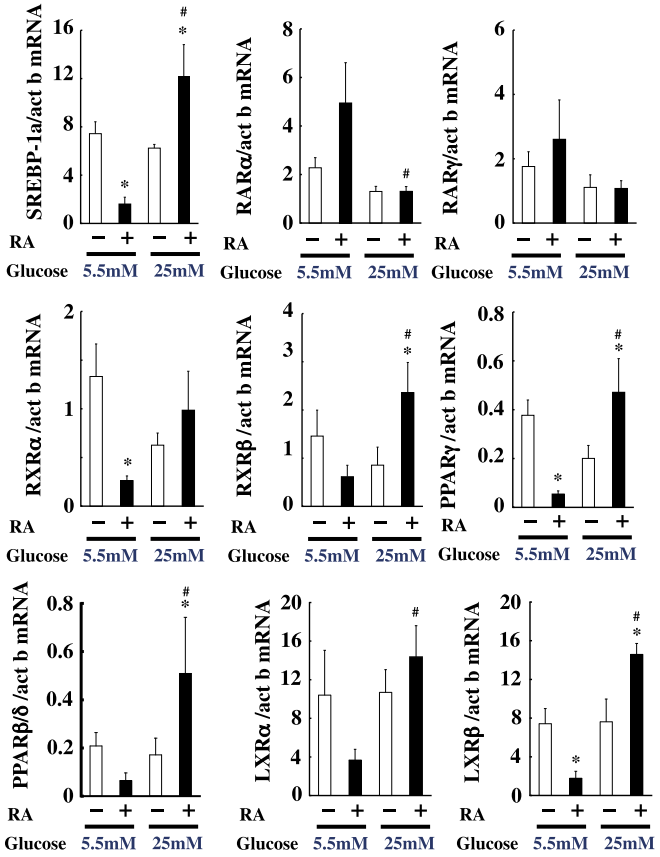

FIG. 1 Effects of retinoic acid on expression of nuclear receptor genes in 3T3-L1 cells cultured for 24 h in normal and high-glucose medium. 3T3-L1 cells were cultured with DMEM containing 5.5-mM glucose or 25-mM glucose, either in the presence (+) or absence (−) of retinoic acid (RA, 1 μM) for 24 h after the addition of the adipogenic differentiation cocktail. Expression of nuclear receptors, RARα, RARγ, RXRα, RXRβ, PPARγ, PPARβ/δ, LXRα, and LXRβ and of SREBP-1a were quantified by real time PCR and normalized to the level of β-actin (*act b*) mRNA expression. Statistical significant differences ($P < 0.05$) are indicated by * (control vs. RA treatment) and # (5.5 mM glucose vs. 25 mM glucose).

dependent on sterol regulatory element-binding protein (*SREBP*)-1-mediated fatty acid synthase (*Fas*) expression. Analyses of expression profiles of the nuclear receptor related to RA action (Fig. 1) suggest that liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR), rather than RARs, were involved in the regulation of SREBP-1 expression. Further analyses allow us to conclude that LXR enhanced PPARγ and LXRβ induction in a RA-dependent and high glucose-dependent manner and those sequentially activated expression of SREBP-1a, which then mediate *Fas* gene expression (Fig. 2).

In this chapter, we summarized the current knowledge about regulatory mechanisms of RA-dependent and glucose-dependent gene expressions and their crosstalk to understand control of carbohydrate metabolism by RA, especially TF cascade as seen in Fig. 2.

Outline of vitamin A metabolism

Vitamin A itself cannot be synthesized in mammals and therefore must be ingested from a diet (Noy, 2000; Zhang et al., 2015; Napoli, 2017). In postfeeding period, chylomicrons carry absorbed vitamin A as retinyl esters in the circulation. After changing chylomicrons to

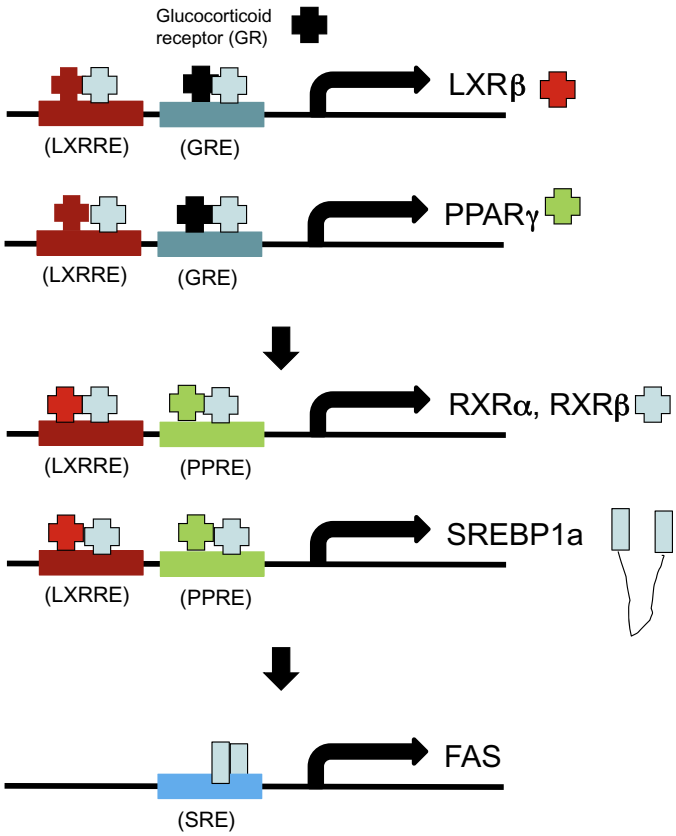

FIG. 2 Sequential induction of transcription factors enhances *Fas* gene expression, which supports retinoic acid-induced lipid accumulation in 3T3-L1 cells cultured in high-glucose medium. This figure represents the schematic diagram based on the results by Abd Eldaim et al. (2017).

chylomicron remnants, the latter are taken up by hepatocytes, and retinyl esters are converted to retinol. Newly formed retinol can be either stored in hepatic stellate cells (Ito cells) in the form of retinyl esters or secreted into the blood with retinol-binding protein 4 (RBP4). Retinol-RBP4 circulates in the bloodstream in a 1:1 molar complex with transthyretin that is also synthesized in and secreted from the liver. The retinol-RBP4-transthyretin complex prevents retinol-RBP4 elimination by the kidney.

RBP4 delivers retinol to target tissues. Uptake of plasma retinol by peripheral cells is mediated by the stimulated by retinoic acid 6 (STRA6) gene product (Kelly and von Lintig, 2015). It is a high-affinity cell surface receptor for RBP that forms transmembrane pore to transport retinol into a cell. Retinol is not biologically active per se and binds one of cellular retinol-binding proteins, CRBP1, to promote efficient use of retinol in the cell (Noy, 2000; Napoli, 2017). Thereafter, retinol is reversibly oxidized to retinaldehyde (retinal) by retinol dehydrogenase (RDH) activity, and then, retinal is irreversibly oxidized to RA by retinal dehydrogenases (RALDH) (Moise et al., 2006; Napoli, 2017). Synthesized RA can be directed by cellular RA-binding protein (CRABP) 2 or fatty acid-binding protein type 5 (FABP5) to the nucleus for activation of specific TFs (nuclear receptors): RARs or PPARβ/δ, respectively. RA is also delivered to catabolic enzymes like CYP 26A1 by CRABP1 and CRABP2 (Napoli, 2017).

Retinal is active in the visual cycle (Noy, 2000). RA modulates overexpression of 500 genes through RAR-mediated gene transcription. RAR consist of three subfamily proteins: RARα, RARβ, and RARγ and bind both all-trans-RA (*at*RA) and its isomer, 9-cis-RA (di Masi et al., 2015; Zhang et al., 2015). RAR associates with retinoid X receptors (RXRs) to form RAR/RXR heterodimer that regulates transcription of a variety of target genes through its interaction with RA response element (RARE) in the promoter region of the respective genes. However, in the absence of ligand, corepressor protein is bound to RAR/RXR heterodimer to repress transcription. Binding of ligands to RAR/RXR results in dissociation of corepressor and recruitment of coactivator protein, which, in turn, promotes transcription of the target genes such as uncoupling protein 1 (*Ucp1*) and hormone-sensitive lipase (*Hsl*).

There are three RXRs (RXRα, RXRβ, and RXRγ) that bind to 9-cis-RA with high affinity, but not to all-trans-RA. In addition to forming RAR/RXR heterodimers, RXRs form homodimers and regulate transcription following binding to RAREs located in the promoters of target genes. Administration of RXR-specific agonist to diabetic rats increased the following gene expression: carnitine palmitoyl transferase I (*Cpt1*), stearoyl-CoA desaturase 1 (*Scd1*), and fatty acid translocase (*Cd36*) in the liver.

RXRs can associate with other nuclear receptors including PPARs, thyroid hormone receptor (TR), farnesoid X receptor (FXR), and LXR to form heterodimers. Responding to the cognate ligands, heterodimers bound to respective responsive elements activate or repress gene transcription.

TFs involved in carbohydrate metabolism

Carbohydrate response element-binding proteins (ChREBPs)

ChREBPα is a glucose-regulatable TF that belongs to the basic helix-loop-helix/leucine zipper (bHLH/LZ) TF family and forms heterodimer with Max-like protein X (MLX) (Abdul-Wahed et al., 2017). As a mechanism by which glucose activates this TF, xylulose 5-phosphate, a metabolite of the pentose phosphate pathway, was first proposed to activate ChREBPα through dephosphorylation by protein phosphatase 2A activation. ChREBPα dephosphorylation is suggested to cause sequential dissociation from cytosolic 14-3-3 protein, translocation to the nucleus, and activation of glycolytic and lipogenic genes containing the carbohydrate response element (Kabashima et al., 2003). Recent studies suggest that glucose-6-phosphate, the product of glucokinase, is an allosteric regulator of ChREBPα through their direct binding (Dentin et al., 2012) and that fructose 2,6-bisphosphate, an allosteric regulator of phosphofructokinase-1 and fructose bisphosphatase-1, is mediated glucose-induced recruitment of ChREBPs to its target genes (Arden et al., 2012).

ChREBP activity is also regulated by other posttranslational modifications such as *O*-GlcNacylation, acetylation, and phosphorylation. Glucagon activates protein kinase A (PKA), which, in turn, phosphorylates ChREBP to accelerate the binding to 14-3-3 scaffolding protein (Kawaguchi et al., 2001, 2002). In addition, PKA phosphorylates phosphofructokinase-2 to decrease fructose 2,6-bisphosphate, suggesting glucagon signal suppresses ChREBP activity. In response to fasting, AMP-activated protein kinase (AMPK) phosphorylates ChREBP, which reduces its binding ability to DNA. Further, AMP and ketone

bodies can act as an allosteric inhibitor of ChREBP dissociation from 14-3-3 (Sato et al., 2016). On the other hand, in response to high-glucose concentrations, ChREBP in nucleus can be modified by *β-N*-acetylglucosaminyltransferase and *O*-GlcNacylation of ChREBP enhances DNA binding and protein stability (Guinez et al., 2011).

It is suggested that ChREBP alone or in concert with LXRs induce expression of glycolytic and lipogenic enzymes in the liver. As the ChREBP promoter contains a binding site for LXR, both LXR and TRβ are supposed to regulate the glucose-dependent ChREBP expression in the liver (Gauthier et al., 2010). However, HNF4α is identified as a key TF that activates the expression of both ChREBP isoforms in response to glucose (Meng et al., 2016). HNF4α also contributes to glucose-inducible *Fas* gene expression through physical interaction with ChREBP (Adamson et al., 2006). Moreover, Caron et al. (2013) have shown the presence of complex of ChREBP, HNF4α, FXR, and the transcriptional coactivators having an intrinsic acetyltransferase activity (p300 and CREB-binding protein, CBP), in the selective region of liver-type pyruvate kinase (*L-Pk)* gene promoter at high-glucose concentrations without FXR ligand. In contrast, FXR activation by a bile acid results in the release of ChREBP, p300, and CBP from the region without affecting FXR and HNF4α binding and concomitant recruitment of the transcriptional corepressor, silencing mediator of retinoid and thyroid receptor (SMRT). Thus ChREBP activity on the expression of *L-Pk* gene can be abrogated by bile acid through FXR activation.

ChREBPβ mRNA is a splicing variant highly expressed in white adipose tissue. Its product has less amino acids than the ChREBPα product, and it lacks 14-3-3 interacting domain and low-glucose inhibitory domain, suggesting constitutive active protein. However, ChREBPβ seems to act as negative feedback regulator, after induction by ChREBPα (Herman et al., 2012; Jing et al., 2016).

Sterol response element-binding proteins (SREBPs)

SREBPs are TFs from the bHLH/LZ TF family, encoded by *Srebp-1* and *Srebp-2*. SREBP-1 is responsible for the genes required for de novo lipogenesis. SREBP-2 regulates the genes of cholesterol metabolism. SREBPs synthesized as inactive precursors are localized in the endoplasmic reticulum membranes through two central hydrophobic transmembrane segments having N-terminal TF region. Through its C-terminal region, SREBPs are associated with SREBP cleavage-activating protein (SCAP) that also interacts with insulin-induced genes (INSIGs). In cells with low levels of sterols, the SREBP-SCAP complex is dissociated from INSIGs and move to the Golgi where SREBP undergoes cleavages to release its N-terminal region, a mature active form. Active SREBP is then translocated to the nucleus and binds to specific sterol regulatory element in DNA sequences, thus upregulating the synthesis of enzymes involved in lipid and sterol biosynthesis. Sterols in turn inhibit the process of SREBP cleavage, and therefore, synthesis of additional sterols is reduced through a negative feedback loop (Soyal et al., 2015).

Insulin enhances SREBP-1c activity at several steps. Insulin promotes SREBP-1c proteolytic processing through its phosphorylation by Akt/PKB, a downstream effector of insulin action and suppression of the gene encoding *INSIG2A*. Insulin also controls nuclear localization of SREBP-1c by inducing mammalian target of rapamycin-mediated phosphorylation of lipin,

which inhibits nuclear localization of SREBP-1c when dephosphorylated. In addition, insulin enhances stability of nuclear SREBP-1c via glycogen synthase kinase 3 β-mediated phosphorylation (Soyal et al., 2015). SREBP-1c activity is also controlled at transcriptional levels. Since there are two functional LXR response elements on the SREBP-1c promoter and SREBP-1c is not induced by insulin in mice deficient in LXRα/β, the functional LXRs are necessary for its induction of SREBP-1c by insulin (Shimano, 2001). In addition, SREBP-1 functions as an autoregulatory positive feedback TF (Shimano, 2001).

Glucagon suppresses *SREBP*-1c mRNA expression by activating PKA signaling pathway (Tang et al., 2009) Lately, it is reported that the fasting-induced TF, Krüppel-like factor 15 (KLF15), a key regulator of gluconeogenesis, forms a complex with LXR/RXR, specifically on the *Srebp-1* promoter. This complex recruits the corepressor receptor-interacting protein 140, resulting in reduced *Srebp-1* and thus downstream lipogenic enzyme expression. Thus KLF15 enables rapid switching between lipogenesis and gluconeogenesis during fasting by controlling *Srebp-1* expression (Takeuchi et al., 2016).

Liver X receptors (LXRs)

The LXRs are known as oxysterol-activated nuclear TFs containing two Zn finger motifs in the DNA-binding domain. LXRs consist of two subtypes, LXRα and LXRβ. LXRα is predominantly expressed in lipogenic tissues including the intestine, adipose, kidney, and adrenals, whereas LXRβ is ubiquitously expressed. Both subtypes form heterodimer with RXRs and regulate the expression of genes involved in cholesterol homeostasis directly or control lipogenesis and glucose metabolism indirectly through the expression of TFs including ChREBP and SREBP-1 (Gauthier et al., 2010; Laurencikiene and Ryden, 2012; Schulman, 2017). In the absence of ligands, LXR/RXR is in a nonactive state, binding to a cognate LXRE with corepressors such as nuclear corepressor 1 (NCoR1) or SMRT. Binding of ligand induces conformational changes of LXRs that enables recruitment of coactivators such as PPARγ coactivator-1 (PGC-1α) to replace corepressors and, in turn, the direct activation of gene transcription (Gabbi et al., 2014). Interestingly to note, Mitro et al. (2007) have demonstrated that D-glucose and D-glucose-6-phosphate bind and stimulate the transcriptional activity of both LXRα and LXRβ. Glucose at physiological concentrations expected in the liver induces the expression of LXR target genes with efficacy similar to that of oxysterols. Cholesterol homeostasis genes that require LXR for expression are upregulated in the liver and intestine of fasted mice refed with a glucose diet, indicating that glucose is an endogenous LXR ligand.

Hepatocyte nuclear factor 4α (HNF4α)

HNF4α is orphan nuclear factor containing a Zn finger DNA-binding domain and shows significant sequence similarities to the RXRα. However, no transcriptional active heterodimer is formed with any of the other nuclear receptors identified. HNF4α forms a homodimer to bind a relatively degenerate consensus DNA sequence and is considered to be a master regulator of liver-specific gene expression, such as microsomal triglyceride transfer protein (*Mtp*) and apolipoprotein B (*ApoB*), both are essential for the formation and secretion of very-low-density lipoprotein (Hayhurst et al., 2001). HNF4α activity is regulated both at the

transcriptional and posttranscriptional levels by different mechanisms. Several kinases including PKA, protein kinase C, AMPK, and p44/p42 MAPK were shown to phosphorylate HNF4α at different sites and decrease its transcriptional activity (Vető et al., 2017). Moreover, HNF4α interacts with SHP resulting in direct competition with coactivator binding and consequent repression of the transcription of target genes (Zhang et al., 2011). It is also reported that phosphorylation of coactivator PGC-1α by p70 ribosomal protein S6 kinase 1 specifically interferes with the interaction between HNF4α and PGC-1α and blocks coactivation of the gluconeogenic target genes (Lustig et al., 2011).

Peroxisome proliferator-activated receptors (PPARs)

PPARs are known as a ligand-activated nuclear TFs containing Zn fingerlike DNA-binding domain. PPARs consist of three subtypes, PPARα, PPARβ/δ, and PPARγ, and each subtype forms heterodimer with RXRs and regulates the expression of genes by binding to specific DNA sequences, peroxisome proliferator response elements. In the unliganded conformation, however, PPAR/RXR dimer forms the complex with corepressors, SMRT and NCoR, to suppress transcription. In the presence of agonists such as long-chain fatty acid and its derivatives, an exchange of corepressors to coactivators including SRC-1, CBP/p300, and PGC-1 activates transcription of PPAR target genes (Nakamura et al., 2014).

PPARα is richly expressed in the liver, heart, skeletal muscle, brown adipose tissue, and kidney. PPARα activates transcription of the genes involved in peroxisomal and mitochondrial β-oxidation, fatty acid transport, and hepatic glucose production, the latter being rodent specific (Xu et al., 2002; Pawlak et al., 2015).

PPARβ/δ is expressed ubiquitously. In skeletal muscle, PPARβ/δ is the most dominant subtype expressed, and the protein is detected in oxidative type 1 fibers more abundantly than glycolytic type 2 fiber. PPARβ/δ is also activated by RA (Napoli, 2017). In preadipocytes, where CRABP2 and RARα are expressed, RA increases Cyp26a gene expression 10-fold. Although the expression of RARα and CRABP2 was decreased, the expression of PPARβ/δ and FABP5 was increased after induction of differentiation. RA and a PPARβ/δ ligand, GW0742, induced mRNA expression of insulin-dependent glucose transporter, Glut4, in mature adipocytes compared with preadipocytes (Wolf, 2009).

PPARγ is mainly present in adipose tissue, the large intestine, and macrophages. PPARγ agonists such as thiazolidinediones are shown to accelerate adipogenesis and functions as an insulin sensitizer. In contrast, PPARγ knockout mice fail to generate adipose tissue when fed a high-fat diet. Therefore PPARγ regulates the genes related to adipogenesis and insulin sensitivity in fat cells (Janani and Kumari, 2016).

Crosstalk between retinoid- and glucose-dependent pathways to regulate gene expressions

RA regulation of HNF4α expression, thereby affects expression of downstream TFs, PPAR, LXR, SREBP-1, and ChREBP

The relationship between retinoid metabolism and HNF4α has been indicated. HNF4α can regulate the intracellular transport of RA by activating the transcription of CrbpII (Nakshatri

and Chambon, 1994). On the other hand, RA strongly inhibits α-fetoprotein expression through the RARE-mediated downregulation of HNF1 and HNF4 expression in Hep3B cells (Magee et al., 1998).

Recently reported a novel transcriptional repressor, hairy and enhancer of split 6 (Hes6) gene promoter, is specifically activated by the RAR in response to *at*RA. Hes6 subsequently represses HNF4α-activated PPARγ2 gene expression by direct inhibition of HNF4α transcriptional activity (Kim et al., 2014). Further, in the absence of RAR ligand, SHP represses Hes6 expression through RAR, and decreased level of Hes6 then derepresses HNF4α-regulated PPARγ2 expression. Thus it is most likely that RA controls HNF4α protein by suppressing its RARE-dependent transcription and HNF4α activity through Hes6 repressor.

As aforementioned, expressions of the TF related to carbohydrate (glucose and lipid) metabolism are regulated by each other: HNF4α activates both PPARγ and ChREBP genes (Kim et al., 2014; Meng et al., 2016). PPARγ activates transcription of LXRα gene (Laffitte et al., 2001). LXRα increases SREBP-1 and ChREBP transcription (Shimano, 2001; Gauthier et al., 2010). Interestingly there are hierarchies among the receptors, and the diagram of their relationship reveals RA-RAR is on top of the cascade (Fig. 3). At the second row, there are steroid hormone family receptors including HNF4α, PPAR, LXR, and FXR. At the bottom, both bHLH/Zip-type TFs exist. It is well known that RA through RAR activates *Ucp1* expression and lipolytic genes that lead to increase energy utilization. In contrast, HNF4α, PPAR, LXR, FXR, SREBP, and ChREBP are involved in biosynthetic pathways to store energy (Table 1). To shift from energy storage to energy consumption, it is the most efficient to switch HNF4α activity from on to off, which is located on the second top of the cascade. Indeed, RA-RAR is able to control transcription and function of HNF4α, suggesting the possibility that RA-RAR indirectly governs all the transcriptional factors listed.

As many of gene transcriptions are regulated by multiple factors, actual interrelationship may not be simple like Fig. 3. However, this fundamental notion shown in Fig. 3 is worth considering, taking into account the regulation of carbohydrate and retinoid metabolism, nuclear receptor biology, and their possible linkage.

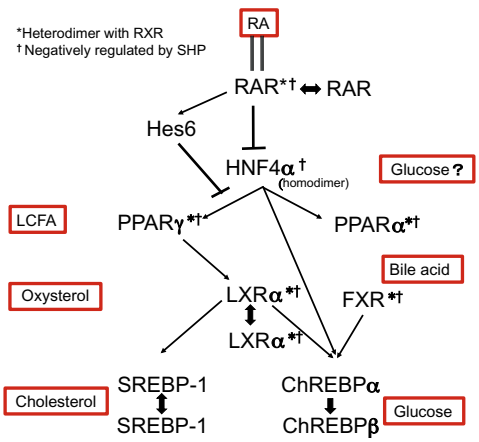

FIG. 3 Relationship among the nutrient-sensing receptors and their autoexpression. This figure represents the diagram of hierarchical relationship among transcription factors in their own expression. As discussed in the Section "RA regulation of HNF4α expression, thereby affects expression of downstream TFs, PPAR, LXR, SREBP-1 and ChREBP", activation of RAR can inhibit HNF4α expression directly and PPARγ expression indirectly via Hes6. HNF4α activates ChREBP expression and PPARγ expression. PPARγ activates LXRα expression, which in turn activates expression of LXR, SREBP-1, and ChREBP. SREBP-1 and ChREBP have self-inducibility. Therefore we propose the possibility that inhibition of the upstream of the cascade such as HNF4α by RA-dependent RAR activity influences downstream transcription factors.

TABLE 1 Target genes for respective transcription factors

RAR: uncoupling protein 1 (Ucp1)[20], hormone-sensitive lipase (Hsl)[20], glucokinase (Gck)[1, 2], phosphoenolpyruvate carboxykinase (Pck1)[3], Hes6[4], (repress) HNF4α[5]

RXR: carnitine palmitoyl transferase I (Cpt1)[21], stearoyl-CoA desaturase 1 (Scd1)[22], fatty acid translocase (Cd36)[23]

HNF4αa: ChREBP[7], PPARγ[4], SHP[8], cellular retinol-binding protein 2 (Crbp2)[9], α-fetoprotein[5], apolipoprotein (Apo)a2[10], Apob[10], Apoa4[10], Apoc2[10], Apoc3[10], Apoe[24], microsomal triacylglycerol transfer protein (MTP)[10], CYP7A1[11]

LRH-1: scavenger receptor class B type 1 (SR-BI)[25], steroidogenic acute regulatory protein (StAR)[26], a-fetoprotein[27], Gck[28], CYP7A1[11], CYP8B1[11], SHP[8]

FXR: SHP[8], PPARα[29], Apoc2[30], (repress) SREBP-1c[31], ChREBP[12], L-Pk[12]

PPARα: PGC-1a[36], fatty acid-binding protein 1 (L-Fabp)[32], Cd36[32], Acyl-CoA oxidase[33], 3-ketoacyl-CoA thiolase[34], fatty acid transport protein (Fatp)[35]

PPARβ/δ: L-Fabp[32], Cd36[32], Glut4[13]

PPARγ: LXRα[14], SHP[8], fatty acid-binding protein 4 (aP2)[37], Pck1[38], lipoprotein lipase (Lpl)[39], Ucp1[40], Cd36[41], Glut4[42]

LXR: LXR[14], ChREBP[15], Srebp-1[16], SHP[8], ABCA1[43], ABCG1[44], ABCG5/ABCG8[45], Apoe[46], CYP7A1[47], acetyl-CoA carboxylase (Acc)[48], fatty acid synthase (Fas)[49]

SREBP-1: Srebp-1[16], ATP-citrate lyase[50], acetyl-CoA synthetase[51], Acc[52], Fas[53], Scd1[54], glycerol 3 phosphate acyltransferase[55], SHP[8]

SREBP-2: low-density lipoprotein (LDL) receptor[56], HMG-CoA reductase[56], HMG-CoA synthetase[56]

ChREBP: ChREBP[17, 18], L-Pk[12], Acc[57], Fas[19, 57], Scd1[57], glucose-6-phosphatase catalytic subunit[57]

KLF15: Glut4[58], acetyl-CoA synthetase 2[59], Pck1[60], alanine aminotransferase 1[61]

Reference in the text: (1) Cabrera-Valladares et al. (2001), (2) Li et al. (2014), (3) Lee et al. (2002), (4) Kim et al. (2014), (5) Magee et al. (1998), (6) Wang et al. (2007), (7) Meng et al. (2016), (8) Zhang et al. (2011), (9) Nakshatri and Chambon (1994), (10) Hayhurst et al. (2001), (11) Goodwin et al. (2000), (12) Caron et al. (2013), (13) Wolf (2009), (14) Laffitte et al. (2001), (15) Gauthier et al. (2010), (16) Shimano (2001), (17) Herman et al. (2012), (18) Jing et al. (2016), (19) Adamson et al. (2006).
References not in the list: (20) Mol. Cell. Biol. (MCB) 29, 3286–96, (21) Int. J. Biochem. Cell Biol. 44, 2019-27, (22) Lipids 46, 1013-20, (23) Immunology 106, 53-9, (24) J. Biol. Chem. (JBC) 220, 22577-85, (25) EMBO Rep. 3, 1181-7, (26) J. Clin. Endocrinol. Metab. 89, 3042-7, (27) MCB 16, 3853-65, (28) J. Clin. Invest. (JCI) 122, 2817-26, (29) Mol. Endocrinol. 17, 259-72, (30) Mol. Endocrinol. 15, 1720-8, (31) JCI 113, 1408-18, (32) Biochem. J. 355, 481-8, (33) EMBO J. 11, 433-9, (34) BBRC 269, 347-51, (35) JBC 273, 16710-4, (36) MCB 29, 6257-67, (37) Proc. Natl. Acad. Sci. U.S.A. (PNAS) 83, 3786-90, (38) MCB 15, 351-7, (39) EMBO J. 15, 5336-48, (40) MCB 16, 3410-9, (41) Cell 93, 241-52., (42) JBC 278, 30614-23, (43) JBC 275, 28240-5, (44) JBC 275, 14700-7, (45) JBC 277, 18793-800, (46) PNAS 98, 507-12, (47) JBC 272, 3137-40, (48) J. Lipid Res. 47, 2451-61, (49) JBC 277, 11019-25, (50) JBC 275,12497-502, (51) Am. J. Physiol. Endocrinol. Metab. 282, E222-30, (52) PNAS 93, 1049-53, (53) JBC 271, 32689-94, (54) JBC 273, 22052-8, (55) JBC 272, 7298-305, (56) JCI 101, 2331-9, (57) PNAS 101, 7281-6, (58) JBC 277, 34322-8, (59) JBC 279, 16954-62, (60) BBRC 327, 920-6, (61) Cell Metab. 15, 311–23.

RA modulates SHP expression that affects several transcriptional activities linked to metabolism

SHP is a nuclear receptor but lacks the conserved DNA-binding domain. By forming heterodimer with various nuclear receptors or interacting with cofactors, SHP functions as a repressor and antagonist of coactivator recruitment to a target TF (Zhang et al., 2011). Its expression may be intricately controlled at transcription level, as the SHP promoter contains binding sites to HNF4α, LXRα, PPARγ, SREBP-1c, FXR, steroidogenic factor-1, liver receptor

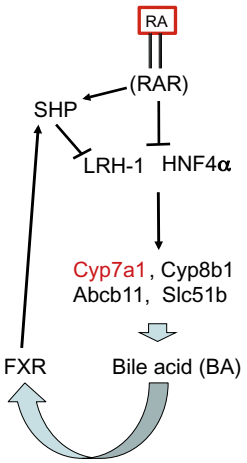

FIG. 4 Proposed pathway for retinoic acid- and bile acid-induced suppression of bile acid synthesis. This figure represents the schematic diagram of small heterodimer partner (SHP)–mediated suppression of bile acid synthesis. As discussed in Section "RA modulates SHP expression that affects several transcriptional activities linked to metabolism", it is known that LRH-1 and HNF4α stimulate bile acid through the induction of Cyp7a1, Cyp8b1, Abcb11, and Slc51b. Synthesized bile acids activate FXR to produce SHP that inhibits LRH-1 and HNF4α. Activated RAR can suppress HNF4α and LRH-1 via SHP expression.

homologue-1 (LRH-1), c-Jun, estrogen receptor (ER) α, ER-related receptor γ, E2A gene products (E47, E12, and E2/5), adaptor protein, pregnane X receptor, the core circadian component CLOCK-BMAL1, and upstream stimulatory factor-1. Although no RAREs have been found in the proximal region of SHP promoter, nontransformed hepatic cell line AML12 treated with RA and the liver from mice supplemented with *at*RA for 7 days increase SHP protein (Mamoon et al., 2014; Yang et al., 2014).

RA supplementation also causes decreased in mRNA expression of Cyp7a1, Cyp8b1, Abcb11, and Slc51b genes related to bile acid synthesis and transport and of Lrh-1 and Hnf4α genes, TFs for Cyp7a1 (Yang et al., 2014). Involvement of SHP in this process is not known. However, findings that bile acid-activated SHP inhibits the activity of LRH-1 (Goodwin et al., 2000) suggest that RA-induced SHP may contribute to the inhibition of CYP7A1 and CYP8B1 by repression of LRH-1 and HNF4α (see Fig. 4).

RA induces PEPCK expression through ATF-2 activation (Lee et al., 2002), while RA increases glucokinase (*L-GK*) mRNA in neonatal and adult cultured hepatocytes (Cabrera-Valladares et al., 2001), possibly mediating RARE through activated RARs (Li et al., 2014). SHP is shown to decrease mRNA levels of gluconeogenic enzymes via SHP-mediated repression of HNF4α and Foxo1 (Yamagata et al., 2004) and *L-GK* gene expression by inhibiting the transcriptional activity of LXR and PPAR (Kim et al., 2009), independently on RA action.

RA is an inhibitor of the antioxidant response element (ARE)–binding TF, Nrf2

Reactive oxygen species (ROS) is generated in normal cellular metabolism and is altered depending on cellular metabolic states. However, its harmful effects, known as oxidant stress, appear when it is overproduced or is not scavenged by antioxidant systems. NF-E2-related factor 2 (Nrf2) is a basic region leucine zipper-type TF that forms heterodimer with small Maf and bind the antioxidant response element (ARE) in the promoter region of the target genes including NADPH/quinone oxidoreductase (Fatehi-Hassanabad et al., 2010). Nrf2 activity is controlled by Kelch-like ECH-associated protein 1 (Keap1). Keap1 binding to Nrf2

accelerates ubiquitination of Nrf2, resulting in degradation of Nrf2 by proteasome. Upon a stress signal like hyperglycemia-induced ROS, modification of cysteine residue in Keap1 molecule leads to the suppression of the proteasome-mediated degradation and to nuclear translocation of Nrf2. It should be noted that genetic activation of Nrf2 signaling by Keap1 gene hypomorphic knockdown ($Keap1^{flox/-}$) markedly suppresses the onset of diabetes when $Keap1^{flox/-}$ mice were crossed with diabetic *db/db* mice (Uruno et al., 2013).

RA antagonizes the expression of Nrf2 target genes through activation of RARα (Wang et al., 2007) and also through RXRα (Wang et al., 2013). In addition, there is a report showing the repression of RAR/RXR in cardiomyocytes by high glucose-induced ROS (Singh et al., 2012). Thus we proposed a model of the interaction between RA signaling and Nrf2 activation (Fig. 5). In normal glucose conditions, Nrf2 protein is physically associated with RAR and/or RXR to prevent Nrf2 binding to ARE and also with Keap1 to accelerate Nrf2 ubiquitination and degradation. Upon the high oxidative stress condition, ROS represses RAR/RXR resulting in upregulation of Nrf2 availability. ROS also modify cysteine residue of Keap1, leading to Nrf2 nuclear translocation and activation of the target genes.

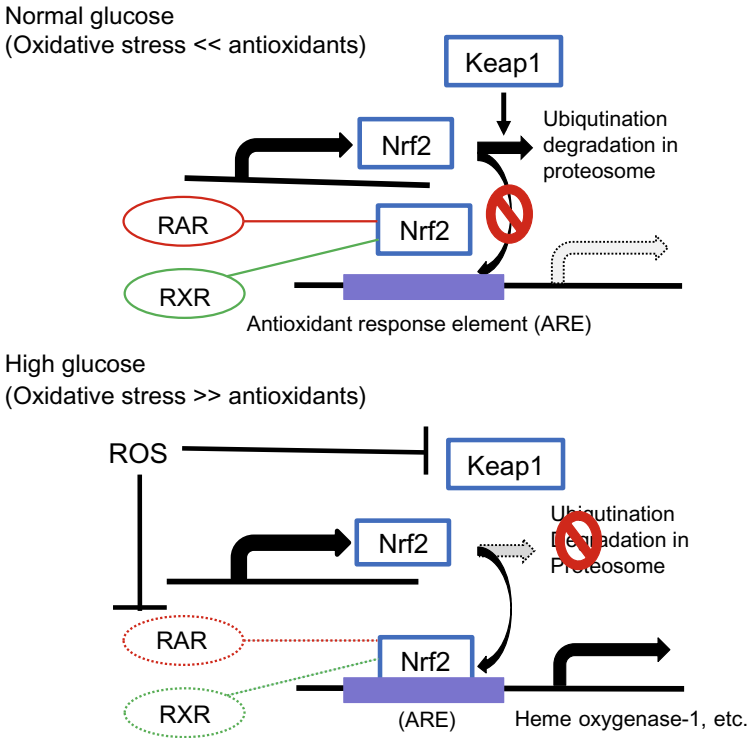

FIG. 5 Proposed interaction between RAR/RXR and Nrf2-Keap1. This figure represents the schematic diagram of proposed interaction between retinoic acid signaling and Nrf2-dependent antioxidant system. In the normal glucose condition, Nrf2 is associated with RAR or RXR to suppress the Nrf2 activity and also with Keap1 protein to be received ubiquitination and proteasomal degradation. Reactive oxygen species (ROS) produced by high-glucose condition suppresses RAR and Keap1 activities, leading to Nrf2 activation.

CRABP2 is a substrate for Sirt1, NAD^+-dependent protein deacetylase, in embryonic stem cells

Activation of Sirt1 (sirtuin 1) upon autophagy is glucose concentration dependent (Chang et al., 2015). Under glucose starvation, but not amino acid starvation, cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is phosphorylated by AMPK and then transferred to the nucleus, where it interacts with and activates Sirt1. In the fasted liver, Sirt1 also interacts with and deacetylates transcriptional coactivator, PGC-1α, to induce gluconeogenic genes and repress glycolytic genes (Rodgers et al., 2005). In contrast, high-glucose condition leads to lower activation of AMPK and Sirt1, because of lowered AMP/ATP and NAD^+/NADH ratios. High glucose also significantly downregulates both the mRNA and protein levels of Sirt1 in Raw264.7 cells (Jia et al., 2015).

There is the report showing that RA-mediated acetylation of CRABP2 is essential for its nuclear accumulation and subsequent activation of RA signaling in embryonic development. Sirt1 interacts with and deacetylates CRABP2, regulating its subcellular localization. Sirt1 deficiency induces hyperacetylation and nuclear accumulation of CRABP2, leading to enhanced RA signaling both in vivo and in vitro (Tang et al., 2014). However, precious roles of acetylation/deacetylation of CRABP2 in RA effects on energy metabolism remain to be elucidated.

Modulation of RA synthesis affects energy metabolism

RA is synthesized from retinol in the cell, and CRBP1 serves as a chaperone of retinol to converting retinal by RDH and retinal conversion to RA by RALDH. RA induces *Pck1* expression to accelerate gluconeogenesis in the liver. Refeeding, oral gavage with glucose, or injection with insulin decreased *Rdh1* and *Rdh10* mRNA 50% or greater in the mouse liver. Concentration of *at*RA in the liver was decreased 44% 3 h after reduced *Rdh* expression. These results indicate that insulin inhibits RA biosynthesis through the inhibition of FoxO1-induced *Rdh10* gene expression (Obrochta et al., 2015).

In the pancreas of Rbp1-null (CRBP1-deficient) mouse, retinol concentration increased with ectopic expression of Rpb2 mRNA encoding CRBP2. Both would contribute to increased 9-cis-RA, a RXR agonist that attenuates glucose-stimulated insulin secretion (GSIS) and insulin biosynthesis in β-cell (Kane et al., 2011). Overexpression of dominant negative form of RARα caused a decrease in plasma insulin and insulin secretion in response to glucose challenge, suggesting that RAR signaling is required to maintain GSIS (Brun et al., 2015).

RALDH1-deficient mice are protected from diet-induced obesity. The null mice display reduced hepatic glucose production with impaired gluconeogenic pathway. In addition, RALDH1 deficiency also results in repressed triacylglycerol synthesis, accompanied with a significant decrease in ChREBP expression, without affecting SREBP-1c expression (Kiefer et al., 2012).

Cellular retinol is partly metabolized by retinol saturase (RetSat) mainly located in endoplasmic reticulum. RetSat converts retinol to 13,14-dihydroretinol that is sequentially metabolized to 13,14-dihydroretinoic acid by RDH and RALDH. These dihydroretinoids are selective RAR/RXR agonist (Moise et al., 2006) and the 9-cis-isomer of 13,14-dihydroretinoic acid is identified as an endogenous ligand for RXR/RXR (Ruhl et al., 2015). Ablation of RetSat expression in preadipocytes is dramatically inhibited adipogenesis, but this ablation was not

overcome by 13,14-dihydroretinol, the putative product of RetSat reaction. Ectopic RetSat with an intact, but not a mutated, FAD/NAD dinucleotide-binding motif increased endogenous PPARγ transcriptional activity and promoted adipogenesis. RetSat was not required for adipogenesis when cells were treated with exogenous PPARγ ligands (Schupp et al., 2009), indicating RetSat has additional PPARγ ligand-generating yet unknown enzymatic activity.

Liver-specific depletion of RetSat in dietary obese mice lowers hepatic and circulating triacylglycerol and normalizes hyperglycemia, accompanied with reduction of ChREBP activity. Defects upon RetSat depletion are rescued by ectopic expression of ChREBP, but not by 13,14-dihydroretinol. Thus RetSat is a critical regulator of liver metabolism functioning upstream of ChREBP (Heidenreich et al., 2017).

Aldose reductase is the regulator of epigenetic control of RAR-mediated transcription

Histone deacetylase 3 (HDAC3) recruits to the genome causing histone hypoacetylation induced by deacetylase activity is associated with gene silencing. HDAC3 requires association with the deacetylase activation domain of NCoR1 and SMRT, for its stability and activity (Watson et al., 2012). The HDAC3-NCoR1/SMRT complex represses several nuclear receptors including TR and RAR. Upon ligand binding, the HDAC3-NCoR1/SMRT complex dissociates, and activator complexes move to bind the receptors and activate gene transcription. Lately, aldose reductase is identified as the factor involved in the regulation of repressor complex dissociation, although it is the rate-limiting enzyme of the first step of polyol pathway that catalyzes conversion of glucose to sorbitol. Aldose reductase decreases HDAC3-corepressor complex formation, resulting in specifically derepression of PPARγ- and RAR-, but not TR- and LXR-mediated transcription (Thiagarajan et al., 2016).

Glossary

Aldose reductase Aldose reductase is a cytosolic enzyme that catalyzes conversions of glucose to sorbitol and galactose to galactitol in a NADPH-dependent manner, as the first step of the polyol pathway.

Coactivators Transcriptional coactivating proteins (coactivators) interact with several transcription factors and act to increase the expression of their target genes. CREB-binding protein/E1A-binding protein p300 (CBP/p300), peroxisome proliferator-activated receptor gamma (PPAR-γ) coactivator 1 (PGC-1) and steroid receptor coactivator-1 (SRC-1) are well-characterized coactivators, and all contain intrinsic histone acetyltransferase (HAT) activity.

Farnesoid X receptor (FXR) FXR is the nuclear receptor that primarily controls cellular bile acid levels through the suppression of cholesterol 7 alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid synthesis from cholesterol, indirectly. FXR enhances expression of *small heterodimer partner (SHP)* gene product that functions as an inhibitor of the CYP7A1 gene transcription.

Hairy and enhancer of split-6 (Hes6) Hes6 is a basic helix-loop-helix (bHLH) transcription factor, encoded by a member of the family of mammalian homologues of *drosophila hairy and enhancer of split (Hes) gene.* Hes6 negatively regulates transcription of the genes that require a bHLH protein for its transcription, although Hes6 cannot bind directly to DNA.

Histone deacetylase 3 (HDAC3) HDAC3 is an enzyme that catalyzes deacetylation of acetylated histone. This post-translational modification of histone tail causes chromatin remodeling, resulting in repressing in gene expression. HDAC3 is found exclusively in the cell nucleus, possibly associated with nuclear receptor corepressor complex containing NCoR1 and SMRT.

Krüppel-like factor 15 (KLF15) KLF15, formerly known as kidney-enriched Krüppel-like factor, is a zinc finger transcription factor protein. KLF15 is increased by fasting and glucocorticoid signaling and decreased by feeding and insulin stimulation.

Max-like protein X (MLX) MLX is a protein that belongs to the family of bHLH/leucine zipper (bHLH/LZ) transcription factors. MLX forms heterodimers with ChREBP.

Nrf2-Keap1 system Kelch-like ECH-associated protein 1 (Keap1) is a NF-E2-related factor 2 (Nrf2)–binding protein that accelerates ubiquitination of Nrf2, resulting in degradation of Nrf2 by proteasome. Upon a stress signal, modification of cysteine residue in Keap molecule leads to the suppression of the ubiquitination and nuclear translocation of Nrf2. In the nucleus Nrf2 and small Maf, heterodimer complex activates transcription of the gene having the antioxidant response element (ARE) such as heme oxygenase-1.

Small heterodimer partner (SHP) SHP is a nuclear protein but cannot bind directly to DNA. SHP functions as a corepressor and antagonizes coactivator recruitment to target transcription factors with which it interacts.

Small Maf Small Mafs, encoded by musculoaponeurotic fibrosarcoma oncogene, are basic region leucine zipper-type (bZip) transcription factors that can bind to DNA and regulate gene transcription. They form homodimer and heterodimer with other specific bZip transcription factors such as Nrf2.

Sirt1 (Sirtuin 1) Sirt1 is the most conserved mammalian NAD^+-dependent protein deacetylase. The substrates for SIRT1 include tumor suppressor p53, forkhead transcription factors (FOXOs), PGC-1a, p300, NF-kB, and CRABP2.

Ubiquitin-proteasome system A protein substrate enzymatically and sequentially conjugated with ubiquitin (polyubiquitination) is facilitated to transfer into the proteasome where it is degraded.

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CHAPTER

16

Sugars, sweet taste receptors, and brain responses

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OUTLINE

SUMMARY POINTS

- Sweet taste receptors are a heterodimer composed of taste type 1 receptor 2 (T1R2) and taste type 1 receptor 3 (T1R3) that senses sweet taste in taste buds.
- Sweet taste receptors have been identified in multiple organs throughout the body including the gastrointestinal tract where they likely are involved in carbohydrate sensing and release of incretin hormones.
- Sweet taste receptors have also been identified in the hypothalamus where they

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likely are involved in glucose sensing in the brain.

- It is likely that sweet taste receptors are involved in homeostatic processes throughout the body, including chemosensory functions in the gut and transmitting information on energy homeostasis and glucose metabolism throughout the body.

Introduction

Lingual taste receptors can sense the five basic tastes, including the sweet, salty, umami, bitter, and sour. Recent evidence has shown that these taste receptors are also present throughout the body, including the GI tract where they may act as chemosensors to detect the macronutrient content in the gut lumen. These receptors can then elicit the release of gut hormones and neurotransmitters to coordinate secretomotor responses in the GI tract. In addition, taste receptors have been identified in the central nervous system, including the hypothalamus, where they coordinate the body's response to overall energy needs and maintain glucose homeostasis.

Chemosensory cells in the tongue

Humans can distinguish between at least five basic tastes, including sweet, salty, umami, bitter, and sour. Taste processing first occurs at the level of the taste bud, which is an onion-shaped structure composed of an aggregate of 50–100 neuroepithelial cells (Loper et al., 2015). Each of these cells can be categorized into four subtypes based on their morphologic features, protein expression, and signaling characteristics. Type I, or glial-like cells, is the most abundant cell type and likely transmits salty taste (Chandrashekar et al., 2010). Type II cells express G protein-coupled receptors (GPCR) that detect sweet, umami, and bitter tastes (Chandrashekar et al., 2006). Type III, or presynaptic cells, senses sour taste and carbonation (Huang et al., 2006). Meanwhile, type IV cells represent stem or progenitor taste cells (Lee and Owyang, 2017).

Taste receptors

Sweetness detection is mediated by a single receptor that is composed of two distinct GPCR: taste 1 receptor family member 2 (T1R2) and taste 1 receptor family member 3 (T1R3). The sweet taste receptor is formed by a heterodimer of T1R2 and T1R3 that can sense all molecules known to taste sweet to humans, including sugars (glucose, fructose, sucrose, and maltose); artificial sweeteners (e.g., saccharin, aspartame, and cyclamate); sweet amino acids (D-tryptophan, D-phenylalanine, and D-serine); sweet proteins (monellin, brazzein, and thaumatin); and plant metabolites, such as stevioside (Jiang et al., 2005). In addition, T1R3 may form a T1R3/T1R3 homodimer that may also detect monosaccharides and disaccharides at high concentrations, suggesting some overlap in this system.

Sweet taste signaling

Binding of sweet taste compounds to the T1R2/T1R3 receptor interacts with the heterotrimeric G protein (α-gustducin, Gβ3, and Gγ13). The α subunit then dissociates from the βγ subunit to activate phospholipase C-β2 (PLCβ2), leading to 1,4,5-inositol triphosphate-mediated release of intracellular Ca^{2+} and subsequent opening of a transient potential ion channel, transient receptor potential cation channel subfamily M member 5 (TRPM5). This signaling cascade results in membrane depolarization, release of ATP, and activation of sensory afferent neurons involved in taste perception (Fig. 1) (Neiers et al., 2016).

Several bioactive peptides, including glucagon-like peptide-1 (GLP-1), glucagon, neuropeptide Y (NPY), peptide YY (PYY), cholecystokinin (CCK), vasoactive intestinal peptide (VIP), and ghrelin, are expressed by taste cells. Although the functional significance of expressing these peptides in taste buds is still unknown, their presence suggests that processing and modulating taste information occurs at the level of the taste bud.

Chemosensors in the gastrointestinal tract

Chemosensory cells must be present in the epithelial lining and have direct access to intraluminal content in the gastrointestinal tract. Potential candidates include enterocytes, brush cells, and enteroendocrine cells. One of the most intriguing discoveries recently has been the identification of taste receptors in extraoral sites, such as the gastrointestinal tract,

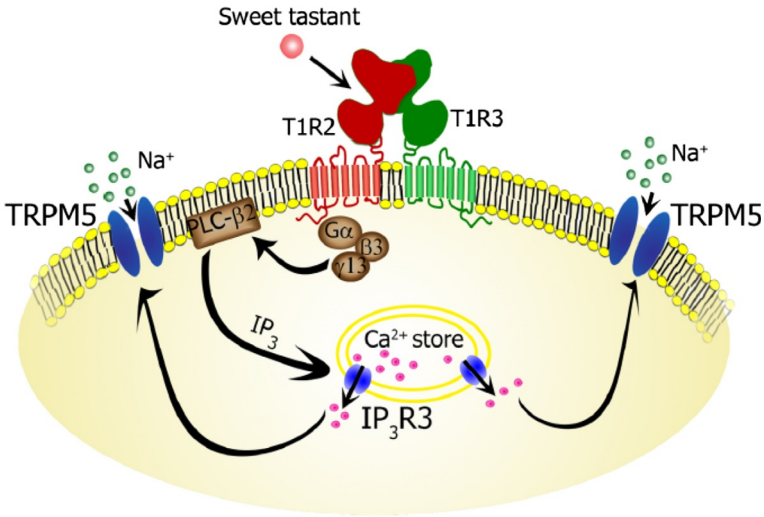

FIG. 1 Principal pathway for taste transduction. The binding of sweet tastants to the T1R2/T1R3 receptor results in the dissociation of the heterotrimeric G protein (α-gustducin, Gβ3, and Gγ13), leading to an increase in the phospholipase C-β2 (PLC-β2) activity, which causes the inositol 1,4,5-triphosphate (IP_3) receptor, type 3 (IP_3R_3)-mediated release of calcium from intracellular stores. The last component of the transduction mechanism is the transient potential ion channel, TRPM5, whose opening leads to membrane depolarization. *Reproduced with permission from Neiers, F., Canivenc-Lavier, M.-C., Briand, L., 2016. What does diabetes "taste" like? Curr. Diab. Rep. 16(6), 49.*

pancreatic islet cells, and the central nervous system (CNS) (Lee and Owyang, 2017). This strongly suggests that taste receptors are involved in nutrient sensing, regulation of glucose homeostasis, and nutrient intake.

Enteroendocrine cells

Enteroendocrine cells (EECs) comprise a small proportion (<1%) of the total number of epithelial cells in the gastrointestinal (GI) tract. However, collectively EECs form the largest endocrine organ in the body (Furness et al., 2013). Most data suggest that EECs are the primary chemosensory cell in the GI tract. At least 12 subtypes of EECs have been identified, which secrete a wide range of peptides involved in regulation of satiety, energy metabolism, and GI motility (Table 1). Although EECs were initially classified by the peptide secreted, recent evidence has shown that EECs are capable of expressing multiple different gut hormone precursors suggesting that the dogma of "one cell type-one hormone" likely is not accurate (Engelstoft et al., 2013). In addition, EECs show a gradient in their distribution pattern except

TABLE 1 Enteroendocrine cells of the mammalian gastrointestinal tract

Cell	Products	Luminal receptors	Locations	Principal effects
A (X-like) cells and subtypes	Ghrelin, nesfatin-1	T1R1-T1R3; T2Rs	Stomach	Appetite control, growth hormone release
Enterochromaffin cells[a,b]	5-HT (5-HT is also contained in subgroups of I, K and L cells)	FFARs 2, 3; TRPA1; toxin receptors; TLRs	Stomach, small and large intestine	Facilitation of intestinal motility reflexes and secretion; triggering of emesis and nausea in response to toxins
I cells	CCK (5-HT)	T2Rs; FFA1; GPR120; LPAR5; CaSR; TRPA1; TLRs	Proximal small intestine	Activation of gallbladder contraction and stimulation of pancreatic enzyme secretion
K cells, and subtypes	GIP	GPR119, GPR120; FFAR1	Proximal small intestine	Stimulation of insulin release
L cells, and subtypes[b]	GLP-1, GLP-2, PYY, oxyntomodulin (5-HT)	T2Rs; T1R2-T1R3; FFARs 1–3; GPR119, LPAR5, GPR120; CaSR	Distal small intestine, colon	Stimulation of carbohydrate uptake, slowing of intestinal transit, appetite regulation, insulin release
P cells	Leptin	Nutrient receptors	Stomach	Appetite regulation, reduction of food intake

[a] *ECL cells do not contact the lumen.*
[b] *Sweet taste receptor molecules have been identified within L cells and EC cells.*
Several of the enteroendocrine cell types, notably A, K, and L cells, have subgroups or gradients along the intestine that contain different combinations of products: subgroups of I and L cells contain 5-HT.
CaSR, calcium-sensing receptor; *CCK*, cholecystokinin; *ECL*, enterochromaffin-like; *FFAR*, free fatty acid receptor; *GIP*, gastric inhibitory polypeptide; *GLP*, glucagon-like peptide; *GPR*, G protein-coupled receptor; *5-HT*, 5-hydroxytryptamine; *LPAR*, lysophosphatidic acid receptor; *PYY*, peptide YY; *T1R*, taste 1 receptor family; *T2R*, taste 2 receptor family; *TLR*, Toll-like receptor; *TRP*, transient receptor potential.
Adapted with permission from Furness, J.B., Rivera, L.R., Cho, H.-J., Bravo, D.M., Callaghan, B., 2013. The gut as a sensory organ. Nat. Rev. Gastroenterol. Hepatol. 10(12), 729–40.

for enterochromaffin cells (5-hydroxytryptamine, 5-HT). For example, EECs present in the stomach include D cells (ghrelin and somatostatin), G cells (gastrin), and ECL cells (histamine). Meanwhile, I cells (CCK) and K cells (gastric inhibitory peptide, GIP) are present mainly in the proximal small intestine, while L cells (glucagon-like peptide 1, GLP-1; peptide YY, PYY) are predominantly found in the ileum and colon (Steinert and Beglinger, 2011). EECs can be further classified as "open cells" with microvilli extending to the lumen or "closed cells," which do not reach the lumen (Sternini et al., 2008). EECs, particularly open cells, are in direct contact with intraluminal contents and, as such, are particularly well suited to act as chemosensors in the GI tract.

Sweet taste receptors in the GI tract

Components of the sweet taste signaling pathway have been identified within EECs, notably L cells producing the incretin hormones GLP-1 and PYY and K cells, which secrete GIP (Fig. 2) (Jang et al., 2007; Margolskee et al., 2007). Glucose uptake by the sodium-glucose-

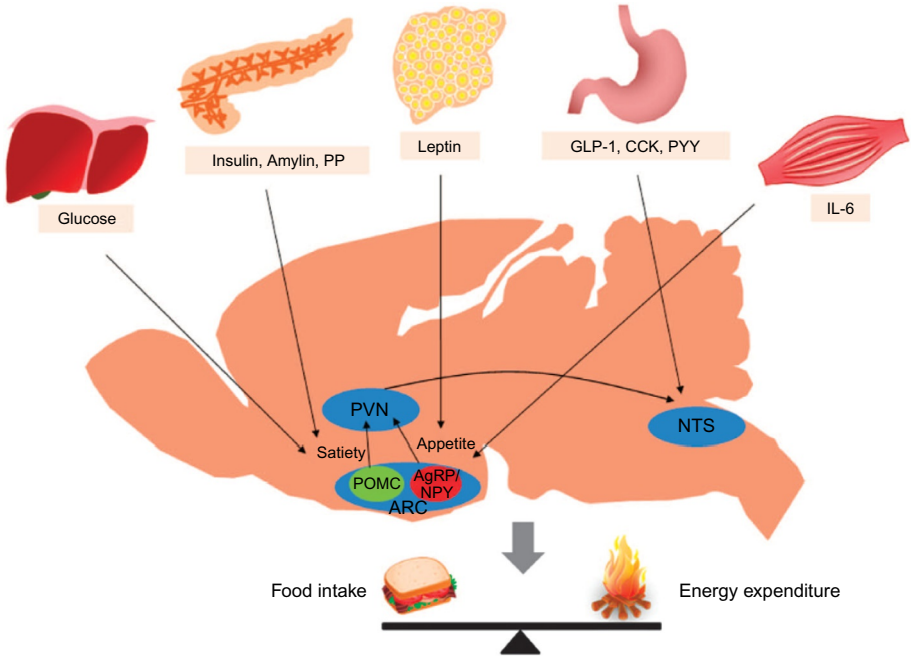

FIG. 2 Integration of peripheral metabolic signals and the central nervous system maintains energy homeostasis. The brain integrates metabolic signals from peripheral tissues such as the liver, pancreas, adipose tissue, gut, and muscle. Specialized neuronal networks in the brain coordinate adaptive changes in food intake and energy expenditure in response to altered metabolic conditions. Neuropeptide Y/agouti-related protein and proopiomelanocortin-producing neurons in the hypothalamic arcuate nucleus primarily sense the body energy state. These neurons project to other hypothalamic nuclei and to the nucleus of the solitary tract in the brain stem to control multiple aspects of the homeostatic regulation of energy balance. *ARC*, arcuate nucleus; *CCK*, cholecystokinin; *GLP-1*, glucagon-like peptide-1; *IL-6*, interleukin-6; *PP*, pancreatic polypeptide; *PVN*, paraventricular nucleus; *PYY*, peptide YY. *Reproduced with permission from Roh, E., Song, D.K., Kim, M.-S., 2016. Emerging role of the brain in the homeostatic regulation of energy and glucose metabolism. Exp. Mol. Med. 48, e216.*

linked transporter 1 (SGLT1) leads to depolarization of the K/L-cell plasma membrane, opening of voltage-gated Ca^{2+} channels, and subsequent release of incretin hormones. GLP-1, GIP, and PYY stimulate pancreatic insulin secretion, slow gastrointestinal motility, and decrease appetite (Wren and Bloom, 2007). The highest expression of sweet taste receptors has been found in the proximal small intestine (Dyer et al., 2005).

Accumulating evidence supports the role of sweet taste receptors in carbohydrate sensing in the GI tract. It has long been known that orally administered glucose is much more effective in increasing insulin release compared with intravenous delivery (Mcintyre et al., 1964). The mechanism for this "incretin effect" has largely been unknown until recently. Sweet taste signaling proteins, including T1R2, T1R3, α-gustducin, PLCβ2, and TRPM5, have been identified in mouse and human L cells and K cells (Jang et al., 2007; Steinert et al., 2011). Immunofluorescence studies have demonstrated that T1R3 and α-gustducin colocalize with L/K cells in the duodenum as well as GLP-1 and PYY containing colonic cells (Jang et al., 2007; Steinert et al., 2011). In addition, the carbohydrate glucose and fructose and the artificial sweetener sucralose are able to increase SGLT1 expression and elicit GLP-1 secretion from mouse (GLUTag) and human (NCI-H716) L cells in vitro as well as mouse small intestinal explants (Jang et al., 2007; Margolskee et al., 2007). Meanwhile, knockout mice lacking α-gustducin or T1R3 do not show upregulation of SGLT1 expression and are defective in secreting GLP-1 in response to intraluminal glucose. Lastly, the administration of a sweet taste receptor antagonist, lactisole, in humans significantly reduced glucose-stimulated GLP-1 and PYY secretion (Steinert et al., 2011). Taken collectively, these results suggest that sweet taste receptor proteins expressed in L/K cells are able to sense intraluminal glucose concentration and trigger release of incretin hormones, which leads to enhanced expression of SGLT1 and glucose uptake likely through paracrine mechanisms.

However, some evidence suggests that glucose may directly stimulate intestinal L cells, which coexpress SGLT1 (Reimann et al., 2008). In addition, the relevance of the sweet taste receptor system in gut hormone secretion and glucose homeostasis has been questioned. For example, the effects of the sweet taste receptor antagonist lactisole were attenuated in the presence of a mixed liquid meal consisting of proteins, fats, and other carbohydrates (Gerspach et al., 2011). In addition, artificial sweeteners showed no effects on GLP-1 or ghrelin release in mice or humans (Steinert et al., 2011). However, since carbohydrates and artificial sweeteners may bind at different locations in the sweet taste receptor heterodimer, they may activate different signaling cascades leading to variable physiologic effects.

Central regulation of food intake and energy balance

Key hypothalamic neuronal circuits

The hypothalamus is a key brain region in the regulation of appetite, satiety, and energy balance. The hypothalamus receives and integrates neurohumoral input from the periphery, including the gut, pancreas, and adipose tissue, to coordinate energy homeostasis. The arcuate nucleus (ARC) is a fundamental component of the hypothalamic regulatory circuit. The ARC is adjacent to the median eminence (ME), which is rich in fenestrated capillaries and leads to a leaky blood-brain barrier. Thus this key area of the hypothalamus is situated to

detect circulating neurohumoral signals, for example, glucose, insulin, and leptin, from the periphery, which provide input regarding overall energy balance (Rodríguez et al., 2010). Two distinct neuronal populations within the ARC have been identified with functionally antagonistic effects on appetite and energy regulation (Wren and Bloom, 2007). One group of neurons in the medial ARC coexpresses neuropeptide Y (NPY) and agouti-related peptide (AgRP), which stimulate appetite and preserve weight. A second group of neurons in the lateral ARC coexpresses proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) to promote decreased appetite and weight loss. These neurons are first-order neurons that regulate body energy state by sensing alterations in key hormones, including leptin, insulin, ghrelin, and nutrient levels (Schwartz et al., 2000). POMC neurons project to second-order hypothalamic neurons, such as the paraventricular hypothalamic nucleus (PVN), the ventromedial hypothalamus (VMN), dorsomedial nucleus (DMN), and the lateral hypothalamus (LH). Differential activity between orexigenic AgRP/NPY-expressing neurons and anorexigenic POMC/CART-expressing neurons is critical to energy homeostasis and body weight control (Fig. 2) (Roh et al., 2016).

Upon ingestion or high energy states, POMC undergoes posttranscriptional processing to produce the anorexigenic neuropeptide α-melanocyte-stimulating hormone (α-MSH), which is released from presynaptic terminals of POMC-containing neurons. α-MSH can then bind to second-order neurons via melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R) to promote increased energy expenditure and decreased food intake. Inactivation of the MC4R leads to hyperphagia and obesity in mice (Huszar et al., 1997). Meanwhile, MC4R mutations are seen in ~6% of severe early-onset obesity cases in humans (Tao, 2005). These observations point to the importance of melanocortin pathways in preserving normal body weight and protection against obesity.

In contrast, during periods of fasting, AgRP/NPY-containing neurons produce AgRP, which can compete with α-MSH for binding to MC3Rs and MC4Rs and thus prevents anorexigenic effects on second-order neurons (Ollmann et al., 1997). AgRP/NPY neurons also corelease NPY, which stimulates food intake and decreases energy expenditure via Y1 and Y5 receptors (Yulyaningsih et al., 2011). Furthermore, these neurons directly inhibit POMC neurons in the ARC by release of γ-aminobutyric acid (GABA) (Cowley et al., 2001).

Neurons within the PVN synthesize and release several neuropeptides, including corticotrophin-releasing hormone, thyrotropin-releasing hormone, somatostatin, vasopressin, and oxytocin to produce catabolic effects (Roh et al., 2016). These neurons in the PVN also control sympathetic outflow to peripheral organs (Foster et al., 2010). Destruction of the PVN leads to hyperphagia and obesity in rodents, which suggests an important inhibitory role in appetite and weight gain (Leibowitz et al., 1981).

The VMN also plays an important regulatory role in maintaining body weight and energy balance. While receiving neuronal projections mainly from the ARC, the VMN also projects axons to the ARC, DMN, LH, and brain stem regions. Neurons within the VMN produce the anorexigenic neuropeptide brain-derived neurotrophic factor (BDNF) upon activation of MC4R (Xu et al., 2003). Destruction of the VMN results in hyperphagia and obesity in rodents (Shimizu et al., 1987).

In contrast to the PVN and VMN, destruction of the LH results in hypophagia and weight loss and thus is considered a feeding center (Milam et al., 1980). The LH is composed of two neuronal populations producing orexigenic neuropeptides, including melanin-concentrating

hormone (MCH) and orexin (or hypocretin), which are likely downstream targets of AgRP and NPY (Broberger et al., 1998).

The hypothalamus also regulates energy expenditure by thermogenesis whereby energy may be dissipated in the form of heat mainly through brown adipose tissue (BAT) (Kajimura and Saito, 2014). Activation of MC4R in the hypothalamus leads to increased activity of the sympathetic nervous system, release of norepinephrine, and activation of β3-adrenergic receptors in adipocytes in brown adipose tissue and inguinal fat pads. This subsequently increases the expression of uncoupling protein 1 in the mitochondria of BAT to stimulate thermogenesis. Hypothalamic regions, including the prooptic area, VMN, DMN, and ARC, control sympathetic outflow and are key regulators of BAT thermogenesis (Seoane-Collazo et al., 2015).

Extrahypothalamic neuronal circuits

The nucleus of the solitary tract (NTS) located in the brain stem is another key area that regulates appetite and body weight and receives input from the hypothalamus. POMC neurons from the ARC innervate NTS via MC3R and MC4R to suppress food intake (Zheng et al., 2010). Similarly, oxytocin-expressing neurons from the PVN innervate the NTS to modulate food intake (Blevins et al., 2004). Meanwhile the NTS also receives orexigenic signals from hypothalamic orexin neurons to stimulate appetite and food intake (Parise et al., 2011).

Food intake can also be modulated by hedonic properties of eating. This is largely controlled through the mesolimbic reward system, including the ventral tegmental area (VTA) and the nucleus accumbens (NAc). These regions receive input from hypothalamic regions and are thus sensitive to peripheral signals of energy status. Orexin and glutamate coexpressing neurons from the LH activate dopaminergic neurons in the VTA in the setting of decreased glucose levels (Sheng et al., 2014). Furthermore, inhibitory GABA-expressing neurons from the LH innervate the VTA that increases food intake and compulsive eating behaviors (Jennings et al., 2015).

Brain regulation of glucose metabolism

Glucose is a key energy source for the brain. As such, there is a complex and highly integrated system to regulate normoglycemia in the brain. Glucose-sensitive neurons are found throughout the brain. Nuclei in the hypothalamus, such as in the ARC, VMN, and LH as well as areas in the brain stem, are particularly enriched with glucose-sensing neurons (Fig. 3). Glucose-sensing neurons are categorized into glucose-excited (GE) neurons, which are activated by an increase in extracellular glucose levels and glucose-inhibited (GI) neurons, which are stimulated by decreased extracellular glucose concentration. GE neurons are concentrated in the VMN, the ARC, and the PVN, while GI neurons are located in the LH, ARC, and PVN. In addition, both neuronal types are expressed in the dorsal vagal complex in the brain stem, including the area postrema, the NTS, and the dorsal motor nucleus of the vagus (DMV), as well as the ventral part of the medulla (Roh et al., 2016).

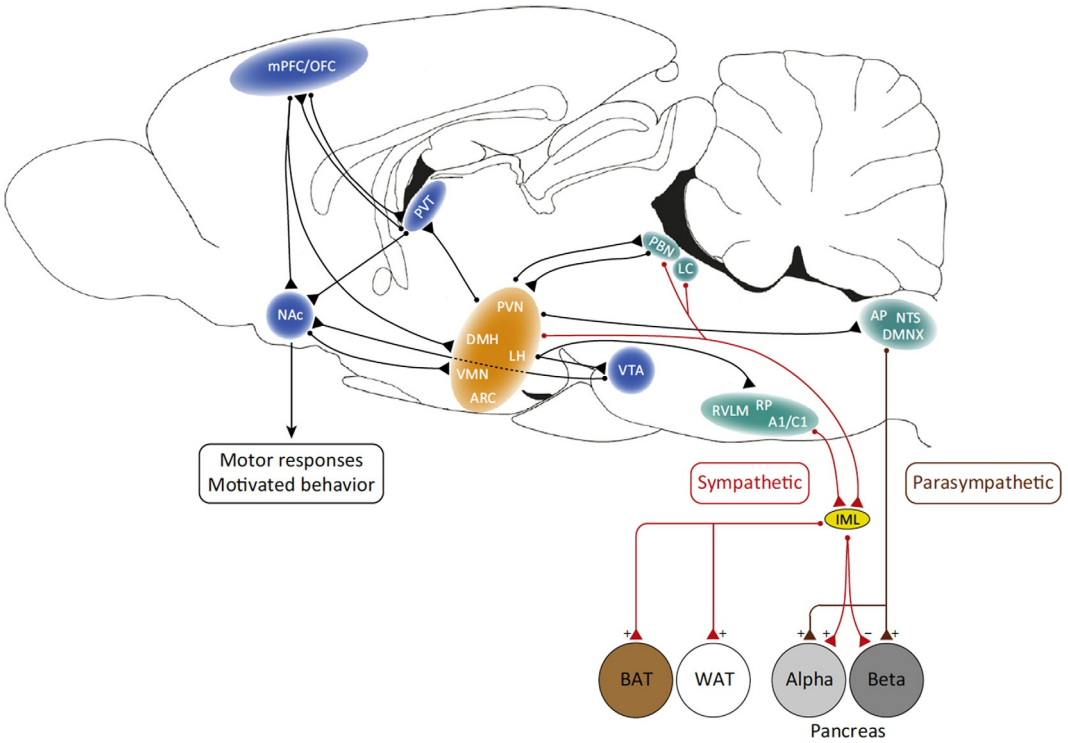

FIG. 3 Glucose-sensing cells in homeostatic and hedonic control centers. Glucose-sensing cells, both glucose excited (GE) and glucose inhibited (GI), have been identified in the brain stem structures (green): in the dorsal vagal complex that comprises the area postrema (AP), the nucleus of the tractus solitarius (NTS), and the dorsal motor nucleus of the vagus (DMNX); the basolateral medulla that includes the A1/C1 catecholaminergic neurons, the rostral ventrolateral medulla (RVLM), and the raphe pallidus (RP); the locus coeruleus (LC); and the parabrachial nucleus (PBN). In the hypothalamus (orange), glucose-sensing neurons are found in the arcuate nucleus (ARC) and in the ventromedial (VMN), lateral (LH), dorsomedial (DMH), and paraventricular (PVN) nuclei. The mesolimbic dopamine system, which comprises the ventral tegmental area (VTA), the major source of dopamine, the nucleus accumbens (NAc), the medial prefrontal cortex (mPFC), and orbitofrontal cortex (OFC), is a system influenced by inputs from the paraventricular nucleus (PVT). This system integrates external stimuli through the mPFC and OFC and internal metabolic cues including glucose levels, through interaction with hypothalamic and brain stem nuclei, as depicted by connecting lines. The NAc plays an important role in integrating this information to direct motor and behavior responses. Glucose-sensing cells in the hypothalamus and brain stem control the activity of peripheral metabolic organs through autonomic nervous system activity. Sympathetic efferents (red) that project to peripheral organs via the spinal intermediolateral cell column (IML, yellow) are under the control of hypothalamic nuclei, PBN, and LC, as well as of basolateral medulla glucose-sensing neurons. Parasympathetic efferents (brown) originate in the DMNX and are under the control of glucose-sensing neurons present in the NTS and several hypothalamic nuclei. *Reproduced with permission from Steinbusch, L., Labouèbe, G., Thorens, B., 2015. Brain glucose sensing in homeostatic and hedonic regulation. Trends Endocrinol. Metab. 26(9), 455–66.*

Tanycytes are specialized glial cells with glucose-sensing properties. Tanycytes can be subdivided into α and β tanycytes. α tanycytes project to the VMN, DMH, and ARC, while β tanycytes line the floor of the third ventricle and project to the ARC and the ME that regulates permeability of the ME and the ARC (Fig. 4) (Steinbusch et al., 2015).

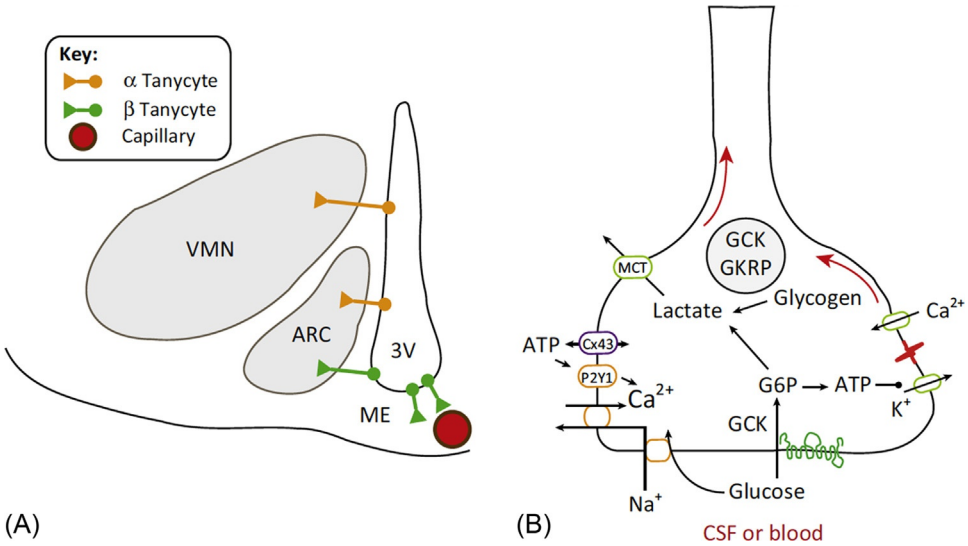

FIG. 4 Tanycytes are glucose-sensing cells. (A) Tanycytes reside in the ventral part of the third ventricle and have projections through the ventromedial nucleus (VMN) and arcuate nucleus (ARC) (α tanycytes in orange) and through the capillaries present in the median eminence (ME) (β tanycytes, green). (B) Different glucose-sensing modalities have been evidenced in tanycytes. In one modality, glucose is taken up by glucose transporter GLUT2, phosphorylated by glucokinase (GCK), leading to the closure of the K_{ATP} channel and entry of Ca^{2+}. In another modality, glucose is taken up by the SGLT1 Na^+/glucose cotransporter, leading to the increase in ATP production and release through connexin-43 (Cx-43) hemichannel and activation of P2Y1 purinergic receptor to induce Ca^{2+} waves; intracellular Ca^{2+} can also be increased through a sodium-induced Ca^{2+} entry mechanism. Tanycytes could also release lactate in the extracellular space because they express monocarboxylate transporters (MCT1 and 4), possibly influencing the metabolism of neighboring neurons. *GKRP*, glucokinase regulatory protein; *3 V*, third ventricle. *Reproduced with permission from Steinbusch, L., Labouèbe, G., Thorens, B., 2015. Brain glucose sensing in homeostatic and hedonic regulation. Trends Endocrinol. Metab. 26(9), 455–66.*

Glucose-sensing mechanisms

Glucose-sensing neurons likely employ signaling pathways similar to pancreatic β cells. For glucose-excited neurons, glucose is taken up by the glucose transporter GLUT2 and undergoes phosphorylation by glucokinase (GCK), which results in an increase in the ATP/ADP ratio, closure of K_{ATP} channels, membrane depolarization, and Ca^{2+} influx through voltage-gated Ca^{2+} channels (Steinbusch et al., 2015). Another mechanism by which glucose-excited neurons may be activated involves glucose uptake by the Na^+/glucose cotransporter SGLT1 leading to membrane depolarization (O'Malley et al., 2006). Glucose-sensing cells can then influence both the sympathetic and parasympathetic branches of the autonomic nervous system to regulate glucose metabolism by controlling glucagon and insulin secretion, thermogenesis in brown adipose tissue, lipolysis in white adipose tissue, catecholamine release, and hepatic gluconeogenesis (Ruud et al., 2017).

Activation of glucose-inhibited neurons by hypoglycemia stimulates AMP-dependent protein kinase (AMPK), increased nitric oxide (NO), and cGMP levels, which further activates AMPK. This results in suppression of the cystic fibrosis transmembrane regulator (CFTR)

chloride channel activity (Fioramonti et al., 2010). GLUT2 neurons in the NTS employ a similar pathway but result in closure of K^+ leak channels rather than CFTR channels. In addition, activation of orexigenic neurons in the LH lead to closure of K^+ leak channels by glucose metabolism-independent mechanisms. The majority of glucose-inhibited neurons are found ventromedially in the hypothalamus, while glucose-excited neurons are located laterally (Verberne et al., 2014).

Tanycytes may have multiple mechanisms for glucose sensing (Fig. 4). They express GLUT2, GCK, and K_{ATP} channels that suggest tanycytes use signaling pathways similar to GE neurons. Tanycytes also express monocarboxylate transporters (MCTs) 1 and 4, which can promote lactate efflux in the presence of glucose, and subsequently activate neighboring cells (Cortés-Campos et al., 2011). Furthermore, tanycytes are stimulated by glucose leading to increased intracellular Ca^{2+} levels, ATP release through connexin-43 hemichannels, and activation of P2Y purinergic receptors (Orellana et al., 2012).

Recently, several components of the sweet taste receptors, including T1R2, T1R3, α-gustducin, and TRPM5 were discovered in the brain, particularly in the hypothalamus including the ARC (Ren et al., 2009). Expression of these sweet taste receptors could be modulated by metabolic states. Hypothalamic expression of T1R2 is increased after 24 h fasting (Ren et al., 2009). Meanwhile, treatment of hypothalamic cell lines with leptin or high concentrations of glucose resulted in downregulation of T1R2 and T1R3 (Herrera Moro Chao et al., 2016). Similarly, there is decreased expression of T1R2 and T1R3 in the hypothalamus in obese mice either from leptin deficiency (ob^-/ob^-) or high-fat diet (Herrera Moro Chao et al., 2016). These data indicate that sweet taste receptor expression is involved in glucose sensing in the hypothalamus and associated with overall energy status.

A recent study utilized the artificial sweetener sucralose to examine the function of the sweet taste receptor in the ARC (Kohno et al., 2016). Since sucralose is a nonnutritive molecule, its effects are likely to be mediated purely by signaling at the sweet taste receptor. These authors demonstrated that Ca^{2+} signaling in isolated neurons from the ARC was increased in a dose-dependent manner by sucralose and suppressed by the sweet taste receptor antagonist, gurmarin. The majority of these neurons were non-POMC leptin-responsive neurons. Icv injection of sucralose into mice led to a dose-dependent decrease in food intake. These results suggest that sweet taste receptors also act as glucose-sensitive neurons in the brain leading to anorexigenic signals and reduced food intake.

Intriguingly, functional MRI studies in humans suggest that carbonation may attenuate brain processing of sweet stimuli (Di Salle et al., 2013). CO_2 is detected by type I and type III taste cells. Meanwhile, imaging studies have demonstrated that different tastes activate distinct cortical fields in the mammalian gustatory cortex, which points to the existence of a gustotopic map in the brain (Chen et al., 2011). However, these results suggest that carbonation may have the net effect of decreasing perception of sweet tastes, leading to overconsumption of energy-rich foods and thus increasing the propensity for obesity and other metabolic disorders.

Hypothalamic descending pathways

Glucose-sensing neurons in the hypothalamus project to sympathetic and parasympathetic efferent neurons in the brain stem and spinal cord. Neurons in the PVN and LH directly synapse with sympathetic preganglionic motor neurons in the spinal cord and sympathetic

catecholaminergic premotor neurons in the rostral ventrolateral medulla of the spinal cord (Shafton et al., 1998). Meanwhile, neurons in the ARC, VMN, and DMH directly project to the DMV (ter Horst and Luiten, 1986; Sim and Joseph, 1991). These neurons can then project to the pancreas and adrenal gland to increase glucagon and norepinephrine secretion to counteract against glucoprivation (Verberne and Sartor, 2010).

Neurons in the PVN and LH directly synapse with parasympathetic motor neurons, particularly in the NTS and DMV area (Loewy et al., 1994). Activation of orexigenic pathways to the DMV results in increased pancreatic parasympathetic activity (Wu et al., 2004). This suggests that the PVN and LH are major hypothalamic centers regulating descending parasympathetic pathways to control glucose homeostasis.

Central actions of gut hormones

Insulin

Insulin has central effects in the hypothalamus to regulate glucose metabolism. Both POMC and AgRP/NPY neurons in the ARC express insulin receptors. Previous studies have suggested that activation of insulin receptors on POMC neurons results in hyperpolarization of POMC neurons (Williams et al., 2010). However, more recent experiments using whole-cell recording demonstrated that purified insulin leads to activation of POMC neurons via transient receptor potential channel subfamily 5 (TRPC5) while inhibiting NPY/AgRP neurons via activation of K_{ATP} channels (Qiu et al., 2014). These discrepant results may be explained by heterogeneity of the neuronal population. Meanwhile, intracerebroventricular (icv) injection of insulin decreased food intake and increased *c-fos* expression in arcuate POMC neurons. In mice lacking insulin receptors in AgRP neurons, insulin showed an impaired ability to suppress hepatic glucose production, while insulin-induced adipose tissue lipolysis was impaired in knockout mice lacking insulin receptors in POMC neurons (Shin et al., 2017). This suggests insulin has specific actions depending on neuronal population with NPY/AgRP neurons regulating hepatic glucose production while POMC neurons control adipose tissue lipolysis and prevent high-fat diet-induced hepatic steatosis.

Insulin receptors are also expressed on neurons in the VMN. Insulin signaling inhibits steroidogenic factor 1 (SF-1) expressing neurons in the VMN mediated by IP_3 leading to activation of K_{ATP} channels. Meanwhile, mice with targeted genetic deletion of the insulin receptor specifically in SF-1 neurons showed no changes in body weight or glucose homeostasis. However, when these mice were challenged with a high-fat diet, they were protected against weight gain and impaired glucose tolerance (Klöckener et al., 2011).

Finally, insulin also acts on dopaminergic neurons in the mesolimbic reward system. Insulin stimulates dopaminergic neurons in the VTA and substantia nigra (Könner et al., 2011). Icv injection of insulin decreases sucrose intake and intake of high-fat diet (Figlewicz et al., 2008; Figlewicz et al., 2004). Meanwhile, mice with targeted genetic deletion of the insulin receptor in catecholaminergic neurons of the midbrain resulted in an obese phenotype with hyperphagia (Könner et al., 2011). In addition to acting on hypothalamic nuclei to regulate feeding behavior, these results suggest that insulin has direct effects on higher neuronal circuits that are involved in modulating the hedonistic aspects of eating.

Leptin

Leptin also plays an important role in modulating brain responses to energy balance and glucose metabolism. Leptin is mainly produced by adipocytes in response to insulin stimulation and is proportional to whole-body fat stores (Russell et al., 2001). Similar to insulin, leptin stimulates POMC neurons and inhibits AgRP/NPY neurons in the ARC resulting in decreased food intake and increased energy expenditure. However, leptin and insulin act on different subpopulations of neurons in the ARC (Williams et al., 2010). Leptin signaling in the VMH via SF-1 neurons improves glucose homeostasis while in the DMH results in increased sympathetic activation of brown adipose tissue and increased energy expenditure (Fig. 5).

Mice genetically deficient in the leptin gene (*ob/ob*) or the leptin receptor (*db/db*) are characterized by obesity, hyperphagia, insulin resistance, and impaired glucose tolerance (D'souza et al., 2014; Coleman, 1978). Meanwhile, treatment with leptin in *ob/ob* or *db/db* mice results in improvement in obesity and metabolic derangements (Pelleymounter et al., 1995). Although these effects were initially attributed to effects on reduced body weight, it is now

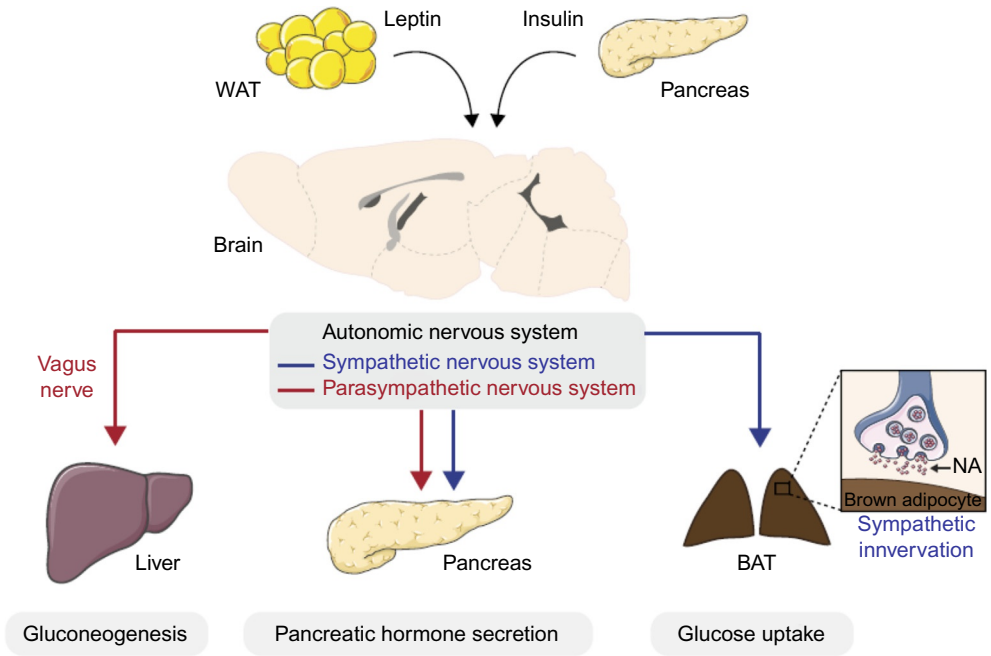

FIG. 5 Pathways involved in the control of glucose homeostasis. The central nervous system contains high density of receptors for the WAT-derived hormone leptin and receptors for the pancreatic hormone insulin. Leptin and insulin act on specific brain regions that will in turn modulate glucose utilization and production in peripheral tissue via the ANS. Notably the vagus nerve links brain insulin action and the liver in the control of hepatic gluconeogenesis. At the pancreatic level the ANS is involved in pancreatic hormone secretion. The BAT receives sympathetic innervation, which activity directly control BAT glucose uptake. *ANS*, autonomic nervous system; *BAT*, brown adipose tissue; *NA*, noradrenaline; *WAT*, white adipose tissue. *Adapted with permission from Ruud, J., Steculorum, S.M., Brüning, J.C., 2017. Neuronal control of peripheral insulin sensitivity and glucose metabolism. Nat. Commun. 8, 15259.*

known that leptin signaling in the hypothalamus is critical for maintenance of glucose homeostasis. Icv injection of leptin results in normoglycemia and improves insulin sensitivity in *ob/ob*, high-fat fed, and insulin-deficient rodents but has negligible effects on peripheral leptin levels (Koch et al., 2010; Fujikawa et al., 2010).

Leptin also selectively suppresses sweet taste responses. Chorda tympani (CT) nerve responses, which transmit taste information from the anterior two-thirds of the tongue, were compared in *db/db* and control mice. The *db/db* mice lacking leptin receptors showed greater CT nerve responses to sugars and sweeteners compared with control mice (Sako et al., 1990). However, there were no differences in CT nerve responses to other taste compounds, including NaCl, HCl, and quinine in *db/db* and control mice. Meanwhile, intraperitoneal administration of leptin to control mice resulted in decreased CT nerve responses to sucrose and saccharin (Kawai et al., 2000). However, these effects of leptin were not seen in *db/db* mice.

Leptin acts on taste receptor cells to suppress sweet taste sensitivity. The functional leptin receptor, Ob-Rb, has been shown by RT-PCR to be expressed in taste bud cells (Shigemura et al., 2004). Whole-cell patch-clamp recordings demonstrated that leptin activated efflux of K^+ and subsequent hyperpolarization of taste cells (Kawai et al., 2000). Furthermore, extracellular recording of isolated taste cells showed significant reduction to sweet stimuli in approximately half of sweet-responsive cells after application of 10–20 ng/mL leptin (Yoshida et al., 2006). Thus leptin may act to suppress sweet taste sensitivity by modulating responsiveness of sweet taste cells.

Leptin may also affect sweet taste responsiveness in humans as well. Plasma leptin levels show a diurnal variation in humans with leptin levels rising before noon, peaking between 2300 and 0100 h, and then declining until the morning (Sinha et al., 1996). To determine if the threshold for sweet taste similarly shows diurnal variation, recognition thresholds for different taste stimuli and plasma leptin levels were measured under three different meal conditions in 91 nonobese subjects (Nakamura et al., 2008). Under normal meal conditions (meals at 0830, 1230, and 1730), subjects required higher concentrations to detect sweet substances, including sucrose, glucose, and saccharin, when tested in the evening compared with the morning. Meanwhile, under restricted meal conditions with one meal (1730 h) or two meals (1230 and 1730 h) per day, leptin levels were phase shifted, while recognition thresholds for sweet substances shifted in parallel. These findings were not seen with other taste substances, such as NaCl, citric acid, quinine, and monosodium glutamate. These observations may not extend to overweight and obese subjects, potentially due to higher basal levels of plasma leptin (Yoshida et al., 2013).

Endocannabinoids

Cannabinoids, such as *Cannabis sativa*, have been well documented to have an appetite-stimulating effect (Mechoulam et al., 1998). More recently, endocannabinoids, such as anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), have been shown to promote orexigenic effects via CB_1 receptors in the hypothalamus and limbic regions in the brain (Kirkham et al., 2002). CB_1 receptor knockout mice have decreased food intake compared with wild-type mice, while CB_1 antagonist, SR141716A, decreased food intake in wild-type mice only (Di Marzo et al., 2001). Meanwhile, acute administration of leptin to normal rats and *ob/ob* mice decreases AEA and 2-AG levels in the hypothalamus. Moreover, *ob/ob* and *db/db* mice

demonstrated elevated levels of endocannabinoids in the hypothalamus but not in the cerebellum, which is more commonly associated with motor function. Thus endocannabinoids in the hypothalamus likely act as orexigenic mediators and play an important role in modulating appetite but appear to be under negative control by leptin.

Endocannabinoids also likely enhance taste cell sensitivity to sweet substances. Genetically deficient *db/db* mice show greater sensitivity to sweet responses, which is at least partly related to leptin deficiency. However, endocannabinoids may also contribute to these effects via sweet taste receptors. In wild-type mice, ip administration of AEA or 2-AG increased CT nerve responses to sweeteners (sucrose, saccharin, and glucose) in a dose-dependent manner but not to other substances, including NaCl, HCl, quinine, and MSG (Yoshida et al., 2010). These sweet-enhancing effects of AEA and 2-AG were abolished in CB_1 knockout mice or in the presence of AM251, a CB_1 receptor antagonist. Meanwhile, administration of AEA or 2-AG to the basolateral surface of sweet taste cells markedly enhanced responsiveness to sweet substances. In addition, these authors demonstrated using RT-PCR and immunohistochemistry that ~60% of taste cells coexpress CB_1 and T1R3. These observations suggest that endocannabinoids enhance sweet taste sensitivity through sweet taste receptors.

Conclusions

The identification of the sweet taste receptors and its subunits has brought about tremendous advances in our knowledge of the gustatory system. In addition, sweet taste receptors play an important role as chemosensory agents in the gastrointestinal tract. These receptors are also involved in regulating GI hormones, such as GLP-1 and PYY, which transmit information to brain regions that are critical regulators of appetite, satiety, glucose homeostasis, and weight. There remains a tantalizing prospect that modulation of sweet taste receptors and downstream targets may be novel approaches towards treatment of conditions, such as obesity, diabetes mellitus, and other metabolic conditions.

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Conflicts of interest

The authors have declared that no conflict of interest exists.

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CHAPTER

17

The role of D-galactose in the aging heart and brain

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OUTLINE

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SUMMARY POINTS

- This chapter focuses on research into D-galactose-induced cardiac and brain aging and a variety of treatments, specifically hyperbaric oxygen therapy (HBOT).
- D-Galactose is a reducing sugar, and it has been well established that it can be used to induce mimetic cardiac and brain aging in animal models.
- The underlying mechanisms behind D-galactose-induced cardiac aging are increased oxidative stress, apoptosis, inflammation, calcium dyshomeostasis, and impaired autophagy.
- D-Galactose exerted cognitive decline via the several mechanisms, including increased oxidative stress, apoptosis, inflammation, autophagy imbalance, BDNF deficiency, cholinergic system impairment, astrocytes, and microglia activation and decreased antioxidant enzymes.
- HBOT has been widely used clinically, and recently, HBOT has been used to treat D-galactose-induced neurodegeneration.
- This chapter summarizes the various pathways for targeting future antiaging studies.

Key facts of hyperbaric oxygen therapy

- Hyperbaric oxygen therapy, also called HBOT, delivers oxygen to the patients inside a pressurized air chamber.
- Under normal physiological conditions, the hemoglobin in red blood cells carries oxygen and transports it to the whole body.
- In the hyperbaric state, oxygen molecules can easily dissolve in all body fluids providing oxygen to previously unreachable areas by blood supply.
- Hyperbaric oxygen therapy is used to treat medical conditions that profit from increased tissue oxygen availability such as carbon monoxide poisoning, air or gas embolisms, and compartment syndrome.
- Pressure, frequency, period, and duration of HBOT vary depending on different diseases.

Introduction

Globally, more than 36 million people die every year as a result of noncommunicable diseases such as cardiovascular diseases, dementia, diabetes, chronic respiratory diseases, and cancers (Banjare and Bhalerao, 2016). Although aging contributes to this prevalence, gerontologists have struggled to clarify the evolutionary process of aging. The elderly population is growing speedily, and of the age-related diseases, cardiovascular diseases and dementia are the major health problems in this demographic worldwide (Wortmann, 2012; Yazdanyar and

Newman, 2009). At this time, much more research is needed to inform the comprehension of the aging mechanisms that lead to the degenerative/pathological processes in the vital organs such as the heart and the brain and for developing antiaging treatments that can hopefully slow down these undesirable cascades. Unfortunately, human studies in antiaging experiments are complicated due to many preexisting conditions such as diabetes, hypertension, and cancer.

Although in vitro studies of diseases offer useful information regarding the cellular and molecular mechanisms, they have not been able to provide the precise phenotypes pertinent to in vivo models. Therefore, any in vivo models that can exhibit the characteristics similar to those in aging humans will be very useful in enabling investigators in their pursuit of fundamental understanding of the aging process without the need to wait for the natural aging in animals. Currently, there are various types of senescence models that can be induced by X-ray (Bertell, 1977), jet lag (Xu et al., 2009), and D-galactose (Cebe et al., 2014; Kumar et al., 2009). In addition, naturally aging and genetically modified models (insulin/IGF-1 receptor gene daf-2 knockdown or injection of secreted isoform Klotho) are being used in antiaging studies (Masso et al., 2017; Van Raamsdonk, 2018; Yanar et al., 2011). As regards genetically modified models, different timing has been required for distinct pathways of lifespan extension, and the underlying mechanisms are still unclear (Van Raamsdonk, 2018).

Currently, premature aging induced by chronic D-galactose exposure has been shown to have phenotypes similar to those reported in naturally aging rodents (Cebe et al., 2014; Yanar et al., 2011). This model has been used widely to investigate the aging-related mechanisms involving heart diseases (Bei et al., 2018) and neurodegenerative diseases (Qu et al., 2016). As the D-galactose-induced aging model has been the most commonly used model for both cardiac and brain aging processes (Bei et al., 2018; Haider et al., 2015), this chapter explores the mechanisms for aging induction and the aging-related cardiovascular and neurodegenerative diseases in this model.

D-Galactose and the aging process

D-Galactose is an aldohexose, which occurs naturally in the D-form in lactose, gangliosides, cerebrosides, glycoproteins, and glycolipids and a variety of foods such as dairy products and some fruits (Acosta and Gross, 1995). Sodium-dependent glucose cotransporters type 1 transport D-galactose across the luminal side of the enterocytes, while glucose transporter type 2 facilitates the passage of galactose across the plasma membrane (Ferrannini and Solini, 2012; Leturque et al., 2005). The major pathway for D-galactose metabolism is the Leloir pathway in which three enzymes, including galactokinase, galactose-1-phosphate uridyltransferase, and UDP-galactose-4′-epimerase, metabolize D-galactose into glucose, which then enters the glycolytic pathway or is stored as glycogen in liver, muscle, and adipose tissues (Bosch, 2006). This pathway is illustrated in Fig. 1. In both the heart and brain, the uptake of D-galactose is via glucose transport type 1 (GLUT-1) (Shao and Tian, 2015). In healthy adults the normal plasma concentration of D-galactose is <10 mg/dL (Morava, 2014). However, if excess D-galactose accumulates in the body, it can be converted into its toxic metabolite galactitol, which can induce adverse effects associated with the aging process (Xu et al., 2018).

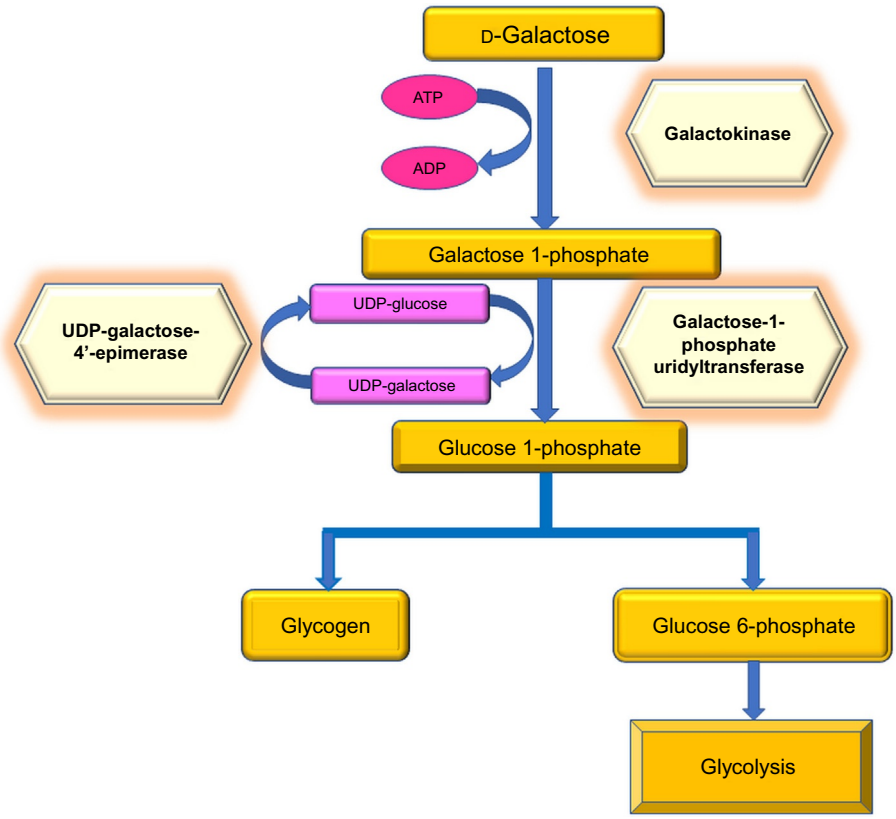

FIG. 1 The Leloir pathway. The major pathway for D-galactose metabolism is the Leloir pathway in which three enzymes galactokinase, galactose-1-phosphate uridyltransferase, and UDP-galactose-4′-epimerase facilitate the metabolism of D-galactose into glucose, which then enters the glycolytic pathway or is stored as glycogen in the liver, muscle, and adipose tissues.

Accumulating evidence demonstrates that an excess amount of D-galactose can upregulate the expression of senescence markers (Cebe et al., 2014; Wu et al., 2017) and decrease telomere length (Bei et al., 2018) in cardiac tissues. A previous in vitro study reported that D-galactose increases the expression of senescence-associated β-galactosidase (SA-β-gal) positive cells (Liu et al., 2012). When D-galactose accumulation occurs, a nonenzymatic reaction between D-galactose and lysine residue of proteins occurs to produce an unstable compound Schiff base. Weeks or months later, this Schiff base becomes stable compounds known as advanced glycation end-products (AGEs) (Hegab et al., 2012). AGEs are one of the senescence markers that have been shown to increase in aging (Frimat et al., 2017; Song et al., 1999). AGEs can bind with its complementary receptors for AGEs (RAGE) in various cell types, and this can activate nuclear factor kappa-B (NF-κB) and other signaling pathways, which are able to produce reactive oxygen species (ROS), thereby speeding up the aging process (Frimat et al., 2017; Hegab et al., 2012). Additionally, increases in SA-β-gal staining and β-gal-positive cell

expression have been seen in a recent study of a D-galactose-induced aging heart model (Chang et al., 2017).

It is known that the tumor suppressor pathways p53 or p16 are usually involved in the mechanism of aging. Expression of p53 and p21 was increased in D-galactose-treated mice (Wu et al., 2017). When senescent cells accumulate in the cardiac tissues, they can decrease the regenerative capacity and induce low-grade inflammation, thus promoting aging and age-related disorders (Chang et al., 2016; Wu et al., 2017). Although cellular aging can be generated as a result of many stress-induced signaling pathways, the increase of ROS caused by systemic administration of D-galactose plays a crucial role in the upregulation of cardiac senescence markers (Sikora et al., 2011). Recently the expression of microRNA 21 (miR-21) was found to be elevated in both naturally aged and D-galactose-induced aged mice when compared with young mice and was associated with decreased telomere length and increased p16 and p19 expression in cardiac tissue (Bei et al., 2018). A list of reported senescence markers in the heart is summarized in Table 1.

For D-galactose-induced brain aging, it has been shown that a pathological dosage of D-galactose can increase ROS production, resulting in the impairment of mitochondrial function and increased oxidative stress, inflammation, and apoptosis in neurons (Kumar et al., 2009; Yang et al., 2016). Chronic injections of D-galactose increase senescence markers in the brain. These markers include AGE, RAGE, SA-β-gal staining, telomere length shortening, telomerase activity, beta-site amyloid precursor protein-cleaving enzyme 1, amyloid beta protein ($A\beta_{1-42}$), and senescence-associated genes (p16, p21, and p53). In addition to changes at the molecular level, long-term administration of D-galactose can induce cognitive dysfunction, which is associated with symptoms of brain aging (Zhu et al., 2014). A previously reported list of senescence markers in the brain is summarized in Table 2.

TABLE 1 A list of cardiac senescence markers in the D-galactose-induced aging heart

Study model	D-Galactose dose, route, and duration	Cardiac senescence markers	Ref
Neonatal SD rat cardiomyocytes	5 g/L for 48 h	• ↑ β-gal-positive cells • ↑ AGEs	Liu et al. (2012)
8-week-old C57BL/6J mice	50 mg/kg/d, s.c., 8 weeks	• ↑ p53 • ↑ p21	Wu et al. (2017)
10-week-old SD rats	150 mg/kg/d, i.p., 8 weeks	• ↑ SA-β-gal staining • ↑ p21	Chang et al. (2017)
10–12-week-old C57BL/six mice	100 mg/kg/d, s.c., 10 weeks	• ↓ Telomere length • ↑ p16 • ↑ p19	Bei et al. (2018)
20-week-old Wistar rats	60 mg/kg/d, i.p., 6 weeks	• ↑ AGE	Cebe et al. (2014)

The D-galactose-induced aging heart showed an increase in β-gal-positive cells, SA-β-gal staining, AGE, p53, p21, p16, and p19 and a decrease in telomere length.
SD rats, Sprague-Dawley rats; *IP*, intraperitoneal; *s.c.*, subcutaneous; *AGEs*, advanced glycation end-products; *SA-β-gal*, senescence-associated β-galactosidase; *β-gal*, β-galactosidase.

TABLE 2 A list of brain senescence markers in the D-galactose-induced aging brain

Study model	D-Galactose dose, route and duration	Brain senescence markers	Ref
12-week-old Kunming mice	200 mg/kg/d, i.p., 8 weeks	• ↑ AGE • ↑ p16, p21, p53 • ↑ $A\beta_{1-42}$	Chen et al. (2016c)
10-week-old Kunming mice	50 mg/kg/d, s.c., 8 weeks	• ↑ AGEs • ↑ RAGE	Lu et al. (2010b)
12-week-old Kunming mice	180 mg/kg/d, s.c., 8 weeks	• ↑ AGE • ↑ RAGE • ↑ AR • ↑ SDH	Yu et al. (2015)
12-week-old Sprague-Dawley rats	120 mg/kg/d, s.c., 6 weeks	• ↑ p53 • ↑ $p19^{Arf}$ • ↑ $p21^{Cip1/Waf1}$ • ↑ SA-β-gal staining • ↓ Telomere length • ↓ Telomerase activity	Zhu et al. (2014)
12-week-old Sprague-Dawley rats	100 mg/kg/d, i.p., 7 weeks	• ↑ BACE-1 • ↑ Aβ • ↑ RAGE	Rehman et al. (2017)

Increased AGE, RAGE, AR, SDH, p16, p21, p53, $p19^{Arf}$, $p21^{Cip1/Waf1}$, SA-β-gal staining, Aβ, and BACE-1 and decreased telomere length and telomerase activity were found in the D-galactose-induced aging brain.
s.c., subcutaneous; *AGEs*, advanced glycation end-products; *RAGE*, AGE receptors; *inj*, injection; *AR*, aldose reductase; *SDH*, sorbitol dehydrogenase; *SA-β-gal*, senescence-associated β-galactosidase; *p53, p19Arf, p21Cip1/Waf1*, senescence-associated genes; *Aβ*, amyloid beta protein; *BACE-1*, β-site amyloid precursor protein-cleaving enzyme 1; *i.p.*, intraperitoneal.

Role of D-galactose in cardiac aging

The underlying mechanisms behind the effects of D-galactose-induced cardiac aging have been extensively investigated. Previous studies have indicated that exogenous D-galactose injections can cause cardiac dysfunction as shown by a reduction in left ventricular ejection fraction and fractional shortening, which was assessed by echocardiography (Bei et al., 2018; Chang et al., 2016). Cardiac dysfunction seen in D-galactose-treated rats and mice is caused by factors including increased oxidative stress, apoptosis, inflammation, calcium dyshomeostasis, and autophagy (Bo-Htay et al., 2018; Ren et al., 2017). This is illustrated in Fig. 2.

D-Galactose administration could increase oxidative stress and decrease antioxidant levels (Cebe et al., 2014; Wu et al., 2017). When large amounts of D-galactose are present, it is converted to galactitol by galactose reductase via the polyol pathway. Excess D-galactose is also oxidized to hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) by the action of galactose oxidase, both of which could accelerate the mechanisms of aging (Aydin et al., 2012;

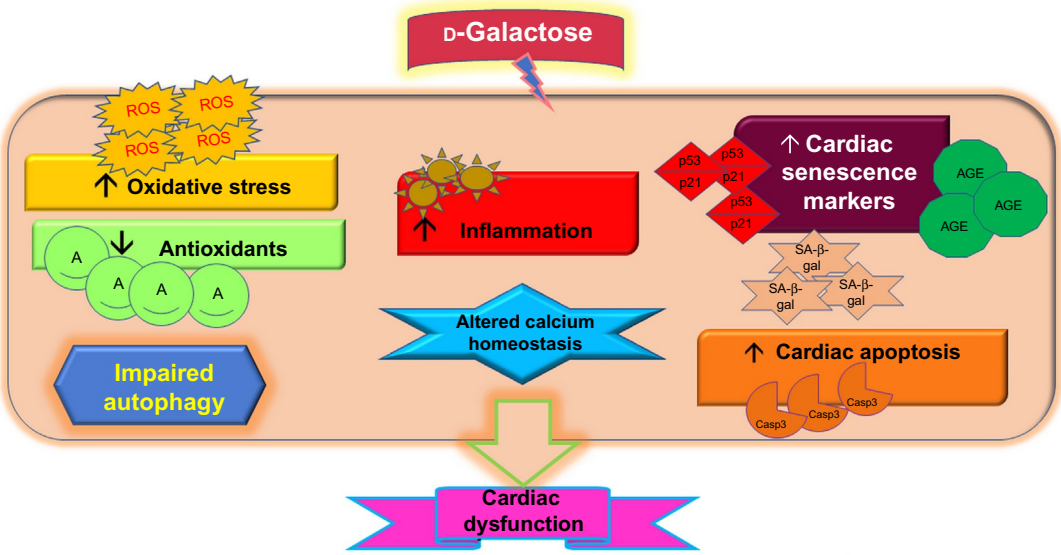

FIG. 2 A summary of the mechanisms of how D-galactose induces cardiac aging. Exogenous D-galactose injections led to increased cardiac aging markers, oxidative stress, inflammation, apoptosis, calcium dyshomeostasis, and impaired autophagy, resulting in cardiac dysfunction.

Yanar et al., 2011). Excess ROS released by the metabolism of D-galactose can increase protein, lipid, and DNA peroxidation, resulting in the damage of proteins, lipids, and DNA in cardiac cells (Cebe et al., 2014). Also reduced levels of endogenous antioxidant enzymes such as SOD, glutathione peroxidase (Wu et al., 2017), and reduced glutathione levels (Li et al., 2016) have been observed in D-galactose-induced aged rats and mice. DNA oxidation was observed in D-galactose-treated rats in addition to the protein and lipid peroxidation, as indicated by increased levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in myocardial tissues (Cebe et al., 2014). Guanosine is the DNA nucleobase most vulnerable to oxidization by ROS, the oxidative injury of guanosine potentially leading to the formation of 8-OHdG. This process could be promoted by the increased activity of protein oxidation caused by D-galactose (Cebe et al., 2014).

The second mechanism that leads to cardiac dysfunction in D-galactose-administered aged rats and mice is apoptosis. D-Galactose administration could increase cardiac apoptosis markers (Guo et al., 2017; Sun et al., 2014). It is widely accepted that cardiac apoptosis involves two main pathways, the intrinsic and extrinsic pathways (Wang and Youle, 2009). Data from a recent study have shown that increased cardiac apoptosis was seen in mice given D-Galactose via an extrinsic pathway (Chang et al., 2016). This may well be due to the formation of AGEs from excess D-galactose through the Maillard reaction (Hegab et al., 2012). When AGEs combine with their receptors, RAGEs, ROS production occurs catalyzed by NADPH oxidase. Then, p38 MAP kinase becomes activated and leads to the translocation of nuclear transcription factors (NF-κB) into the nucleus, resulting in the activation of inflammatory cascades and tumor necrosis factor alpha (TNF-α) (Hegab et al., 2012).

D-Galactose administration can also stimulate cardiac apoptosis via the intrinsic pathway. Previous studies have demonstrated that the injection of D-galactose can decrease Bcl-2 and increase Bax protein expression, leading to a reduced ratio of Bcl-2 and Bax, resulting in increased release of mitochondrial cytochrome *c* (Cyt *c*) (Guo et al., 2017; Sun et al., 2014).

The alteration in calcium homeostasis has been shown to be associated with cardiac dysfunction in a D-galactose-induced aging model. D-Galactose administration could reduce the expression of the calcium removal protein, which consequently increased calcium removal time and increased intracellular calcium levels in in vitro and in vivo investigations (Liu et al., 2012; Sun et al., 2014). A reduction in the activity of sarco/endoplasmic reticulum calcium adenosine triphosphate (SERCA) could delay the removal of cytosolic calcium level. In addition, an increased activity of the SERCA-inhibitory protein, phospholamban (PLB), also reduced the elimination of cytosolic calcium. It has been demonstrated that D-galactose increases PLB activity through the reduced phosphorylation of PLB at serine 16 level, thus leading to a decreased activity of SERCA and finally resulting in diastolic dysfunction in D-galactose-induced aging cardiomyocytes (Liu et al., 2012).

Increased oxidative stress caused by the abnormal metabolism of D-galactose led to galactose oxidation and AGE generation, resulting in the overproduction of ROS in cardiac tissue, which could contribute to cardiac hypertrophy in D-galactose-induced aging rats (Chang et al., 2017). Furthermore the interaction of AGE-RAGE in cardiac tissue can induce the transcription of inflammatory genes, leading to inflammation via NF-κB nuclear translocation, resulting in increased cardiac inflammatory cells in D-galactose-induced aging mice (Frimat et al., 2017; Liang et al., 2017). Interestingly, at a cellular level, aging is associated with reduced cellular autophagy and progressive accumulation of damaged proteins and undigested materials, leading to heart failure. A recent study has reported that impaired autophagy is seen in the ischemia/reperfusion (I/R) model of naturally aging rat hearts and the hypoxia/reoxygenation (H/R) model of D-galactose-induced aged cardiomyocytes. This was indicated by decreased LC3-II and Beclin 1 expression and increased p62 expression (Chen et al., 2016b).

Currently, only a few studies have investigated the role of D-galactose in cardiac mitochondria. D-Galactose caused mitochondrial elongation through downregulation of the mitochondrial fission GTPase dynamin-related protein 1 (Drp1) in D-galactose-treated aged H9c2 cells (Ren et al., 2017). In contrast, previous studies investigating the effects of D-galactose on cardiac mitochondrial energy production (Guo et al., 2017) and mitochondrial complex 1 activity (Chang et al., 2014) demonstrated that D-galactose did not decrease the mitochondrial energy production and mitochondrial complex 1 activity in the heart. At this time, more experiments are required to elucidate the effects of D-galactose on mitochondrial functions in the heart.

Role of D-galactose in brain aging

In the D-galactose-induced aging model, it has been shown that D-galactose could cause cognitive decline similar to that found in aging people (Rehman et al., 2017). There are several factors influencing the cognitive function in D-galactose-induced brain aging. These include oxidative stress, apoptosis, inflammation, autophagy, brain-derived neurotrophic factor

(BDNF), cholinergic system, and astrocytes and microglia cell activation (Shwe et al., 2018). Generally, these factors are connected to each other.

It has been shown that the D-galactose-induced oxidative stress mechanism mainly operates at mitochondrial level in the brain (Kumar et al., 2009). Oxidative stress markers detected in D-galactose-induced brain aging models include malondialdehyde (MDA), inducible nitric oxide synthase (iNOS), total NOS, AOPP, ROS, H_2O_2, and 8-oxoguanine (Du et al., 2012; Zhang et al., 2010).

Following the systemic injection of D-galactose, surplus D-galactose combines with amines to form AGEs, which act on its receptor RAGEs, causing neuronal cell injury and cognitive decline (Hsieh et al., 2009). In addition, excess D-galactose is reduced via galactose reductase to produce galactitol, resulting in osmotic stress and a reduction in mitochondrial electron transport chain activity with increased oxidative stress production, leading to mitochondrial dysfunction (Hsieh et al., 2009). Furthermore, D-galactose could increase the number of mitochondrial DNA mutations and reduce the number of mitochondrial respiratory chain complex enzymes in the brain (Kumar et al., 2009).

In addition to leading to increased oxidative stress, D-galactose reduced antioxidant enzymes including catalase, superoxide dismutase, glutathione, and glutathione peroxidase, reducing total antioxidant capacity. An imbalance between ROS and antioxidants favors mitochondrial dysfunction, which plays an important role in apoptosis and the inflammation processes associated with aging (Kumar et al., 2009).

D-Galactose has been shown to activate both the intrinsic and extrinsic pathways associated with apoptosis in the brain (Salehpour et al., 2017). It has been shown that D-galactose suppressed the expression of antiapoptotic Bcl-2, elevated apoptotic Bax levels, and accelerated the capacity of the mitochondria to release Cyt *c*, which caused caspase-3 and caspase-9 activation (Salehpour et al., 2017). These findings indicated that D-galactose mediated the apoptosis (Qian et al., 2008) and enhanced inflammation in the brain (Yu et al., 2015).

Interestingly, inflammation plays a major role in the extrinsic pathway of apoptosis (Salehpour et al., 2017). D-Galactose enhances neuroinflammation by stimulating the transcription of NF-κB, leading to cognitive dysfunction (Rehman et al., 2017). Different inflammatory markers have been used in D-galactose-induced senescence animal models including nuclear factor (NF-κB), cyclooxygenase (COX-2), iNOS, NOS-2, tumor necrosis factor alpha (TNF-α), and interleukins (IL-1β and IL-6) (Gao et al., 2015; Yang et al., 2016; Yu et al., 2015).

It has been shown that the production of inflammatory markers increased microglia cells and astrocytes activation, resulting in cognitive dysfunction (Srikanth et al., 2011). Astrocytes and microglia cell activation played crucial roles in aging-related neurological diseases (Benner et al., 2004; Wu et al., 2002). The specific marker for activated microglia cells is glial fibrillary acidic protein and CD11b for astrocytes. In D-galactose-induced senescence models, astrocytes and microglial cell activation were observed in the hippocampus, prefrontal cortex, and whole brain (Lu et al., 2010b; Rehman et al., 2017; Zhu et al., 2014).

The mechanism underlying D-galactose-induced senescence has been associated with the impaired autophagy pathway. Several studies have pointed out that autophagy declines with aging (Cuervo et al., 2014; Ott et al., 2016). Autophagy is a compensatory mechanism that blocks apoptosis and maintains homeostasis in the body (Yuan et al., 2018). It has been shown that an imbalance in autophagy, controlled by the Rheb-mTOR pathway in the hippocampus, contributed to cognitive decline in the D-galactose-induced aging brain (Wang et al., 2018).

Kou and his colleagues indicated that spatial learning and memory impairment are associated with decreased levels of autophagy-related proteins, LC3 and Beclin 1 in the hippocampus of D-galactose-induced aging rats (Kou et al., 2017). In summary, D-galactose impairs the autophagic process by decreasing autophagy-related proteins, LC3 and Beclin 1, and by regulating the Rheb-mTOR pathway (Kou et al., 2017; Wang et al., 2018).

It has been proven that the cholinergic system and brain-derived neurotrophic factor (BDNF) play a key role in cognitive function especially in neurodegenerative disorders (Lu et al., 2010a). Increased D-galactose levels could increase acetylcholine esterase activity and decrease acetylcholine levels when compared with the controls. In addition, acetylcholine modulates synaptic plasticity through BDNF in the hippocampus (Czubak et al., 2009). A previous study showed that D-galactose caused a reduction in the BDNF level by decreasing the level of phosphorylated cAMP response element-binding protein (CREB), resulting in cognitive dysfunction (Rehman et al., 2017).

All of earlier findings indicated that D-galactose mediated cognitive dysfunction through the mechanisms of increased oxidative stress, apoptosis, inflammation, autophagy imbalance, BDNF deficiency, cholinergic system impairment, astrocytes and microglia activation, and decreased antioxidant enzymes (Shwe et al., 2018). The mechanisms of D-galactose-induced brain aging are summarized in Fig. 3.

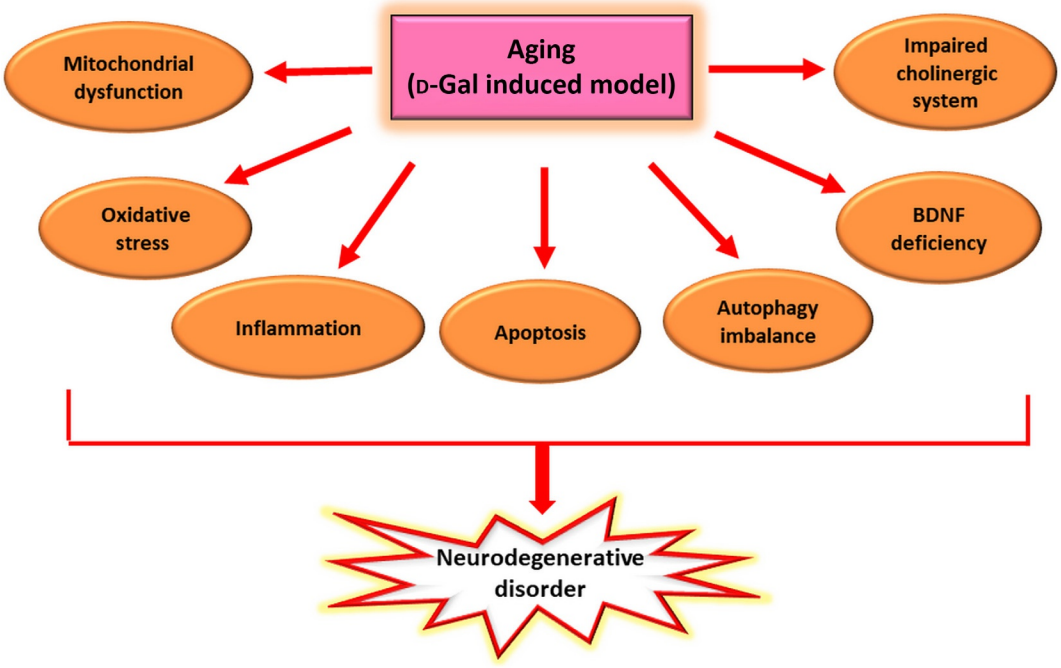

FIG. 3 The underlying mechanisms of D-galactose-induced brain aging. Increased oxidative stress, mitochondrial dysfunction, apoptosis, inflammation, autophagy imbalance, BDNF deficiency, and cholinergic system impairment caused by excess D-galactose administration can result in neurodegenerative disorders.

Interventions to improve the detriment in cardiac and brain aging induced by D-galactose

A diversity of interventions has been investigated in D-galactose-induced cardiac and brain aging, including antioxidants, hormones, HBOT, and other alternative treatments.

Antioxidants

It has been shown that several antioxidants including selenium, vitamin E, and anthocyanins exert beneficial effects on the heart in D-galactose-induced cardiac oxidative stress (Li et al., 2016). *Alpinate oxyphyllae fructus* could decrease hypertrophy of cardiac muscles and ameliorate cardiac dysfunction (Chang et al., 2016; Chang et al., 2017). *Ginkgo biloba* extract (EGB761) could decrease the cardiac aging markers and cause intracellular calcium overload in D-galactose-treated cultured cardiomyocytes (Liu et al., 2012). In some studies concerning D-galactose-induced brain aging, antioxidants like carvedilol and catalpol have been shown to improve respiratory enzyme complex activities in the brain mitochondria (Kumar et al., 2009; Zhang et al., 2010). In addition, ginsenoside and ursolic acid are antioxidants that have the ability to improve cognitive function in D-galactose-treated mice and rats (Lu et al., 2010b; Zhu et al., 2014).

Hormones

It has been proposed that the hormone melatonin may be efficacious in reducing cardiac apoptosis by decreasing Cyt *c* release, increasing Bcl-2 expression, and also increasing cardiac mitochondrial ATP production in D-galactose-induced aging rats (Guo et al., 2017). Melatonin has been shown to ameliorate the D-galactose-induced cognitive decline in the brains of C57BL/6N mice via the RAGE/NF-K B/JNK pathway (Ali et al., 2015). Fibroblast growth factor 21 (FGF21) is a hormone that is produced by many organs and plays a crucial role in glucose metabolism and improves insulin sensitivity (Li et al., 2018). FGF21 has been shown to improve cognitive function in D-galactose-induced aging mice (Yu et al., 2015).

Hyperbaric oxygen therapy

Hyperbaric oxygen therapy, also called HBOT, is the treatment aimed to deliver oxygen to the patients inside a pressurized air chamber (Bennett et al., 2016). It is now widely used as a therapeutic approach in several medical conditions including air or gas embolism, carbon monoxide poisoning, gas gangrene, decompression sickness, and diabetically derived illness (Yan et al., 2015). HBOT has been shown to have minor and temporary side effects such as barotrauma and changes in vision (hadanny et al., 2016), but these are considered acceptable compared with its many benefits. Currently, HBOT is a standard therapy for carbon monoxide poisoning and decompression sickness used clinically by giving oxygen to patients in a chamber where atmospheric pressure is increased and controlled (Chen et al., 2016a, c). In addition, HBOT has been used clinically to improve neurological disorders (Chen et al., 2016c; Shapira et al., 2018). Several studies used HBOT as an adjunctive therapy to get more

enhanced improvements in ischemic conditions such as myocardial infarction and cerebral ischemia (Chen et al., 2016a, c; Dave et al., 2003). In the brain, HBOT has been shown to decrease ROS production while upregulating the antioxidants in hippocampal tissues in D-galactose-induced aging mice (Chen et al., 2016c). HBOT also abrogated the mitochondrial dysfunction in the motor cortex and spinal cord, resulting in a delay in the onset of motor neuron disease (Dave et al., 2003).

Previous studies have demonstrated that HBOT could reduce the formation of AGEs and inflammation in D-galactose-treated mice (Chen et al., 2016c). HBOT alleviated the number of microglia and increased their branching, leading to increased Aβ clearance and reduced secretion of inflammatory cytokines in triple-transgenic Alzheimer's disease mice (Shapira et al., 2018). HBOT also significantly attenuated β-amyloid protein 1–42 expression and restored the expression of BDNF (Chen et al., 2016c). HBOT has also been found to minimize oxidative stress by decreasing mitochondria-mediated apoptosis signaling in an Alzheimer's disease mice model (Chen et al., 2017).

Clinically, HBOT has also been used to treat traumatic brain injury and posttraumatic stress disorder (Guedes et al., 2016). Furthermore, HBOT could decrease senescence-related proteins in D-galactose-treated mice (Chen et al., 2016c). These findings indicate that HBOT provided neuroprotective effects (Chen et al., 2017). The underlying mechanisms and the roles of HBOT in D-galactose-induced brain aging models are summarized in Fig. 4. Despite these potential benefits of HBOT on the brain, limited information is available regarding its effects on the heart in the D-galactose-induced aging model. Future studies are needed to elucidate the role of HBOT in D-galactose-induced aging heart.

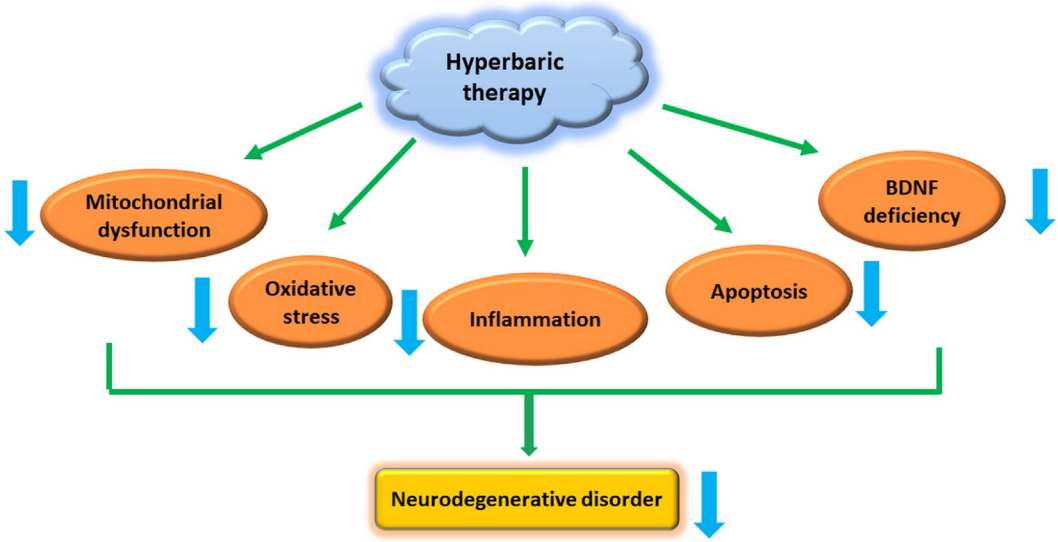

FIG. 4 Effects of HBOT on D-galactose-induced aging in the brain. HBOT alleviated D-galactose-induced mitochondrial dysfunction, oxidative stress, inflammation, apoptosis, and BDNF deficiency, leading to improvement in neurodegenerative disorders.

Alternative treatments to improve D-galactose-induced aging in the heart and brain

In the heart the beneficial effect of the hydrogen sulfide (H_2S) donor, sodium hydrosulfide (NaHS), has been demonstrated by the attenuation of the expression of senescence marker expression in D-galactose-induced cardiac aging mice (Wu et al., 2017). H_2S also increased autophagy and decreased apoptosis, thus resulting in increased cell viability in D-galactose-induced aged H9c2 cells subjected to hypoxia/reoxygenation (H/R) injury (Chen et al., 2016b). In addition, data from a recent study have shown that the knocking out of the miR-21 gene could prevent cardiac dysfunction in D-galactose-treated aging mice. In that study, 10–12 weeks old miR-21 knockout mice were subcutaneously injected with D-galactose for 10 weeks, and the results indicated that cardiac function including the left ventricular ejection fraction percentage were preserved compared with D-galactose-administered mice (Bei et al., 2018). In addition, increased telomere length and decreased senescence proteins such as p16 and p19 expression were found in miR-21 knockout mice subjected to D-galactose compared with mice treated with D-galactose alone.

In the brain the benefits of transcranial low-level laser therapy (LLLT) have been demonstrated in limiting the impact of aging. LLLT at both red and near-infrared wavelengths is a noninvasive therapy, which has also been successfully used to treat different neurodegenerative disorders (Lapchak, 2012). Mitochondria are the main site of photon absorption at a cellular level, and Cyt *c* oxidase is the key enzyme in photoneuromodulation, which is responsible for mitochondrial electron transfer leading to increased ATP production (Karu and Afanas'eva, 1995). LLLT could also enhance ATP production and ameliorate mitochondrial dysfunction, oxidative stress, and apoptotic process, thus restoring the cognitive function in D-galactose-treated BALB/c mice (Salehpour et al., 2017).

Conclusion

Due to a rapid increase in the aging population worldwide, degenerative diseases in both the heart and the brain are inevitable. Due to this, increase intervention in the aging process becomes vital. In order to provide optimal intervention, understanding the pathophysiology of the aging heart and brain is essential. D-Galactose-induced aging model can be used to study the mechanisms of aging in both the heart and brain. The underlying mechanisms in D-galactose-mediated cardiac and brain aging include mitochondrial dysfunction, increased oxidative stress, decreased levels of antioxidants, increased apoptosis and inflammation, alteration in calcium homeostasis, and a reduction in brain-derived neurotrophic factors. Despite the existence of reports regarding the interventions used in this model to improve the pathophysiological changes in the heart and brain, much has yet to be learned before they can be applied clinically. The pathways described in this chapter are possible targets for prevention and better treatment in aging-related diseases in the future.

Glossary

Advanced glycation end-products (AGEs) AGEs are stable compounds formed from a nonenzymatic reaction with glucose or galactose and the lysine residue of proteins.

Hyperbaric oxygen therapy (HBOT) HBOT is a therapeutic intervention that transports oxygen to the patients inside a pressurized air chamber.

Receptors for advanced glycation end-products (RAGE) RAGEs are receptors for advanced glycation proteins present in various organs such as the heart, brain, and kidneys.

Schiff base Schiff bases are stable compounds formed from a nonenzymatic reaction between glucose or galactose and the lysine residue of proteins.

Senescence-associated β-galactosidase (SA-β-gal) SA-β-gal is a hydrolase enzyme that is currently widely used as a marker for aging both in in vivo and in vitro studies, usually detectable at pH 6 in aging cells.

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Conflict of interests

The authors declare no conflict of interest.

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CHAPTER

18

Effects of dietary Salba (*Salvia hispanica* L.) on glucose metabolism in an experimental model of dyslipidemia and insulin resistance

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OUTLINE

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Key Facts

- N-3 fatty acids play a role in the metabolic syndrome.
- *Salvia hispanica* L. (Salba) seed is rich in 18:3, n-3 fatty acids.
- *Salvia hispanica* L. (Salba) seed might reduce the metabolic syndrome factors.
- A sucrose-rich diet induces metabolic abnormalities in rats that mimic the metabolic syndrome in humans.
- Effects of *Salvia hispanica* L. (Salba) seed on glucose homeostasis and insulin resistance in the heart and skeletal muscle of rats fed a sucrose-rich diet.

Introduction

Dietary long-chain polyunsaturated fatty acids of the n-3 family (n-3 LCPUFAs) such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) play an important role in the maintenance of health (Calder, 2014). In recent years the study of plant-derived n-3 PUFAs began to gain importance. Different epidemiological and clinical studies have suggested that a higher concentration of α-linolenic acid (18:3 n-3, ALA)—the natural essential fatty acid precursor of n-3 LCPUFAs (EPA; docosapentaenoic acid, DPA; and DHA)—is associated with a reduced risk of cardiovascular disease (CVD), with improving dyslipidemia, insulin sensitivity, and glucose tolerance and with modifying the response to insulin action in key tissues. Additional health benefits include reductions of blood pressure, inflammatory, and coagulation markers (Rajaram, 2014). One of the botanical oil sources rich in ALA is the seed of *Salvia hispanica* L., commonly known as chia seed, which also contains considerable amounts of proteins, fiber, minerals, and antioxidant activity. This chapter focuses on the effect of dietary chia seeds upon the reversion and/or improvement of the altered glucose metabolism in an experimental animal model of dyslipidemia, insulin resistance, and visceral adiposity.

Origin, composition, and usage of *Salvia hispanica* L.

Salvia hispanica L., commonly known as chia, is an annual herbaceous plant belonging to the Lamiaceae family of about 900 species of green plants, characterized by its high nutritional and therapeutically potential. Native to Central American countries such as Guatemala and Mexico, its possible development is guaranteed by warm temperatures of 15–30°C and high rainfall (Coates and Ayerza, 1998; Coelho and Salas-Mellado, 2014). The ancient indigenous Aztec civilization consumed chia seed roasted and grounded incorporated to many foods as a source of energy. The word chia derives from the Nahuatl word "chian" with means oily. The name *Salvia hispanica* was given by the Swedish botanist Carl Linnaeus (1707–78), who confused the wild-growing plant coming from the New World with a native plant from Spain. The maximum height of the plant is 1 m with leaves of about 4–8 cm long and 3–6 cm wide. The flowers are white or purple containing oval seed mottle-colored with brown, gray, black, and white (Marcinek and Krejpcio, 2017). The white seed color is a recessive trait governed by a single gene, *scc* (Cahill and Provance, 2002). This trait has been selected by some cultivators with

the brand-name *Salvia hispanica* product Salba (Salba Corporation, Buenos Aires, Argentina, Agrisalba), which has a more stable content of omega-3 fatty acids, especially α-linolenic acid (ALA), than generic *Salvia hispanica* seeds. The lipid content in chia seeds varies from 25% to 40%, with 60% of the total lipids made up of ALA and 20% composed of linoleic acid (18:2 n-6 LA) (Bushway et al., 1984). After removing oil from the chia seed, there remains a significant concentration of dietary fiber (33.9 g/100 g) and protein of high quality (17 g/100 g) (Pereira da Silva et al., 2016). Of the total fiber, the greatest fraction (53.45 g/100 g) comprises insoluble fiber, which plays a role in satiety and proper bowel function (Vazquez-Ovando et al., 2009). Moreover, *Salvia hispanica* L. is also considered as a "functional food" since the nutritional elements described earlier are also supplemented by calcium, magnesium, iron, and bioactive compounds such as phytochemicals and antioxidants that have beneficial effects on human health. Particularly, *Salvia hispanica* L. seeds are a promising source of antioxidants due to the presence of polyphenols, chorogenic and caffeic acid, myricetin, quercetin, and kaempferol (Marcinek and Krejpcio, 2017). Fig. 1 summarizes *Salvia hispanica* L. (Salba) composition.

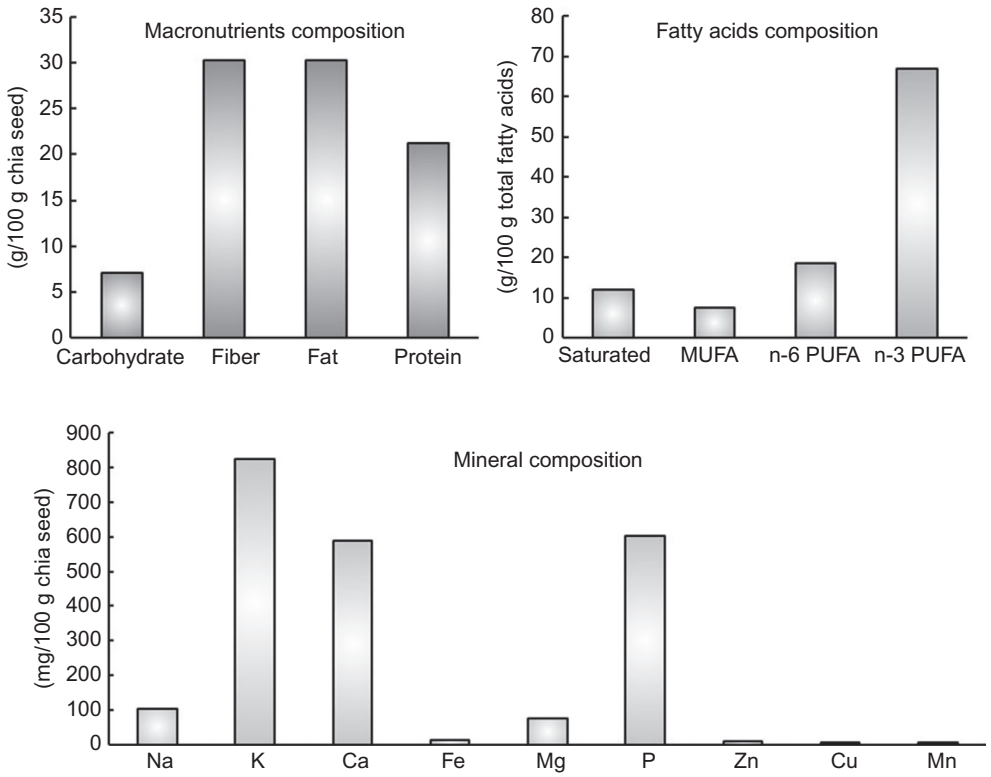

FIG. 1 *Salvia hispanica* L. variety Salba seed. Agrisalba S.A. Buenos Aires Argentina. Vitamin content (mg/100 g chia seed): vitamin C, 5.4; thiamine, 0.7; riboflavin, 0.2; niacin, 7.2; vitamin B6, 0.1. Antioxidant content in chia seed extracts (mol/kg chia seed): caffeic acid, 6.6×10^{-3}; chlorogenic acid, 7.1×10^{-3}; quercetin, $0.2\ 1 \times 10^{-3}$; myrecetin, $3.1\ 1 \times 10^{-3}$; kaempferol $1.1\ 1 \times 10^{-3}$.

Application to health promotion and disease prevention or improvement

Insulin resistance (IR) is a major hallmark implicated in the development of obesity, type 2 diabetes, hypertension, dyslipidemia, and coronary heart disease, disorders included in the metabolic syndrome (MS) (Hauner, 2002). During insulin-resistant states, normal amounts of insulin are inadequate to inhibit liver glucose output and to produce normal glucose uptake from adipose and muscle tissues. The development of IR is linked to both genetic and environmental factors (e.g., life styles and diets) (Storlien et al., 1997). Dietary factors that contribute to the development of IR include the amount and type of fat and its fatty acid composition (saturated and trans-fatty acids), high sucrose/fructose diet, or a combination of both (Western diet) (Lee et al., 2006,). On the other hand, it has been recognized that the adjustment of the quantity and quality of dietary lipids—for instance, increasing n-3 LCPUFAs from marine or plant sources such as EPA, DHA, and ALA—plays an important role in the prevention or treatment of adverse effects of the MS (Clarke, 2001; Barceló-Coblijin and Murphy, 2009). In this vein, it is well known that these fatty acids could act as potent hypolipidemic agents preventing the development of dyslipidemia in both rodents and humans (Connor, 2000; Lombardo and Chicco, 2006; Pan et al., 2012). Besides, they could improve the altered adiposity, glucose homeostasis, and insulin sensitivity (Lombardo and Chicco, 2006).

As mentioned earlier, the seeds of *Salvia hispanica* L. (chia seeds) contain the richest botanic source of ALA. In this regard, results reported by Vuksan et al. (2007) about well-controlled type 2 diabetes demonstrated that the consumption of the Salba grain improves cardiovascular risk factors by reducing blood pressure, inflammatory, and coagulations markers. In another study using a double-blind, placebo-controlled, randomized, crossover study design, the same group of researchers (Vuksan et al., 2010) showed that Salba supplementation attenuates postprandial glycemia and increases satiety in a dose-dependent manner in healthy subjects. Furthermore, Ho et al. (2013) showed that the effectiveness of both ground and whole Salba seeds in the attenuation of postprandial glycemia in healthy individuals was correlated to the amount of Salba incorporated in bread. Lately, a study comparing flax with Salba seeds showed that despite the similarities in nutritional composition, Salba-chia appears to have the ability to convert glucose into a slow-release carbohydrate and affect satiety to a greater extent than flax, possibly due to the higher fiber viscosity (Vuksan et al., 2017a, b). A recent study (Vuksan et al., 2017a, b) revealed that a 6-month addition of Salba-chia to a calorie-restricted diet, in conjunction with the standard medical care, resulted in small but significant weight loss, accompanied by a reduction in waist circumference and C-reactive protein in overweight and obese participants with type 2 diabetes. Other double-blind, randomized studies (Nieman et al., 2012; Nieman et al., 2009) assessed the effectiveness of milled or whole chia seeds in altering disease risk factors in overweight or obese postmenopausal healthy women using a metabolomics approach. Table 1 depicted principal studies of dietary *Salvia hispanica* L. on human health.

Besides, animal studies have shown a reduction in plasma triglyceride (Tg), free fatty acid (FFA), and total cholesterol and an increase in HDL cholesterol when chia seed or oil was used during 3–4 weeks as a source of dietary fat in both normal or dyslipidemic rats (Ayerza and Coates, 2007; Chicco et al., 2009; Ayerza and Coates, 2005). Recently, in rats fed a high fat-high fructose diet during 16 or 32 weeks, Poudyal et al. (2012a, b, 2013) showed that chia oil improved heart left ventricular dimensions, hypertension, and glucose tolerance as well as insulin sensitivity.

TABLE 1 The beneficial effects of dietary *Salvia hispanica* L. in human studies

Authors and references	Population under study and supplementation form	Results
Vuksan et al. (2007)	Randomized single-blind trial: type 2 diabetes (men and women, $n = 20$) aged 18–75 years feeding with 37 ± 4 g ground Salba-chia seeds included to bread/day during 12 weeks	↓ Systolic blood pressure ↓ C-reactive protein ↑ Plasma EPA
Vuksan et al. (2010)	Healthy men and women ($n = 11$) supplemented with 0, 7, 15, or 24 g Salba-chia seeds into bread/day. One day, blood test	↓ Postprandial glycemia compared with controls
Ho et al. (2013)	Healthy volunteers ($n = 13$) in a randomized double-blind trial feeding with 50 g bread with 0, 7, 15, or 24 g Salba-chia seed added. One day, blood test	↓ Postprandial glycemia compared with controls
Vuksan et al. (2017a, b)	Healthy participants ($n = 15$, male and female) age: 23.9 ± 3 years were randomized to receive a 50 g glucose challenge, alone or supplemented with either 25 g ground Salba-chia or 31.5 g flax, on three separate occasions. One day, blood test	↓ Blood glucose area under the curve over 120 min ↓ Peak glucose ↑ Time to peak ↑ Satiety
Vuksan et al. (2017a, b)	Randomized double-blind controlled trial: patients overweight or with type 2 diabetes ($n = 77$) followed 6-month calorie-restricted diet receiving received 30 or 36 g/1000 kcal/day of Salba-chia on oat brand	↓ Body weight ↓ Waist circumference ↓ C-reactive protein
Nieman et al. (2009)	Overweight/obese participants in a single-blind trial ($n = 76$) supplemented with 25 g chia seeds in 250 ml water twice a day during 12 weeks (placebo 37 g, chia seeds 39 g)	↑ Plasma ALA No influences on: blood pressure, inflammatory markers or body composition
Nieman et al. (2012)	Overweight women in a randomized double-blind trial ($n = 62$, aged 49–75 years) supplemented with 25 g whole or ground chia seeds/day during 10 weeks	↑ Plasma ALA and EPA No influences on: blood pressure, inflammatory markers or body composition

↑ increase; ↓ decrease.

Characterization of rats fed a long-term sucrose-rich diet

There are different experimental nongenetic animal models focused on the development of diet-induced metabolic and physiological alterations mimicking several aspects of MS in humans (for a review, see Lombardo and Chicco, 2006). In this regard, several studies including our own have shown that normal rats fed a high-carbohydrate (sucrose/fructose) diet for a short period of time (3–5 weeks) developed hypertriglyceridemia, normoglycemia, hyperinsulinemia, and hypertension (Reaven et al., 1979; Pagliassotti et al., 1994; Lombardo et al., 1983; Mayer et al., 2008). However, if the diet was extended from 3 to 6 months, a steady state of hypertriglyceridemia and high plasma FFA levels, moderate hyperglycemia, and worsening whole body insulin resistance could be observed. These alterations were

accompanied by visceral adiposity and a slight overweight (Soria et al., 2001; Chicco et al., 2003; D'Alessandro et al., 2015). At this point the endocrine pancreas showed a significant increase of islet number and β-cell area, as well as changes in the profile of islet cell distribution, without an increase in the pancreatic content of immunoreactive insulin. Islet isolated from these rats showed an altered biphasic pattern of glucose-stimulated insulin secretion (Lombardo et al., 1996). In addition to ectopic fat deposition in liver, heart, and pancreas tissues, an increase of lipid storage accompanied by an impairment of nonoxidative pathways of glucose metabolism was observed in the skeletal muscle of rats fed a sucrose-rich diet (SRD) (Chicco et al., 1991; Hein et al., 2010; Hein et al., 2012; D'Alessandro et al., 2006, 2013). The temporal metabolic changes described may reflect the early start of type 2 diabetes mellitus, because many patients have chronically elevated plasma FFA and Tg levels, altered peripheral insulin sensitivity, and the loss of the first peak of insulin response to glucose. Table 2 presents a summary of the effects produced by a long-term sucrose feeding to rats.

On the other hand, relatively few studies have examined the effectiveness of changing the quality and/or quantity of dietary nutrients in reversing/improving a stable dyslipidemia and insulin resistance as described earlier. In this sense, most investigations analyzed the replacement of the fat source (Lombardo and Chicco, 2006) (e.g., oils rich in 18:2 n-6 by oils rich in 20:5 n-3 and 22:6 n-3) or protein source (e.g., animal origin by vegetable origin) (Oliva et al., 2009), while only few, partial studies analyzed the administration of whole chia seed or oil as a source of dietary fat, a nutritional tool that could revert or improve insulin resistance induced by the chronic feeding of a high carbohydrate diet. Therefore, we investigated the mechanism/s underlying the possible beneficial effects of dietary chia seed on improving/reversing both the altered glucose homeostasis and insulin resistance present in rats chronically fed a SRD before the source of dietary fat (corn oil rich in LA 18:2 n-6) was isoenergetically substituted by chia seed from month 3 to 6 of the feeding period. This study focuses on two target tissues of insulin action: skeletal and heart muscle.

Effects of dietary *Salvia hispanica* L. (Salba) seed on the altered glucose metabolism and insulin resistance in skeletal muscle

The ectopic deposition of fat in nonadipose tissue (skeletal or heart muscle among others) is considered a key factor in the development of IR. The skeletal muscle is qualitatively the most important peripheral tissue for whole-body glucose utilization. Insulin stimulates glucose uptake, glycolysis, and glycogen synthesis in this tissue. Furthermore, lipid accumulation in skeletal fibers has been linked to IR and directly or indirectly alters insulin signaling. Chicco et al. (2009) showed that chia seed reduced the increased Tg content in the gastrocnemius muscle of SRD-fed rats. More recently, Oliva et al. (2013) reported that the normalization of dyslipidemia by dietary chia seed induced a metabolic shift that was reflected in the skeletal muscle of the SRD group by a reduction of the accumulation of fatty acid derivatives (e.g., LCACoA and DAG). The decrease of LCACoA and DAG could play a key role in the normalization of the increased membrane-associated nPKCθ protein mass level of the SRD-fed rats and in the improvement of insulin action in the skeletal muscle. Yu et al. (2002) demonstrated that LCACoA by its esterification to DAG stimulated the nPKCθ activity. nPKCθ disrupted

TABLE 2 Main effects of a long-term sucrose intake on rats glucose and lipid metabolism

	SRD versus control
Blood pressure	↑[1,2,3]
Plasma	
Glucose	↑[1,4–13]
Insulin	N[1,5–13]
FFAs	↑[1,6–13]
Triglyceride	↑[1,6–13]
Peripheral insulin resistance	↑[5,7]
Liver	
Lipogenesis	↑[11,14]
VLDL secretion rate	↑[1,15]
Triglyceride pool size	↑[11,14]
Insulin resistance	↑[5,12]
Endocrine pancreas	
Islet number	↑[9]
β-Cell area	↑[9]
Islet cell distribution	↑[9]
Insulin content	N[9]
Glucose-stimulated insulin secretion	Altered[1,7]
Skeletal and heart muscle	
Lipid content (TG, LCACoA, DAG)	↑[7,10,13, 16,17]
Glycogen storage	N/↓[17,10,13,16, 17,18]
Glucose oxidation	↓[7,10,13,17,18]
Insulin resistance	↑[5, 13,17,18]
Adipose tissue	
Adipocyte cell size	↑[6,8]
Triglyceride pool size	↑[6,8]
Visceral fat	↑[6,8]
Insulin resistance	↑[6,8]

↑ *increase;* ↓ *decrease; N: normal.*

[1] Lombardo and Chicco (2006);+ [2] Mayer et al. (2008); [3] Creus et al. (2016); [4] Reaven et al. (1979); [5] Pagliassotti et al. (1994); [6] Soria et al. (2001); [7] Chicco et al. (2003); [8] D'Alessandro et al. (2015); [9] Lombardo et al. (1996); [10] Chicco et al. (1991); [11] Hein et al. (2010); [12] Hein et al. (2012); [13] D'Alessandro et al. (2006); [14] Rossi et al. (2013); [15] Chicco et al. (2009); [16] Lombardo et al. (1983); [17] D'Alessandro et al. (2013); [18] Montes et al. (2000).

the insulin signal via serine or threonine phosphorylation of the insulin receptor, insulin receptor substrate-1 (IRS-1), and potentially other proteins such as glycogen synthase (GS). Further, Samuel et al. (2010) showed that the ability of fatty acids to interfere with insulin signaling and glucose transport into skeletal muscle involved an increase of fatty acid derivatives such as LCACoA and DAG. Under insulin stimulation (euglycemic hyperinsulinemic clamp studies), dietary chia seed was able to revert the altered cell surface recruitment of GLUT4, the activity of GS, and the increase of glycogen and glucose-6-P concentration. Furthermore, glucose phosphorylation and the oxidative pathway of glucose metabolism were normalized after chia seed administration, reversing or improving insulin action in the skeletal muscle of SRD-fed rats.

As mentioned earlier, chia seed reverts dyslipidemia. In this vein, in a previous study, we demonstrated that the activities of key enzymes involved in hepatic lipogenesis and oxidative mitochondrial and peroxisomal fatty acid oxidation were coordinately decreased and increased accompanied by a parallel decrease and increase in the protein mass levels of mature sterol regulatory element-binding protein-1 (SREBP-1) and peroxisome proliferator activated receptor-α (PPAR-α), respectively, leading to a reduction in liver Tg synthesis, hepatic steatosis, and dyslipidemia in SRD-fed rats (Rossi et al., 2013). Besides, the increased visceral adiposity and adipocyte hypertrophy as well as basal lipolysis were markedly reduced by dietary chia seed. This was accompanied by a normalization of the antilipolytic action of insulin (Oliva et al., 2013). Therefore, these findings suggest that at least one of the mechanisms involved in the whole effect of dietary chia seed on glucose metabolism and insulin sensitivity in the skeletal muscle could be related to the reduction of the availability of plasma Tg and FFA that in turn decreases lipotoxicity in this tissue and normalizes glucose homeostasis and insulin action. Besides, it could also be the result of a subsequent change in fatty acid composition in membrane phospholipids in the skeletal muscle due to the absolute and relative amount of LA and ALA in the diet and the competitive interaction in the metabolism of LA and ALA to long-chain n-6 and n-3 PUFAs (EPA, DPA, and DHA) since they compete by the same elongase and desaturase enzyme activities (Brenna, 2002). N-3 PUFAs could in turn improve insulin sensitivity. Changes in membrane fluidity or in the DAG signaling function could influence insulin secretion and its biological activity (Storlien et al., 1991). In this regard, Poudyal et al. (2013) in rats fed a high-fat/high-sucrose diet supplemented with either chia oil or seed showed that the fatty acid profile of skeletal muscle had a significant increase on n-3 PUFA (C18:3 n.3, C22:5 n-3, and C22:6 n-3) increasing the n-3/n-6 ratio. In addition, a high content of EPA and DHA was observed in the hepatic membrane of rats fed a perilla oil (rich in ALA), although this oil does not contain EPA or DHA (Kim and Choi, 2001). Moreover, a significant increase of plasma ALA, EPA, and DHA levels and the n-3/n-6 ratio in face of a normal whole-body insulin sensitivity was recorded in insulin-resistant rats fed a SRD after chia seed replaced corn oil as the source of dietary fat (Chicco et al., 2009).

A summary of the effects of dietary chia seed administration in the skeletal muscle in the presence of stable dyslipidemia, abnormal glucose metabolism, and insulin resistance achieved by a long-term SRD feeding is depicted in Fig. 2.

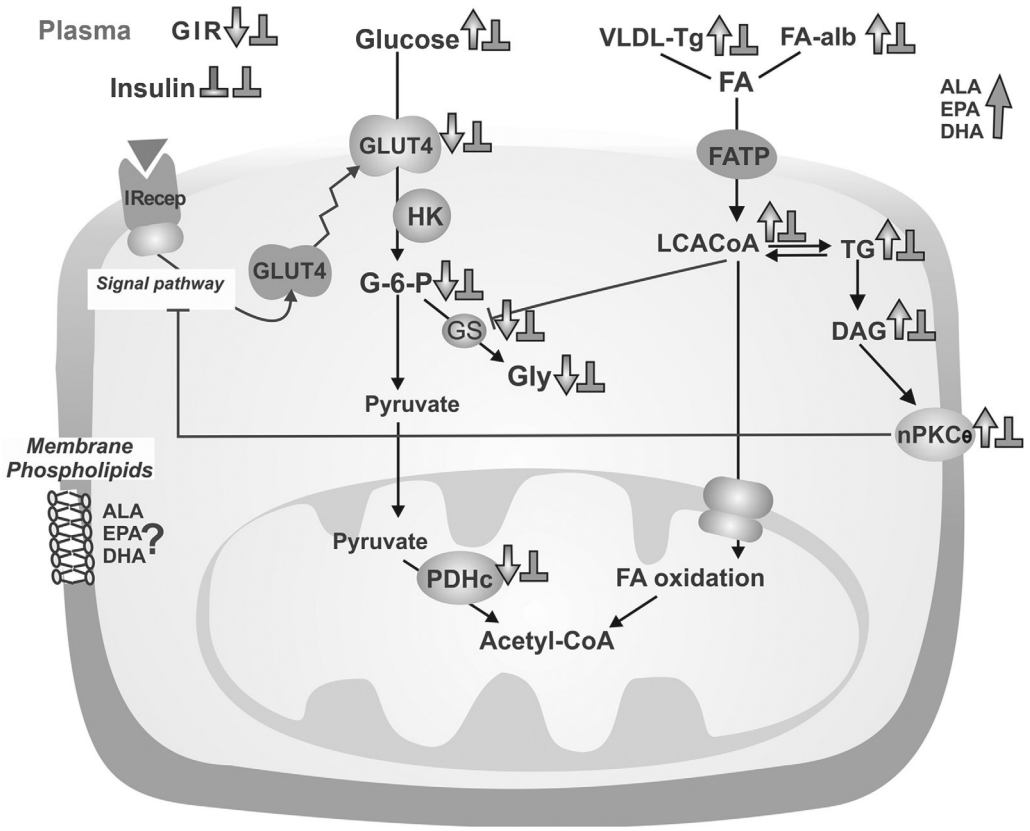

FIG. 2 A normalization of dyslipidemia and plasma glucose was achieved by dietary *Salvia hispanica* L. (Salba) seed. *In the basal state*, dietary chia seed induce a reduction of the intracellular fatty acids derivatives. The normalization of membrane nPKCθ protein mass levels could in turn improve insulin signaling. *Under insulin stimulation* (euglycemic-hyperinsulinemic clamp), dietary chia seed was able to revert the altered cell surface recruitment of GLUT4 and the metabolic pathway of glucose (phosphorylation, oxidation, and storage). All these effects could contribute to the normalization of glucose-stimulated insulin secretion and muscle insulin insensitivity. ⇑ low increase; ⇑ increase; ⇓ decrease; ⊥ normal.

Effect of dietary *Salvia hispanica* L. (Salba) seed on the metabolic fate of glucose and its interaction with lipid metabolism in the heart muscle of a dyslipemic-insulin resistant rat model

Long-lasting hypertriglyceridemia and altered glucose metabolism also have profound effects on myocardial substrate utilization. In this regard, hyperglycemia and the increased storage of lipid triglyceride and its metabolites (LCACoA and DAG) within the cardiac muscle could interfere with the activation of insulin signaling pathways involved in the

recruitment of GLUT4 to the surface. In previous work with isolated heart muscle of dyslipemic insulin-resistant rat induced by the chronic administration of a sucrose diet, our group reported an altered flux of metabolites characterized by a decreased glucose uptake and increased fatty acid uptake and oxidation (Chicco et al., 1991; Montes et al., 2000). In the same experimental animal model, Creus et al. (2016, 2017) recently observed an increase of FAT/CD36 protein mass levels in the sarcolemma—one of the most important fatty acid transporters in the heart muscle—and a normal protein mass level of GLUT4. In this line, both a reduction of glucose uptake and total GLUT4 protein and IRS-1 protein mass levels were described by Desrois et al. (2004) in the heart of Goto-Kakizaki rats, a model of spontaneous type 2 diabetes mellitus.

The normalization of dyslipidemia and insulin resistance achieved by the chia seed in sucrose-fed rats was accompanied by a decreasing lipid accretion and a normalization of FAT/CD36 protein mass levels in the sarcolemma of the heart muscle. Further, GLUT4 protein mass levels did not change despite a significant increase of IRS-1 protein mass levels that reached values higher than those of the animals fed a control diet (Creus et al., 2016, 2017). On the other hand, under insulin stimulation, the hormone was able to increase the recruitment to the cell surface of GLUT4 over the normal levels and to reverse the impaired insulin stimulated FAT/CD36 translocation to the plasma membrane when chia seed was present in the sucrose-rich diet (Creus et al., 2016). In this sense, Bonen et al. (2009) suggested a possible link of impaired GLUT4 trafficking to the plasma membrane with both the increase of sarcolemma FAT/CD36 and the rate of fatty acid transport and lipid accumulation. In addition, Gray and Kim (2011) reported that an increased intracellular lipid inhibits insulin action by the activation of serine/threonine kinases and phosphorylation of IRS-1.

Another important issue accompanying the decrease of lipid accretion in the heart muscle of the dyslipemic insulin-resistant rat model was the normalization of the impaired glucose phosphorylation and glucose oxidation (estimated by the activity of hexokinase (HK) and pyruvate dehydrogenase complex (PDHc), respectively) when chia seed was the fat source of the diet. In the skeletal muscle of normal rats, it was demonstrated that increased levels of acylCoA inversely correlated with HK activity by allosteric inhibition (Thompson and Cooney, 2000). In addition, the normalization of both PDHc and the improvement of M-CPT1 activities after dietary chia seeds (Creus et al., 2016) could also be one of the possible mechanisms involved in the improvement of glucose oxidation in the heart muscle of SRD-fed rats.

AMP-activated protein kinase (AMPK) plays an important role as a master regulator of cellular energy homeostasis, fuel preference, and flexibility. In this respect, a significant increase of pAMPK protein mass level and pAMPK/AMPK ratio in the heart muscle of SRD-fed animals was recently demonstrated by Creus et al. (2017). Longnus et al. (2005) showed that the activation of AMPK inhibits IRS-1 associated phsphoinositide-3-kinase (PI3K) activity and that AMPK activates atypical PKC and extracellular signal-regulated kinase in the heart. Samovsky et al. (2015) demonstrated a close coordination between AMPK and FAT/CD36 in the regulation of FA metabolism in myocytes. On the other hand, insulin decreased pAMPK (Dyck and Lopaschuk, 2006), and an inhibition of AMPK activity was associated with an increase of acetyl CoA carboxylase activity and a decrease of mitochondrial fatty acid oxidation (Gamble and Lopaschuck, 1997). Interestingly, after chia administration the pAMPK protein mass level returned to values similar to those observed in the rats fed a

control diet, and this was accompanied by normalization of the protein mass levels of FAT/CD36. Therefore, taking into account all these mechanisms, we cannot discard the possibility that a coordinate action between the behavior of AMPK, FAT/CD36, and a decrease of fatty acid availability induced by chia seed might also contribute to the improvement of heart fuel utilization.

On the other hand, the precise underlying mechanism by which dietary chia seed (ALA and/or other components present in the chia such as antioxidants fiber) could be involved in the improvement of both glucose metabolism and its relationship with lipid metabolism is still not completely elucidated in the heart muscle of sucrose-fed rats. At this point, we recently demonstrated (Creus et al., 2016) that the replacement of corn oil by chia seed was able to increase the incorporation of both ALA and DHA in the fatty acid phospholipids of cardiac muscle associated with a significant rise in membrane unsaturation, suggesting an increase in membrane fluidity that could in turn improve insulin-stimulated glucose uptake and sensitivity (Storlien et al., 1997). Similar results were observed by Poudyal et al. (2012a, 2012b) in rats fed a high-fat/high-fructose diet supplemented with chia seed or in rats fed for a short period of time with chia oil reported by Valenzuela et al. (2014). Thus the changes observed in the fatty acid profile in the heart muscle phospholipids of the chia seed group might be another possible pathway involved in the normalization of insulin sensitivity and its action.

A summary of the effects of dietary chia seed administration in the heart muscle of insulin-resistant, dyslipidemic rat achieved by a long-term sucrose-rich diet feeding is depicted in Fig. 3.

Effect of dietary *Salvia hispanica* L. (Salba) seed on glucose homeostasis in rats offspring exposed to a sucrose-rich diet from utero to adulthood

Perturbation of the developmental milieu during early stages of life (in utero an early postnatal life) may also lead to the abnormalities included in the metabolic syndrome in the adult life of the offspring (Reynolds et al., 2015). On this subject, previous work from us showed that maternal sucrose-rich diet during pregnancy and lactation could be associated with a programming effect on glucose homeostasis and hepatic lipid metabolism that predisposed offspring to develop later-life insulin resistance and metabolic disorders, regardless of postnatal life (D'Alessandro et al., 2012, 2014). On the other hand, some studies (Boone-Heinonen et al., 2015) have addressed the possibility that postsuckling environment including dietary manipulation (changes in macronutrient: e.g., type of fatty acid and protein) could be able to attenuate programmed outcomes. Fortino et al. (2017) demonstrated that changing the source of fat in the sucrose diet at weaning (chia seed instead of corn oil) inhibited the development of hyperglycemia and the altered insulin tolerance test in the offspring from sucrose-fed dams that were maintained on chia seed from weaning to adulthood. This suggests a positive effect on glucose homeostasis. However, an impaired glucose response was still present when the offspring were exposed to a glucose challenge. Nevertheless, chia seed was able to prevent the development of liver steatosis and hypertriglyceridemia although plasma FFA and visceral adiposity were still over the control levels (Table 3).

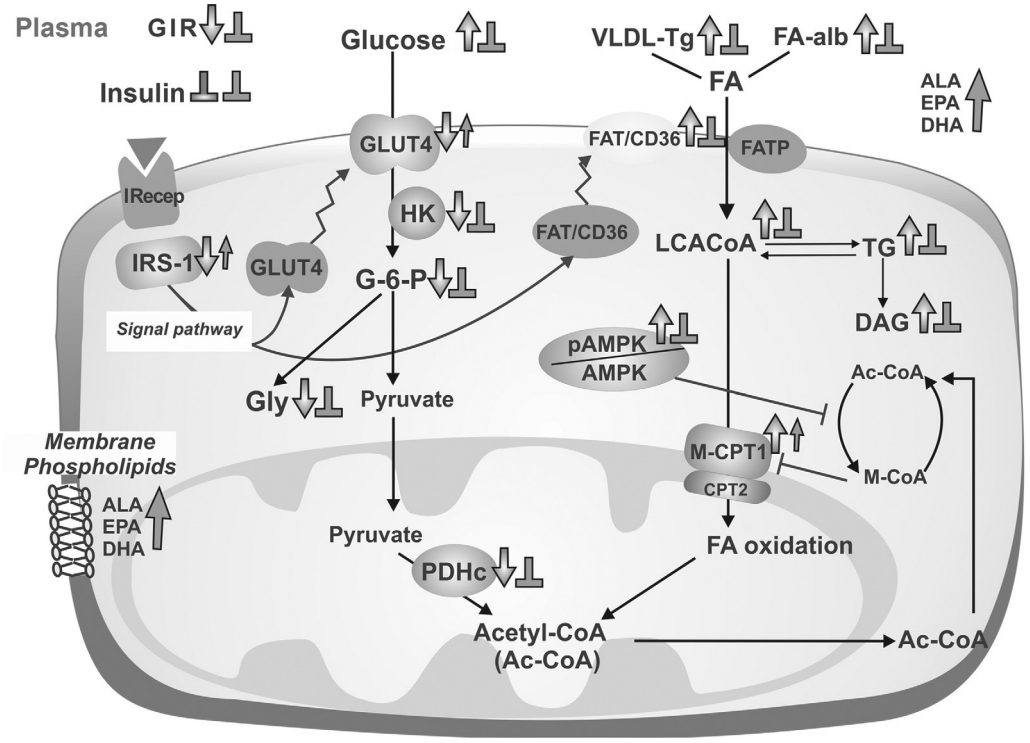

FIG. 3 Dietary *Salvia hispanica* L. (Salba) seed induce the normalization of plasma glucose and fatty acids levels. *In the basal state*, dietary chia seed was able to decrease heart lipid accretion associated with the normalization of FAT/CD36 in the sarcolemma. An improvement of M-CPT1 activity suggests the decrease of mitochondrial fatty acid oxidation that in turn could improve glucose metabolism. Moreover, the normalization of glucose phosphorylation, glucose oxidation, and storage were associated with the increase of IRS-1 protein mass levels without changes in GLUT4 protein mass expression. *Under insulin stimulation* (euglycemic-hyperinsulinemic clamp), the recruitment of GLUT4 to the cell surface was significant increase, reaching values similar to those observed in the control heart. This was associated with the reversion of the impaired insulin-stimulated FAT/CD36 translocation to the plasma membrane. Another possible mechanism that could improve glucose utilization could involve the normalization of pAMPK/AMPK coordinately associated with FAT/CD36 and the decreased fatty acid availability. Finally, changes in the fatty acid composition of membrane phospholipids—increases the n-3/n-6 ratio—could also be involved in the amelioration of heart insulin sensitivity and action. ⇑ low increase; ↑ increase; ⇓ decrease; ⊥ normal.

TABLE 3 Effects of dietary *Salvia hispanica* L. (Salba) seed on glucose homeostasis in rats fed a sucrose-rich diet (SRD) from in utero to adulthood

	SRD	SRD + chia seed
Adiposity	↑	↑/N
Plasma		
Glucose	↑	N
FFA	↑	↑/N
i.v. Glucose tolerance test (kg)	↑	↓
Insulin tolerance test (K_{ITT})	↓	N

↑ *i*ncrease; ↓ decrease; N: normal.

Finally, in the skeletal and heart muscle of this experimental model that mimics the MS, it is possible that all the mechanisms mentioned earlier involving the effects of dietary chia seed acting coordinately could contribute to improving the impaired glucose metabolism and insulin resistance and thus to improving fuel utilization. Besides, recent research suggests that dietary chia seed might mitigate adverse outcomes of glucose metabolism induced in rats by a high-sucrose diet from in utero to adulthood.

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CHAPTER

19

Beneficial applications of glucosamine

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OUTLINE

SUMMARY POINTS

- This chapter focuses on glucosamine, which is a well-known supplement for the treatment of osteoarthritis.
- It has potential health benefits especially in treating inflammatory-mediated diseases.
- The interaction of glucosamine with

multiple molecules and signaling pathways makes it an attractive to explore its use in various diseases including cardiovascular and neurodegenerative diseases, skin disorders, and adjuvant anticancer agent.

- The mechanism of its actions is often through antioxidation and regulation of inflammatory responses by notably inhibition of NF-B pathway and O-GlcNAc modification of target proteins.
- More extensive studies and randomized clinical trials are now needed to explore its use for more preventive and therapeutic situations.

Key facts

- Glucosamine is an essential amino-monosaccharide component of many cellular glycoproteins, glycolipids, and glycosaminoglycans.
- It is one of the most popular over-the-counter (OTC) supplements used in treatment of osteoarthritis.
- Glucosamine has also some other biological and pharmacological properties, including antiinflammatory, antioxidant, anticancer, antiaging, neuroprotective, and cardioprotective activities.
- Because of its biological safety, combined with its efficacy, it can also be as a promising candidate for the prevention and/or treatment of some other inflammatory-related diseases.

Introduction

Glucosamine (GlcN), 2-amino-2-deoxy-D-glucose, was first discovered in 1876 by a German surgeon, Dr. Georg Ledderhose, while working on cartilage (Ledderhose, 1876). But the stereochemistry of this compound was not fully defined until 1939 by the work of Walter Haworth (Haworth et al., 1939). It was first prepared by hydrolysis of chitin with concentrated hydrochloric acid (Ledderhose, 1876) and then developed in sulfate blend. Thereafter, it has been studied extensively and found to have many potential health benefits.

Glucosamine is an amino sugar made from glucose and amino acid glutamine with a molecular weight of 179.17g/mol. Endogenous GlcN is synthesized in the body through a metabolic pathway known as the hexosamine biosynthetic pathway (HBP). It is naturally present in most human tissues with the highest concentrations being in the healthy cartilages (Anderson et al., 2005; Dalirfardouei et al., 2016). Glucosamine is a major precursor of biochemical synthesis of glycoproteins, proteoglycans, and glycosaminoglycans and all amino sugars in the human body. Glycosaminoglycans are complex carbohydrates that are integral parts of connective tissues such as tendons, ligaments, and extracellular matrix of the cartilage (Anderson et al., 2005). There are three commonly forms of GlcN supplements in the market: glucosamine hydrochloride (GlcN.H), glucosamine sulfate (GlcN.S), and *N*-acetyl glucosamine (GlcNAc) (Fig. 1).

These glucosamine compounds are usually obtained from chitin by chemical and enzymatic hydrolysis. Microbial fermentation by filamentous fungi or recombinant bacteria, specifically *Escherichia coli*, has also recently been studied as an alternative to the conventional processes

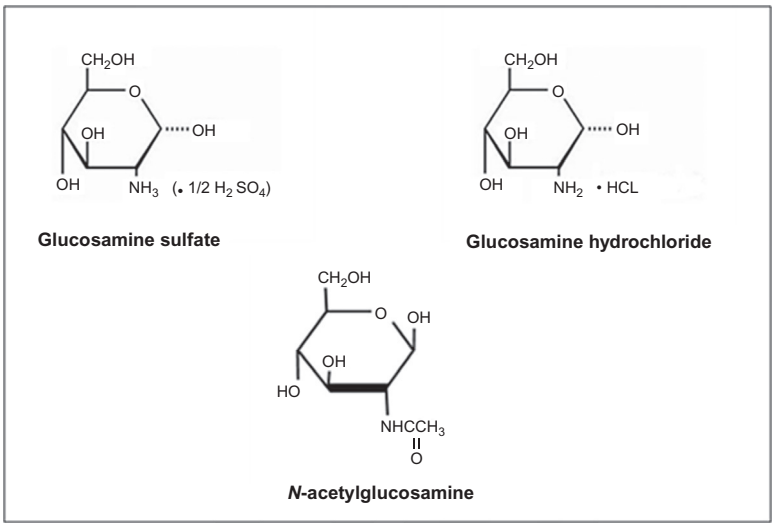

FIG. 1 Chemical structures of glucosamine supplements in the market.

(Dalirfardouei et al., 2016). Although these different chemicals have some similarities, they may not have exactly the same effects when taken as a dietary supplement (Henrotin et al., 2014).

Glucosamine is usually taken orally, and parenteral and topical uses are less common. Approximately 90% of each oral conventional dose (1500 mg/day) is absorbed (Anderson et al., 2005), but the bioavailability of GlcN with oral dosing is low, and it is only 26% of the bioavailability of intravenously administered glucosamine (Barclay et al., 1998). This limited bioavailability is mainly the result of first-pass hepatic metabolism. Hence the development of new formulations and peptide prodrugs of glucosamine with more stability and enhanced gut permeability has received particular attention in recent years (Dalirfardouei et al., 2016).

Glucosamine is a multifunctional and pharmacologically safe dietary supplement commonly taken for rheumatoid arthritis and osteoarthritis. Glucosamine is known recently to modulate the multiple signaling pathways and, thanks to these effects, to have the novel beneficial roles in various diseases including cancer, cardiovascular and neurodegenerative diseases, skin disorders, and bacterial infection (Fig. 2).

It also displayed antioxidant and antiinflammatory activities, which are likely to be responsible for the most of its functions (Dalirfardouei et al., 2016; Yan et al., 2007).

In this chapter, we provide an update on the current applications of glucosamine and also discuss the prospects for future potential beneficial applications of this amino sugar in human health.

Antioxidant mechanisms of glucosamine

Oxidative stress, which contributes to severe metabolic dysfunctions and pathological conditions, occurs in an imbalanced situation between oxidant and antioxidant defense systems. The antioxidant properties of glucosamine have been the focus of pharmacological studies in

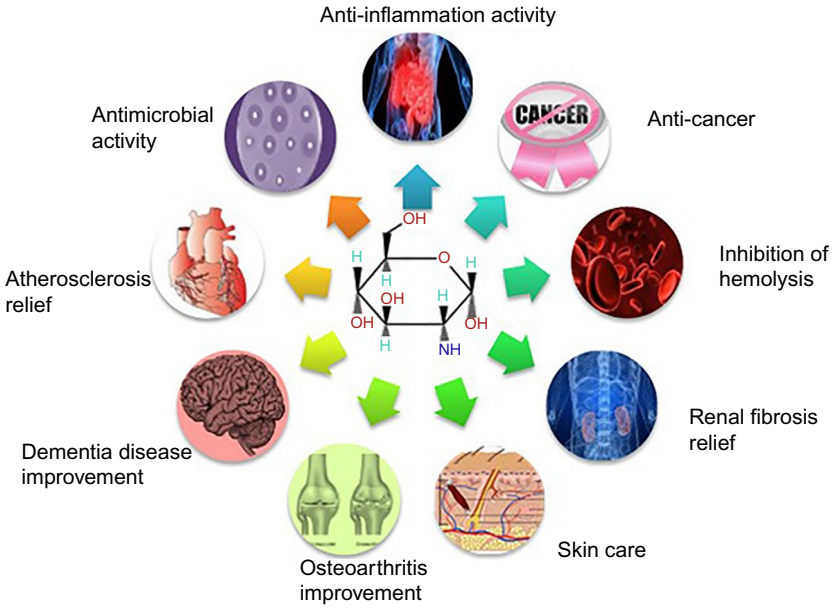

FIG. 2 Potential biological activities of glucosamine and its derivatives (Dalirfardouei et al., 2016).

the recent past. Glucosamine exhibits antioxidant effects through strong free radical-scavenging activity and chelating effect on ferrous ions (Xing et al., 2006; Yan et al., 2007). Glucosamine also stabilizes cell membranes by preventing lipid peroxidation and protects other macromolecules such as protein and deoxyribose from oxidative damage induced by hydroxyl radicals (Mendis et al., 2008). The same results were reported in other experimental models, which indicated glucosamine efficiently protects proteins from oxidation and increases reduced glutathione level in oxidatively stressed erythrocytes and chondrocytes (Jamialahmadi et al., 2014a; Mendis et al., 2008). Glucosamine and its derivatives also are suitable agents to prevent H2O2-induced DNA damage (Jamialahmadi et al., 2014b). In addition, that GlcN can reduce the oxidative stress-induced increase of intracellular ROS in vitro has also been demonstrated (Wu et al., 2014).

Since oxidative stress-induced damage of DNA, proteins, and membrane lipids are associated with various chronic pathological conditions such as cancer and neurodegenerative and cardiovascular diseases, this amino sugar is thought to perform an important role against these free radical-associated diseases.

Antiinflammatory and immunomodulatory activities of glucosamine

Glucosamine has been shown to have antiinflammatory properties in both in vitro studies and animal models. Its antiinflammatory property appears to be mediated mainly by

suppression of NF-kB activation and thereby inhibiting the production of inflammatory mediators and cytokines (Dalirfardouei et al., 2016; Largo et al., 2003; Nagaoka et al., 2011).

Effect of glucosamine on nuclear factor-κB

The nuclear factor-κB is a eukaryotic transcription factor that plays a key role in regulating the expression of genes involved in controlling inflammatory responses, cellular proliferation/growth, and immunity. The functionally active NF-κB is p65/p50 heterodimer, which normally remains inactive by forming a complex with an inhibitory protein, IκB. Exposure of cells to external stimuli activates NF-κB by inducing phosphorylation and proteasomal degradation of IκB. This liberates the activated dimeric NF-κB, which translocates to the nucleus where it regulates the transcription of genes involved in inflammatory responses. There is now growing evidence that aberrant constitutive activation of this transcription factor is associated with many human disorders (Hwang et al., 2013). Several recent studies have shown that GlcN suppressed the production of proinflammatory cytokines via inhibition of NF-κB signaling pathway. It seems that the most described mechanism for inhibitory effects of GlcN on the inflammatory genes expression could work at least in part through the attenuation of NF-kB pathway (Dalirfardouei et al., 2016; Largo et al., 2009). Glucosamine inhibited NF-κB, by preventing the degradation of its inhibitory subunit, Iκ-B. Therefore this transcription factor is unable to translocate to the nucleus where it activates the expression of genes involved in inflammatory responses (Largo et al., 2003). Moreover, it inhibits NF-κB activation through regulating O-linked *N*-acetylglucosamine (O-GlcNAc) modification of p65 or c-Rel in response to LPS. This posttranslational modification seems to be a component of an alternative regulatory mechanism of NF-κB activation (Hwang et al., 2013; Hwang et al., 2010). Furthermore, GlcN treatment reversed transglutaminase 2 (TGase 2)-mediated I-κBα polymerization by direct binding to TGase 2 and inhibiting its activity, which ultimately prevents the NF-κB activation (Jeong et al., 2010).

Effect of glucosamine on cyclooxygenases and matrix metalloproteinases

The antiinflammatory property of glucosamine is due, in part, to suppression of prostaglandin E2 (PGE2) production. PGE2 that is elevated by cyclooxygenase-2 (COX-2) is a principal mediator of inflammation and plays crucial roles in the development of osteoarthritis by induction of MMP production and inhibition of collagen and proteoglycan synthesis in chondrocytes (Chan et al., 2005; Nakamura et al., 2004).

The COX-2 enzyme appears to be the primary COX, which controls PGE2 synthesis in response to inflammation, and its gene expression can be induced by several cytokines and growth factors. As well as COX-2, the families of MMPs also play critical roles in cartilage degradation in OA. In addition, COX-2 and MMPs overexpression has been implicated in the carcinogenesis of many tumors, and so the inhibition of these two enzymes is useful for treating inflammatory diseases including OA and cancer (Dalirfardouei et al., 2016; Hong et al., 2009; Zahedipour et al., 2017).

Different cell culture studies have shown the inhibition of the expression and/or activity of inflammatory mediators such as PGE2, COX-2, MMPs, nitric oxide, and IL-1β by glucosamine derivatives. For example, GlcN.H inhibited the production of PGE2, NO, MMP-1, MMP-3,

and MMP-13 in IL-1-treated chondrocytes and synovial cells (Nakamura et al., 2004) and also suppressed the activation of COX-2 and MMP-13 expression in human skin fibroblasts (Hong et al., 2009). Moreover, GlcN.S has been reported to downregulate the IL-1β-induced COX-2, nitric oxide synthase (iNOS), and microsomal prostaglandin E synthase-1(mPGES-1) expression and PGE2 synthesis (Chan et al., 2005). Most of these genes are under the transcriptional control of the nuclear factor NF-κB signaling pathway, which can be inhibited by glucosamine.

Effect of glucosamine on inducible nitric oxide synthase

Inducible nitric oxide synthase is another enzyme that plays an important role in mediating inflammation. Nitric oxide is a potent proinflammatory mediator synthesized from L-arginine by the nitric oxide synthase (NOS) family of enzymes. In vitro studies, using different cell types, suggest that glucosamine can inhibit lipopolysaccharide (LPS)-mediated iNOS induction and NO production (Chuang et al., 2013; Hwang et al., 2017). Additionally, a decline in iNOS transcript and NO release into the media in cytokine-stressed chondrocytes and cartilage explants after treatment with GLN has been reported (Chan et al., 2005). Downregulation of the iNOS gene by GlcN might be attributed to the inhibition of p38-MAP kinase and transcription factor NF-κB (Rafi et al., 2007). Hua et al. also reported that GlcN could prevent the progression of adjuvant arthritis in rats and significantly reduces NO and prostaglandin E2 levels in plasma (Hua et al., 2005).

Effect of glucosamine on cytokine production

An increasing number of studies have addressed the regulation of cytokine and chemokine production by glucosamine. Tumor necrosis factor alpha (TNF-α), interleukin-1b (IL-1b), and interleukin-6 (IL-6) are primary inflammatory cytokines that play a prominent role in inflammatory responses. Several in vitro and animal researches have demonstrated that GlcN reduces the production of these proinflammatory cytokines by suppressing their mRNA transcription and/or protein expression (Dalirfardouei et al., 2016; Nagaoka, 2014; Zahedipour et al., 2017). Moreover, treatment with glucosamine led to significant decreases in both serum concentrations of IL-8 and colonic expression of IL-1β and TNF-α in colitic mice. In vitro approaches showed that these effects are mainly mediated by an inhibition of the NF-κB activation (Bak et al., 2014).

In another study by Someya et al., glucosamine was shown to inhibit the expression of IL-6, IL-8, IL-24, and TNF-α genes by synovial cells stimulated with IL-1β and demonstrated that O-GlcNAc modification-dependent and O-GlcNAc modification-independent mechanisms were responsible for the impairment of cytokine production (Someya et al., 2016).

Furthermore, glucosamine has epigenetic effects and can prevent cytokine-induced loss of methylation status in the IL-1β promoter, and this is associated with reduced expression of IL-1β (Imagawa et al., 2011). Significant reduction of transforming growth factor β-1 (TGF-β1) and elevation of interleukin-10 (Il-10) serum levels by GlcN has also been reported in an experimental model of OA, suggesting that these cytokines are possible mediators of GlcN chondroprotective effects in OA (Waly et al., 2017).

In addition to the immunosuppressant effects of glucosamine, it has been recently suggested that GlcN has the potential immunoregulatory and immunostimulatory capacities, although details have been different. Glucosamine exhibited an immunoregulatory effects in animal models of autoimmune disorders such as multiple sclerosis and atopic dermatitis (Chien et al., 2015; Jin et al., 2015). It also suppresses the unprimed T-cell responses by a direct inhibitory effect on T-cell proliferation and by interfering with antigen-presenting cell functions (Forchhammer et al., 2003; Ma et al., 2002). Glucosamine may modulate T-cell differentiation and immune homeostasis via N-glycosylation of CD25 and thereby inhibits Th1, Th2, and iTreg cells but promotes Th17 cell development, as well (Chien et al., 2015). Moreover, it can inhibit the cytotoxic effects of natural killer cells and in vitro dendritic cell activation (Bozic et al., 2018; Ma et al., 2002). The evidence favoring glucosamine as an immunostimulator is weak. Sadeghi et al. reported that GlcN plays an immunostimulatory effect by activating T lymphocytes and allogeneic mixed-lymphocyte-reaction (MLR) enhancement and increasing the serum levels of the soluble IL-2 receptor (sIL-2R) in healthy individuals. It may alter the outcome of bone marrow transplantation toward graft-versus-host disease (GVHD) or graft-versus-leukemia (GVL) development. Therefore precautions should be taken when GlcN is prescribed to bone marrow transplanted patients (Sadeghi et al., 2011).

Conclusively, to further clarify and confirm the proposed immunoregulatory and immunostimulatory effects of glucosamine, additional experiments are needed in the future.

Modulation of transcription factors by glucosamine

In addition to the potent antioxidant and antiinflammatory activities, glucosamine can modulate the activity of various important transcription factors, affect multiple signal transduction pathways, and exert pleiotropic cellular effects. Notably, growing attention has been paid to the ability of GlcN to modulate O-GlcNAcylation of key transcription factors. Glucosamine is incorporated into the cells and utilized for the O-GlcNAc modification of target proteins by the action of O-linked-*N*-acetylglucosamine (O-GlcNAc) transferase (OGT) (Hwang et al., 2013; Someya et al., 2016).

It suppresses the activation of the transcription factor NF-κB by regulating O-GlcNAcylation of p65 or c-Rel in response to LPS or IL-1b, which regulates the expression of proinflammatory genes in both microglia and macrophage cells (Hwang et al., 2017; Hwang et al., 2013). It has also reported the GlcN-mediated O-GlcNAc modification of NF-κB and Sp1 transcription factors involved in the downregulation of TNF-α and IL-8 in synovial cells (Nagaoka, 2014; Someya et al., 2016). Yu et al. have recently demonstrated that GlcN inhibited lung cancer cell proliferation, possibly through O-GlcNAc modification-induced downregulation of the PI3K/AKT and MAPK/ERK pathways and their downstream transcription factors, forkhead box O (FOXO)1 and FOXO3 (Yu et al., 2017). The ability of glucosamine to repress expression of HIF-1a, a tumor angiogenic transcription factor, in human oral cancer cells has also been reported (Jung et al., 2012). More recently, Gu et al. revealed that conditions inducing excessive protein *O*-GlcNAcylation, including high concentrations of glucosamine, inhibit bone morphogenetic protein 2 (BMP2)-induced transcriptional activity of Runx2, a critical transcription

factor for osteoblast differentiation and bone formation, and osteogenic differentiation of C2C12 cells (Gu et al., 2018). Apart from modulating transcription factor function by O-GlcNAc modification, GlcN regulates the activity of some other transcription factors such as signal transducers and activators of transcription 3 (STAT3), P53, β-catenin, OCT-4, KLF4, MITF, and CREB and, in turn, the cellular expression profiles by additional mechanisms (Hosea et al., 2018; Ma et al., 2018; Wang et al., 2017).

Therapeutic uses of glucosamine

Osteoarthritis

Osteoarthritis (OA) is the most common chronic joint disorder in the elderly often resulting in significant disability. Glucosamine and its derivatives are the most commonly over-the-counter (OTC) supplements employed for alleviating the symptoms of OA due to their chondroprotective and antiinflammatory properties (Largo et al., 2003; Nakamura et al., 2004). Supplemental glucosamine can normalize the cartilage metabolism by stimulating the synthesis and inhibiting the degradation of glycosaminoglycans and type II collagen by synovial cells and chondrocytes (Henrotin et al., 2014; Igarashi et al., 2011). Glucosamine also can alter cultured OA chondrocyte metabolism by acting on protein kinase C, cellular phospholipase A2, protein synthesis, and possibly collagenase activation (Piperno et al., 2000). Moreover, it reverses the joint degradation and articular function and increases the SIRT1 expression (a putative gene for the normal cartilage metabolism) in chondrocytes. There are additional evidences to indicate the modulation effects of GlcN on bone metabolism in OA, which include enhancement of the ALP activity, collagen synthesis, and osteocalcin secretion in human osteoblastic cell line (Kim et al., 2007). It also increases the mineralization of mature osteoblasts, induces the osteoblastic cell differentiation, and suppresses the osteoclastic cell differentiation, thereby possibly increasing bone matrix deposition and decreasing bone resorption to modulate bone metabolism in OA (Igarashi et al., 2011). Glucosamine has a potential to promote the osteoblast and chondrocyte proliferation, as well (Lv et al., 2018; Ma et al., 2018).

In addition to the chondroprotective action, GlcN exerts the antiinflammatory effects in OA by inhibition of the expression and/or activity of proinflammatory cytokines and mediators via O-GlcNAc modification-dependent and O-GlcNAc modification-independent mechanisms in chondrocyte (Bruyere et al., 2016; Someya et al., 2016). The disease-modifying ability of glucosamine in osteoarthritis has been shown in several clinical trials and systematic reviews, as well. While there have been some inconsistencies in human studies, leading to doubts about its efficacy, recent overviews of the clinical literature have confirmed that, in adequately potent doses, GlcN is indeed useful in osteoarthritis (Herrero-Beaumont et al., 2007; McCarty et al., 2018). Furthermore, GlcN is recommended for the management of mild-to-moderate osteoarthritis by several international guidelines (Bruyere et al., 2016; Henrotin et al., 2014).

According to the meta-analysis of six studies, after 3 years of treatment, glucosamine sulfate showed a small-to-moderate protective effect on minimum joint space narrowing (JSN) in

knee osteoarthritis patients, and it was suggested that GlcN should be taken at least for over 2 or 3 years to delay radiological progression of OA of the knee (Lee et al., 2010). Recently, the results of a 6-year follow-up from the Osteoarthritis Initiative provide future support for the long-term protective structure-modifying effects of combined chondroitin sulfate plus glucosamine sulfate therapy in knee OA subjects. This combined therapy significantly reduced the cartilage volume loss in the global knee; however, it was not associated with symptom improvement as measured by the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) (Raynauld et al., 2016). These two meta-analysis and other studies suggested that longer intervention periods are needed to have a stronger effect of glucosamine. More recently, based on a meta-analysis of 29 studies, Simental-Mendia et al. concluded that oral supplementation with glucosamine or chondroitin significantly reduces pain in Visual Analog Scale (VAS); however, their combination did not show this behavior (Simental-Mendia and Sanchez-Garcia, 2018). Zhu et al. also reported that glucosamine proved a significant effect only on stiffness improvement (Zhu et al., 2018). Another systematic review and meta-analysis showed a marginally favorable effect of GlcN on VAS pain scores, while it was not significant for WOMAC. Interestingly, Japanese Knee Osteoarthritis Measure (JKOM) meta-analysis provides the evidence of efficacy for alleviating knee OA symptoms by the GlcN (Ogata et al., 2018). In addition to these findings, there is still controversy regarding the effectiveness of glucosamine for treatment of OA. For example, Wandel and colleagues reported that compared with placebo, glucosamine or its combination with chondroitin did not reduce joint pain or had no impact on the narrowing of the joint space (Wandel et al., 2010). Recently, a 6-month trial demonstrated a lack of superiority of chondroitin and glucosamine combination therapy over placebo in patients with symptomatic knee OA. The inconsistencies in the clinical research literature with glucosamine can be attributable to the differences in glucosamine formulations and doses, uneven quality of products tested or other possible sponsor-dependent bias, methodologies and trial durations, etc. (Roman-Blas et al., 2017; Vasiliadis and Tsikopoulos, 2017).

Collectively, these observations motivated future well-controlled large clinical trials with longer treatment duration and adequately dosed glucosamine supplementation to provide robust evidence for the therapeutic use of this dietary supplement in OA.

Cancer

Recent scientific evidence suggests the potential beneficial effects of glucosamine against various types of cancer (Table 1).

Glucosamine can interact with multiple molecular targets and modulate numerous cellular signaling pathways (Fig. 3). Therefore, it may have the potential for being used as an adjuvant anticancer agent in cancer chemotherapy.

First, glucosamine exerts antitumor-promoting effects by suppressing the activation of NF-kB and some other transcription factors, which regulates the proinflammatory mediators' synthesis (Zahedipour et al., 2017). Second, glucosamine has been shown to inhibit the proliferation of a variety of tumor cells via the induction of cancer cell apoptosis and cell-cycle arrest (Wang et al., 2017). Additionally, GlcN can activate autophagic cell death in vitro and

TABLE 1 Effects of glucosamine on different cancer cell lines

Cancer type	Biological effects	Ref.
Cervical cancer	Autophagy induction via an mTOR-independent pathway	Shintani et al. (2010)
Renal cancer	Promoting cell-cycle arrest; upregulation of cell-cycle inhibitors	Wang et al. (2017)
Breast cancer	Reduction in COX-2 and IL-8 expression and PGE(2) production; inhibition of cell proliferation and migration; inhibiting N-linked glycosylation; reduction the stemness of cancer cells; chemosensitizer via Tgase-2 inhibition	Chou et al. (2015); Hosea et al. (2018); Kim et al. (2009)
Glioma cancer	Autophagy induction through the stimulation of ER stress	Hwang and Baek (2010)
Oral cancer	Induction of ER stress and apoptosis; downregulation of HIF-1a	Jung et al. (2012)
Prostate cancer	Inhibition of proteasomal activity; induction of apoptosis and cell-cycle arrest; suppression of STAT3, AKT, ERK1/2, and MAPK activity; inhibition of protein N-glycosylation	Chesnokov et al. (2014); Liu et al. (2011); Oh et al. (2007); Tsai et al. (2009)
Lung cancer	Induction of cell-cycle arrest and apoptosis; inhibition of COX-2 activity; inhibition of PGE2 production; suppression of IGF-1R/Akt, PI3K/AKT and MAPK/ERK signal transduction; induction of FOXO1 and FOXO3 O-GlcNAc modification	Jang et al. (2007); Song et al. (2014); Yu et al. (2017)
Colorectal cancer	Reduced risk of colorectal cancer probably by antiinflammatory mechanism	Kantor et al. (2018)
Leukemia cancer	Induction of apoptosis, changing the component ratio of nucleotides; interfering with the glycosylation of glycoproteins	Song et al. (2014); Wang et al. (2006)
Hepatoma	Induction of apoptosis, cell-cycle arrest	Song et al. (2014)
Sarcoma	Suppression the MMPs expression	Pohlig et al. (2016)

in vivo through mTOR-dependent and mTOR-independent signaling pathways (Jiang et al., 2014; Shintani et al., 2010). Third, glucosamine was reported to act as a chemosensitizer through inhibition of transglutaminase-2 (Tgase-2)-mediated NF-κB activation in drug-resistant cancer cells (Jeong et al., 2010; Kim et al., 2009). Fourth, glucosamine may exert its anticancer activity through inhibition of protein N-linked glycosylation. In fact, treatment with GlcN led to the suppression of many important signaling molecules, such as STAT3, AKT, and ERK1/2 that are activated by N-glycosylated membrane receptors (Chesnokov et al., 2014). Most recently, Hosea et al. reported that GlcN treatment decreases the stemness of human breast cancer stem cells by inactivating STAT3 signaling (Hosea et al., 2018). Fifth, GlcN could have a potential antiangiogenic property by downregulation of the expression of

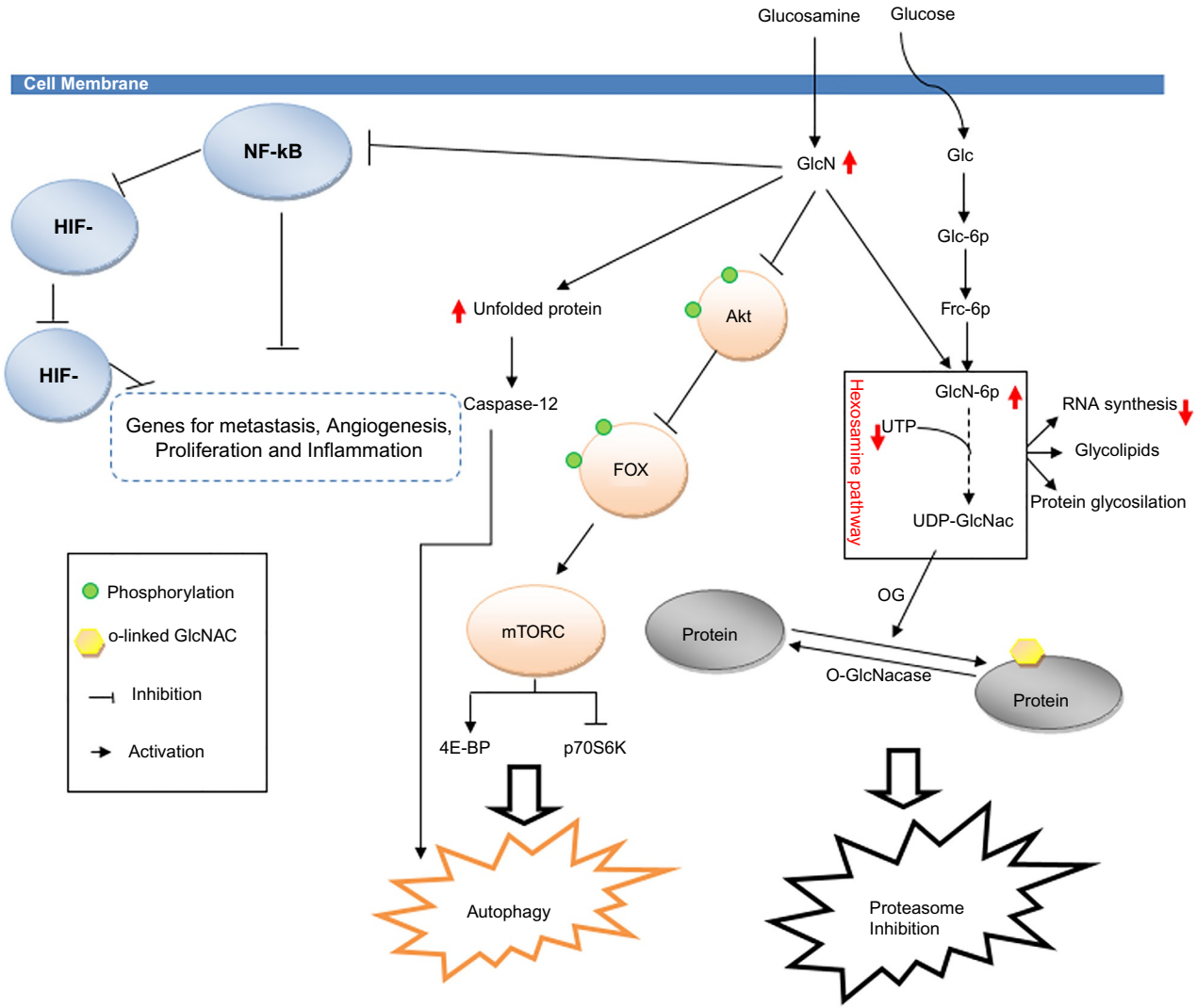

FIG. 3 Schematic of putative molecular mechanisms of glucosamine on cancerous cells. This illustrates that glucosamine can induce autophagy, proteasome inhibition, and NF-κB signaling pathway prevention to exert anti-cancer activity. *FOXO*, Forkhead Box Protein O1; *mTORC*, mammalian Target of Rapamycin Complex; *HIF-1*, Hypoxia-induced factors; *OGT*, *O*-GlcNAc transferase; *Glc*, Glucosamine; *Akt*, v-akt murine thymoma viral oncogene (Zahedipour et al., 2017).

HIF-1 transcription factor (Jung et al., 2012), proangiogenic factors such as metalloproteinases and COX-2, and suppression the production of angiogenic cytokines such as Il-8 (Chou et al., 2015; Rajapakse et al., 2007). Moreover, GlcN inhibits the synthesis of nucleic acids and proteins and also promotes the cellular leakage of nucleotides and their metabolites in tumor cells (Zahedipour et al., 2017). Apart from these well-known activities, this amino sugar also exerts its beneficial effects by its antioxidant and antigenotoxic properties, inhibition of proteasomal activity via O-GlcNAc modification (Liu et al., 2011), and its ability to induce ER stress (Hwang and Baek, 2010).

Together, these studies provide evidence supporting the potential value of glucosamine as an effective and nontoxic anticancer drug, although more work is required to better understanding of the mechanisms by which GlcN exerts its effects.

Beneficial role of Glucosamine in Skin Diseases

Over the last decade, there has been increasing interest in the skin benefits of glucosamine compounds. Glucosamine has the potential for enhancing wound healing, improving skin hydration, and decreasing wrinkles by stimulation of hyaluronic acid synthesis (Bissett, 2006). It also speeds up the wound closure and increases collagen synthesis and the proliferation of skin cells (Ashkani-Esfahani et al., 2012). Another benefit of glucosamine compounds is their usefulness in the treatment of hyperpigmentation disorders. Glucosamine inhibits expression of melanocyte-specific proteins such as tyrosinase by suppressing their glycosylation, which results in inhibition of melanin production and loss of pigmentation in cell culture model systems (Imokawa and Mishima, 1985; Wakabayashi et al., 2013). Recently, Niwano et al. reported the antimelanogenic effect of GlcN is mediated through a deficiency in microphthalmia-associated transcription factor (MITF) expression due to the proteolytic degradation of cyclic AMP-responsive element-binding (CREB) protein in normal human melanocytes (Niwano et al., 2018). Glucosamine has also been suggested as an antiaging agent. It can increase the number of active fibroblasts by promoting their proliferation and stimulates more collagen production by them (Salih and Al-Naimi, 2006). Recently, findings from an ex vivo antiaging model and a clinical trial demonstrated that glucosamine sulfate to have a positive effect on various age-related markers involved in epidermal differentiation or extracellular dermal matrix formation (Gueniche and Castiel-Higounenc, 2017). Glucosamine appears to protect skin by quenching free radicals and reducing inflammation via decrease of COX-2, NF-κB, and Tgase-2 expression (Park et al., 2012). The positive therapeutic effects of glucosamine in combination with low-dose cyclosporine A for the treatment of atopic dermatitis and psoriasis-like dermatitis have also been reported (Jin et al., 2015; Kim et al., 2015).

These studies suggest that glucosamine has the potential to be developed as a potent safe agent for treating skin diseases.

Cardiovascular properties of glucosamine

Glucosamine has potential health benefits in cardiovascular related diseases including cardioprotection against ischemia-reperfusion injury (Champattanachai et al., 2008),

regulation of calcium homeostasis in the heart (Nagy et al., 2006), and improvement of cardiac function following trauma-hemorrhage by increasing protein O-GlcNAcylation and attenuation of NF-κB activation that leads to the decrease of inflammatory responses in the heart tissue (Zou et al., 2009). It also attenuate the process of atherosclerosis via reduction of the susceptibility of LDL to oxidation, attenuating cell growth, influencing the inflammatory environment and the coagulation system, and improvement of vascular endothelial function by modulating intracellular redox state (Katoh et al., 2017; Tannock et al., 2002). Additionally, it can inhibit platelet aggregation and repress the formation of atherosclerotic lesion and infiltration of inflammatory cells into the lesion (Hua et al., 2004; Nagaoka et al., 2011). The impairment of hepatic apolipoprotein B100 (apoB100) production through the induction of endoplasmic reticulum (ER) stress and enhancing apoB100 degradation is another antiatherogenic effect of GlcN (Qiu et al., 2006). Furthermore, GlcN reduces neointima formation by an increase in O-GlcNAc protein modification, stabilizes atheroma plaques, and prevents atherothrombosis, as well (Largo et al., 2009; Xing et al., 2008).

These findings suggest that glucosamine can be introduced as an antiatherogenic agent if its in vivo actions are carefully evaluated in humans in the future.

Central nervous system effects of glucosamine

The available information on glucosamine suggests that its neuroprotective effects might be mainly related to its ability to reduce oxidative stress and inflammatory mediator production. Glucosamine exhibited a neuroprotective effect through the transcriptional repression of inflammatory genes by inhibiting the NF-kB activation in rat brain ischemia/reperfusion injury. In addition, the results showed the microglial activation and/or macrophage accumulation in the presence of GlcN in the postischemic brain (Hwang et al., 2010). A recent study in our laboratory indicated that glucosamine and its acetylated derivative had a cytoprotective property against serum/glucose deprivation (SGD)-induced cell death via antiapoptosis and antioxidant activities (Mousavi et al., 2018). We have also demonstrated the beneficial effect of GlcN on spatial learning and scopolamine-induced memory impairment in an animal model (Jamialahmadi et al., 2013). Additionally, glucosamine can suppress the hypoxia-induced neuroinflammation and learning-memory impairments in adult zebrafish (Lee et al., 2018). These data thus suggest this amino sugar has the potential to be used as a novel therapeutic agent for neurodegenerative disorders.

Conclusion

According to both in vitro and in vivo studies, glucosamine has an enormous potential for a wide variety of human diseases and conditions and has some favorable impact on mortality. Most of these activities can be attributed to its antiinflammatory effect through the inhibition of NF-kB transcriptional activity, antioxidant property, and modulation the O-GlcNAcylation of several cell-signaling proteins. In addition to the biological activities and therapeutic applications that are mentioned in this chapter, glucosamine is gaining attention for its therapeutic effects in osteoporosis (Lv et al., 2018), obesity (Huang et al., 2015), ocular inflammatory disorders like age-related macular degeneration (AMD) and uveitis (Chang

et al., 2008; Chen et al., 2018), inflammatory bowel disease (Nagaoka et al., 2011), allergic asthma, and rhinitis (Jung et al., 2017). Collectively, the pharmacological safety combined with its varied biological properties, warrant further studies and clinical testing of glucosamine to explore its use for more preventive and therapeutic situations.

Glossary

Glucosamine 2-amino-2-deoxy-D-glucose is an essential amino-monosaccharide component of glycoproteins, proteoglycans, and glycosaminoglycans.

Inflammation Inflammation is part of the complex biological response of our immune system to harmful stimuli.

Nuclear factor kappa B (NF-kB) A ubiquitously expressed eukaryotic transcription factor involved in immunity, inflammatory responses, and cell proliferation/death.

O-GlcNAc modification O-GlcNAc modification is one of the principal posttranscriptional modifications, which can influence the transcriptional activity and interaction of transcription factors with other cofactors. Altered O-linked GlcNAc modification levels have been implicated in various pathologies including cardiovascular diseases, diabetes, cancer, and neurodegeneration.

Osteoarthritis One of the most common disabling arthritic diseases that gradually leads to cellular changes, structural defects, and functional impairment of all the joint compartments.

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Further reading

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PART 3

Genetic machinery and its function

CHAPTER

20

Nutrigenomics and personalized nutrition for the prevention of hyperglycemia and type 2 diabetes mellitus

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OUTLINE

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SUMMARY POINTS

- This chapter focuses the effect of the interaction between type 2 diabetes mellitus (T2DM) susceptibility genes and dietary intakes on T2DM and glycemic traits.
- Although a modernization of lifestyles represented by westernized diets are some of the contributing factors to the T2DM epidemic, genetic factors are also important contributing factors to T2DM.
- Importantly, several T2DM susceptibility genetic loci possibly interact with diet to influence T2DM risk.
- Lower glycemic index or glycemic load diet may modify the risk of T2DM conferred by the risk allele of *TCF7L2*, the most common susceptibility gene for T2DM across various ethnic groups.
- This chapter also focuses the roles of gut microbiome in the success of dietary intervention and interpersonal variability in glycemic response to identical meal.
- Resent study has demonstrated that the improvement of glucose tolerance induced by dietary fiber intervention depends on the abundance of *Prevotella*.
- Another study has shown the utility of personalized diet utilizing machine-learning algorithms and gut microbiome features to prevent postprandial hyperglycemia.
- Understanding of the interaction of diet with T2DM susceptibility genes and gut microbiome may contribute to personalized nutrition utilizing genomic information to effectively prevent T2DM and hyperglycemia.

Key fact of genetics of T2DM

- T2DM is a complex disorder caused by multiple genetic and environmental factors.
- Heritability estimates (how much of the variation in traits is explained by genetic factors) of T2DM are 20%–80% (they vary depending on the study).
- More than 100 genetic loci have been identified to be associated with T2DM.
- Accumulated common variants (minor allele frequency $\geq$0.05) with small effect size, but not low-frequency/rare variant (minor allele frequency $<$ 0.05), largely explain the variance in T2DM.
- Large-scale genome-wide association studies have shown that most of the T2DM-associated variants are implicated in impaired insulin secretion, whereas a few of them influenced insulin resistance.

Key facts of gut microbiome

- Gut microbiome has received increased attention over the last decade as a critical factor affecting human health and diseases.
- Recent advances in high-throughput DNA sequencing technologies have provided more detailed information on the human gut microbiome and its roles in the pathogenesis of various diseases including T2DM.

- Several enzymes encoded by the gut microbiota are not present in the host and produce various metabolites. For example, fiber-degrading enzymes encoded by fiber-digesting bacteria metabolize dietary fiber into short-chain fatty acid (e.g., acetate, butyrate, and propionate).
- Metabolites produced by the gut microbiota play roles in host metabolism and immunity, thereby affecting host health and diseases risks.

Background

The prevalence and incidence of type 2 diabetes mellitus (T2DM) have been steadily increasing, which is becoming a major public health problem in both developed and developing countries (Unnikrishnan et al., 2017). Indeed, it has been anticipated that global prevalence of T2DM includes >600 million people in the mid-21st century (Ogurtsova et al., 2017). Considering the current economic cost for T2DM has been steadily increasing even in the most modern countries (American Diabetes Association, 2018), the establishment of a strategy for the prevention of T2DM is urgently needed worldwide.

Although many factors contribute to the development of T2DM, modernization of lifestyles represented by a sedentary lifestyle and westernized diets, characterized by higher intake of total energy, sugar, and animal food, may be some of the main causes of the T2DM epidemic (Hu, 2011). Several longitudinal cohort studies have demonstrated that increased intake of saturate and trans fat (Salmeron et al., 2001; van Dam et al., 2002), animal protein (Malik et al., 2016; Tian et al., 2017), and red meat (Pan et al., 2011; van Dam et al., 2002) and decreased intake of polyunsaturated fat, plant fat (Meyer et al., 2001; Salmeron et al., 2001), and plant protein (Malik et al., 2016; Tian et al., 2017) are associated with a higher incidence of T2DM. Furthermore, increased intake of sugar (sugar-sweetened beverages) (de Koning et al., 2011; Malik et al., 2010), refined carbohydrate, and refined grain (Hu et al., 2012; Nanri et al., 2015) and decreased intake of whole grain and fiber (Aune et al., 2013; de Munter et al., 2007) are also associated with increased risk of T2DM. Importantly the associations of these dietary factors with T2DM risk have not been universally observed across populations (de Souza et al., 2015; Hu et al., 2012; Janket et al., 2003), suggesting that the heterogeneity of the association between dietary factors and T2DM cannot be ignored.

On the other hand, although westernized lifestyles are undoubtedly associated with increase in the prevalence of T2DM, genetic factors are also important factors that contribute to T2DM. Twin studies have demonstrated that the strength of the contribution of genetic factors to T2DM is comparable with that of environmental factors (Kaprio et al., 1992). Furthermore, large-scale genetic association studies have identified several loci associated with T2DM in various populations (Cho et al., 2011; DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium et al., 2014; Kooner et al., 2011; Ng et al., 2014; Voight et al., 2010). Several studies have attempted to investigate the effects of the interactions between single nucleotide polymorphisms (SNPs) and dietary intakes on T2DM (Cornelis et al., 2009; Fisher et al., 2009; Hindy et al., 2012; Lane et al., 2016). In addition to human genetic variations, variations in the human gut microbiome, which is often referred as "our second genome," have emerged as an important contributing factor to T2DM and insulin

resistance (Pedersen et al., 2016; Qin et al., 2012). Importantly, recent studies have demonstrated that the gut microbiome feature determined the success of dietary intervention to improve glucose tolerance and predicted favorable foods for each person regarding their glycemic response (Korem et al., 2017; Kovatcheva-Datchary et al., 2015; Zeevi et al., 2015). As shown in previous studies, there has been considerable heterogeneity of the association between dietary factors and incidence of T2DM. This may be in part explained by host genetic factors and gut microbiota composition. Therefore, understanding of genetic factors and gut microbiome features under this heterogeneity may contribute to personalized nutrition utilizing genomic information to effectively prevent T2DM.

In this chapter, we review recent large-scale genetic association studies that have revealed genetic susceptibility loci and pathophysiology of T2DM. Then, we focus on several T2DM susceptibility genes and introduce several studies examining the interaction between these genes and dietary intakes on T2DM and glycemic traits, particularly focusing on carbohydrate and fiber intakes. Furthermore, we review the roles of the gut microbiome in the pathogenesis of T2DM and discuss the possibility of personalized nutrition utilizing gut microbiome data to prevent T2DM and hyperglycemia.

Genome-wide association studies identify multiple loci for type 2 diabetes

During the past decade, genome-wide association studies (GWASs) have been performed to identify genetic susceptibility loci for T2DM (Cho et al., 2011; DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium et al., 2014; Kooner et al., 2011; Ng et al., 2014; Scott et al., 2007; Sladek et al., 2007; Voight et al., 2010). A GWAS is a robust strategy for detecting novel loci for complex diseases without pedigree information that is required for family-based linkage analysis. Early GWASs suffered from the lack of statistical power to detect novel loci (Scott et al., 2007; Sladek et al., 2007), because large sample sizes are required to detect significant association of SNPs with typically small effect size after multiple comparison adjustments from testing of millions of SNPs (Sham and Purcell, 2014). Furthermore, the cost of high-density SNP arrays is cost inhibitive for the analysis of large sample sizes (Visscher et al., 2012), and it was difficult to combine genotype data obtained from different SNP arrays in each cohort. However, the development of imputation techniques (Marchini et al., 2007) utilizing haplotype reference panels from the International HapMap Project (International HapMap Consortium, 2003) or 1000 Genomes Project (1000 Genomes Project Consortium, 2010) and cost-effective low-density SNP arrays enables large-scale association studies with a low cost. It also enabled metaanalyses combining genotype data from different arrays, contributing to the further identification of T2DM susceptibility loci. Surprisingly, the sample size of T2DM GWAS has been inflated 10-fold this past decade. Indeed, >100 loci have been identified to be associated with T2DM.

Large-scale association analyses provide insights into the pathophysiology of T2DM

Large-scale GWAS not only identified many T2DM susceptibility loci but also contributed to the understanding of pathophysiology of T2DM. It is well known that both impaired

insulin secretion and insulin resistance are implicated in the development of T2DM (Kahn, 2003). This is genetically supported by large-scale association analyses of glycemic traits, which clearly showed that most of the T2DM-associated variants are implicated in impaired insulin secretion, whereas a few of them influenced insulin resistance (Dupuis et al., 2010; Florez, 2008; Voight et al., 2010).

Interestingly, although obesity is well known to be closely associated with insulin resistance and an important risk factor for the development of T2DM, a series of genetic association analyses performed by Scott et al. have highlighted that obesity is not "real" because of insulin resistance and T2DM. The researchers first performed a GWAS of glycemic traits and identified several genetic loci influencing insulin resistance concomitant with worse profiles of high-density lipoprotein and triglyceride but not by increase in body-mass index (BMI) (Scott et al., 2012). They next investigated the association between these insulin resistance-associated variants and detailed body fat distribution and demonstrated that an increased number of risk alleles in these variants were associated with lower BMI and peripheral fat storage and higher levels of liver damage markers that were indicative of hepatic fat deposition (Scott et al., 2014). They also demonstrated that polygenic burden of 53 loci associated with insulin resistance contributed to the pathogenesis of familial partial lipodystrophy type 1 (Lotta et al., 2017), a severe form of insulin resistance characterized by progressive loss of peripheral fat and increased ectopic fat deposition (Jeru et al., 2017). Finally, they combined population-level genetic analyses with experiments in cellular models, revealing that several insulin resistance-associated genes played a role in adipocyte differentiation (Lotta et al., 2017). Thus, large-scale genetic association analyses highlighted that limited peripheral adipose storage capacity and increased ectopic fat deposition rather than overall obesity were the real causes of insulin resistance.

Gene-macronutrient interactions in the development of T2DM and related phenotypes

Several researchers have speculated that the identification of gene–diet interactions might enable personalized nutrition aimed at stratifying dietary interventions by genetic factors. Here, we focus on several T2DM susceptibility genes identified by GWAS and review the studies examining the gene–diet interaction in T2DM or glycemic traits.

Transcription factor 7-like 2 (*TCF7L2*)

TCF7L2 was originally identified as a T2DM susceptibility locus in a family-based linkage analysis followed by association analysis consisting of unrelated individuals (Grant et al., 2006). The GWASs also confirmed that *TCF7L2* was associated with T2DM across various ethnic populations (Cho et al., 2011; DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium et al., 2014; Kooner et al., 2011; Ng et al., 2014; Voight et al., 2010). *TCF7L2* is regarded as the most common susceptibility gene for T2DM across various ethnic groups.

TCF7L2 is an important transcriptional effector of Wnt/β-catenin signaling (Yi et al., 2005), which has critical roles in insulin secretion and β-cell survival and regeneration (Shu et al.,

2008). The T2DM risk allele of *TCF7L2* rs7903146 was associated with impaired insulin secretion, incretin effects, and enhanced rate of hepatic glucose production (Lyssenko et al., 2007). Therefore, the interaction of the *TCF7L2* gene with carbohydrate, dietary fiber, and whole-grain intakes and glycemic index, which are directly related to glycemic response and insulin and incretin secretion, have been of interest.

Fisher et al. reported the interaction between *TCF7L2* rs7903146 and whole-grain consumption in the development of T2DM in 2318 individuals including 724 incident T2DM cases from the subcohort of the European Prospective Investigation into Cancer and Nutrition-Potsdam cohort (Fisher et al., 2009). They detected a significant interaction effect between *TCF7L2* rs7903146 and whole-grain intake on the incidence of T2DM ($P_{\text{interaction}} = 0.016$) and demonstrated that whole-grain intake was inversely associated with risk of T2DM among rs7903146 CC homozygote carriers (hazard ratio [HR] for 50 g portion per day = 0.86; 95% confidence interval [CI] = 0.75–0.99), whereas the risk allele carriers (CT or TT genotypes) negated the protective effect of whole-grain intake (HR for 50 g portion per day = 1.08; 95% CI = 0.96–1.23).

Hindy et al. also detected significant interaction effects of *TCF7L2* rs7903146 with fiber intake on the incidence of T2DM or glycated hemoglobin (HbA1c) levels ($P_{\text{interaction}} = 0.049$ and 0.02, respectively) in a population-based prospective cohort (Malmö Diet and Cancer Study) consisting of 24,799 individuals including 1649 incident cases of T2DM (Hindy et al., 2012). They showed that high intake of dietary fiber was inversely associated with incidence of T2DM only among rs7903146 CC genotype carriers (odds ratio [OR] per quintile = 0.74; 95% CI = 0.58–0.94; $P = .025$), but not among CT (OR per quintile = 1.03; 95% CI = 0.80–1.32; $P = .77$) or TT (OR per quintile = 1.13; 95% CI = 0.62–2.07; $P = .60$) genotype carriers. Furthermore, they demonstrated that each quintile of higher fiber intake was associated with lower HbA1c levels among CC (−0.036% per quintile; $P = 6.5 \times 10^{-7}$) and CT (−0.023% per quintile; $P = .009$) but not among TT (0.012% per quintile; $P = .52$) genotype carriers, even in nondiabetic individuals ($N = 5216$).

On the other hand, Cornelis et al. investigated the interactions between *TCF7L2* rs12255372 and glycemic index, glycemic load, cereal fiber content, and total carbohydrate content of the diet in middle-aged women consisting of 1140 T2DM cases and 1915 controls from the Nurses' Health Study (Cornelis et al., 2009). The glycemic index is a scale that ranks a carbohydrate-containing food or drink by how much it raises blood glucose levels (Jenkins et al., 1981), whereas glycemic load is calculated by multiplying the amount of carbohydrates in consumed food by the glycemic index of the food (Salmeron et al., 1997). They detected significant interactions of the *TCF7L2* genotype between the high and low strata of glycemic index and glycemic load. Indeed, multivariate-adjusted ORs (95% CI) of T2DM associated with the T allele were 1.54 (1.24–1.92) and 1.68 (1.35–2.09) among individuals in the highest tertile group of glycemic index and glycemic load, respectively, whereas corresponding ORs (95% CI) among individuals in the lowest tertile group of glycemic index and glycemic load were only 1.16 (0.93–1.46) and 1.19 (0.94–1.51), respectively.

Taken together, although risk allele carriers of *TCF7L2* might not benefit from high amount of whole-grain or cereal fiber intakes regarding the prevention of T2DM, lower glycemic index or glycemic load diet could modify the risk of T2DM conferred by the risk allele of *TCF7L2*. Therefore, the selection of foods with low glycemic index or glycemic load rather than fiber-rich food may be useful strategy to prevent T2DM in risk allele carriers of *TCF7L2*.

Melatonin receptor 1B (*MTNR1B*)

MTNR1B encodes the MT2 protein that is one of two high-affinity G-protein-coupled receptors for melatonin (Reppert et al., 1995). It is well known that melatonin is a hormone secreted by the pineal gland that shows diurnal variation and regulate sleep–wake cycle (Arendt, 2003). Although the role of melatonin in glucose metabolism and T2DM had been known for a long time, a GWAS of T2DM and functional analysis highlighted *MTNR1B* as a susceptibility gene for elevated fasting glucose and T2DM risk as well as a role of the melatonin system for pancreatic islet function (Bouatia-Naji et al., 2009; Lyssenko et al., 2009; Prokopenko et al., 2009). Lyssenko et al. demonstrated that *MTNR1B* mRNA was primarily expressed in β cells in islets, which was enhanced in nondiabetic individuals carrying the risk allele (G allele of rs10830963) and individuals with T2DM (Lyssenko et al., 2009). Furthermore, the authors acutely incubated clonal β cells (832/13 cells) at low and high glucose concentrations in the presence of 0.1-mM melatonin and demonstrated that melatonin exerted an inhibitory effect on insulin secretion provoked by glucose. This inhibitory effect was also observed in a human study showing that elevated melatonin levels during an oral glucose load during the day caused impaired glucose tolerance (Cagnacci et al., 2001; Rubio-Sastre et al., 2014). Therefore, risk allele carriers with increased expression of *MTNR1B* are likely susceptible to melatonin-induced inhibition of insulin secretion. These are underlying mechanisms linking the *MTNR1B* variant to elevated fasting glucose and T2DM risk.

Because melatonin shows diurnal variation and its secretion peaks at late night to early morning, the circadian rhythm and sleep behavior including bedtime and wake time concomitant with the timing of food intake may modify the risk of hyperglycemia and T2DM caused by melatonin-induced inhibition of insulin secretion. Lane et al. examined the association of *MTNR1B* rs10830963 with measures of sleep or circadian physiology in in-laboratory protocols or cross-sectional studies with sleep quantity, quality, and timing measures (Lane et al., 2016). In the in-laboratory study, they found significant associations between a risk allele (G) of *MTNR1B* rs10830963 and timing of the melatonin rhythm, and this allele was associated with delayed dim-light melatonin offset (mean dim-light melatonin offset times of 7:49 for CC carriers, 9:10 for CG carriers, and 10:32 for GG carriers; $\beta = 1.36$ h; 95% CI 0.28, 2.44; $N = 95$; $P = 0.015$) and a longer duration of elevated melatonin levels (defined as the difference between dim-light melatonin onset and dim-light melatonin offset) (mean duration of 9.7 h for CC carriers, 10.38 h for CG carriers, and 11.07 h for GG carriers; $\beta = 41$ min; 95% CI = 4.2–78; $N = 94$; $P = .032$). In a cross-sectional study consisting of 1513 individuals of multiethnic ancestry, they detected significant interactions of *MTNR1B* rs10830963 with objectively measured weekday sleep midpoint and wake time for T2D risk ($P_{\text{interaction}} = 0.018$ and $P_{\text{interaction}} = 0.029$). In analyses of participants of European descent, a significant association between *MTNR1B* rs10830963 genotype and T2DM was observed in the early risers (wake time of <6:41; $N = 303$; OR = 1.88; 95% CI = 1.04–3.40; $P = .035$), but not in the late risers (wake time of ≥6:41; $N = 320$; OR = 1.25; 95% = CI 0.74–2.13; $P = .406$), which is possibly due to a concomitant shift of breakfast and elevated melatonin levels in the morning, although the authors did not assess the timing of food intakes. This is a remarkable finding because the morning chronotype (early bedtime and wake time) concomitant with habitual intake of breakfast and avoiding late dinner is generally thought to be associated with a better metabolic profile. The *MTNR1B* variant may enable us to establish personalized nutrition with respect to the timing of food intake.

Gut microbiota composition and diversity are associated with T2DM and related phenotypes

The gut microbiome has received increased attention over the last decade as a critical factor affecting human health and diseases (Cho and Blaser, 2012). The evidence linking gut microbiota to human health had been limited for a long time because a limited number of gut microbiota could be cultured under laboratory conditions (Shanahan, 2002). However, recent advances in high-throughput DNA sequencing technologies have provided more detailed information on the human gut microbiome and its roles in the pathogenesis of various diseases including T2DM. The Human Microbiome Project launched in 2007 performed 16S rRNA and metagenomic shotgun sequencing of samples from a healthy human population by using a high-throughput sequencer, characterizing the microbiota from various human body sites including the gut (Turnbaugh et al., 2007).

Qin et al. carried out a two-stage case-control metagenome-wide association study (MGWAS), which is similar analysis to GWAS, based on deep shotgun sequencing of gut bacterial DNA from a total of 345 Chinese patients with T2DM and controls (Qin et al., 2012). They obtained a total of 4,267,985 predicted bacterial genes and validated that the abundance of 52,484 from these genes was significantly different between patients with T2DM and controls in the stage II analysis (false discovery rate [FDR] < 0.025). The MGWAS demonstrated that patients with T2DM were characterized by a moderate degree of gut microbial dysbiosis, a decrease in the abundance of several universal butyrate-producing bacteria, and an increase in various opportunistic pathogens. Furthermore, the authors observed an enrichment in membrane transport of sugars, branched-chain amino acid (BCAA) transport, methane metabolism, xenobiotics degradation and metabolism, and sulfate reduction at the module or pathway level (FDR < 0.05).

Pedersen et al. explored the relationships between the gut microbiome and the fasting serum metabolome in the states of insulin resistance in 277 nondiabetic Danish individuals (Pedersen et al., 2016). They showed that the serum metabolome of insulin-resistant individuals was characterized by increased levels of BCAA: homeostatic model assessment of insulin resistance (HOMA-IR) was positively correlated with the serum levels of leucine, isoleucine, and valine, respectively (Spearman correlation coefficient [SCC] $= 0.40$, 0.44, and 0.49, respectively; $P < 4 \times 10^{-12}$). They also showed that HOMA-IR was positively correlated with the total abundance of all microbial species containing genes for BCAA biosynthesis (SCC $= 0.30$; $P = 5.3 \times 10^{-7}$) and negatively correlated with genes for inward transport of BCAA (SCC $= -0.17$; $P = 4.5 \times 10^{-3}$). This suggests that the gut microbiota regulates circulating levels of BCAA, thereby influencing host insulin sensitivity. Importantly, epidemiological studies demonstrated that increased circulating levels of BCAA were associated with increased risk of insulin resistance and T2DM (Lee et al., 2016; Wang et al., 2011), which may be in part mediated by the gut microbiota.

Prevotella plays a role in dietary fiber-induced improvement of glucose metabolism

Although many factors interact to shape the gut microbial community in each individual, diet is considered one of the most important contributing factors. In fact, enterotype, a

classification of human gut microbial communities into three groups (*Bacteroides*, *Prevotella*, and *Ruminococcus*) (Arumugam et al., 2011), is closely associated with dietary pattern. Wu et al. demonstrated that the *Bacteroides* enterotype was highly associated with Western diet characterized high intake of animal protein and saturated fats, whereas the *Prevotella* enterotype was associated high intake of carbohydrates and simple sugars (Wu et al., 2011). *Prevotella* enterotype is highly prevalent in African (Burkina Faso) children (De Filippo et al., 2010), Hadza hunter-gatherers (Schnorr et al., 2014), and the people of the Amazonas of Venezuela and rural Malawi (Yatsunenko et al., 2012), who typically consume a fiber-rich plant diet. In functional aspects, *Prevotella copri*, the most abundant bacterial species in *Prevotella* enterotype, possesses a number of enzymes and gene clusters essential for fermentation and utilization of complex polysaccharides (Dodd et al., 2011). Therefore, enrichment of *Prevotella* in people who live in rural areas may be a consequence of an adaptive response to their traditional dietary habits.

Interestingly, it has been reported that an abundance of *Prevotella* was associated with dietary fiber-induced improvement in glucose metabolism in humans. Kovatcheva-Datchary et al. compared the gut microbiota composition of healthy individuals who exhibited improved glucose metabolism (responders) following a 3-day consumption of dietary fiber-enriched barley kernel-based bread (BKB) with those who responded least to this intervention (nonresponders) (Kovatcheva-Datchary et al., 2015). They showed that the *Prevotella*/*Bacteroides* ratio was higher in responders than in nonresponders after BKB intervention. Metagenomic analysis showed that the responders had more than twofold higher abundance of *Prevotella copri* than nonresponders and increased enzymes that are essential for the digestion of beta-glucans and other complex polysaccharides after BKB. Furthermore, germ-free mice transplanted with microbiota from the responder exhibited improved glucose metabolism and increased abundance of *Prevotella* and more than twofold higher liver glycogen content compared with germ-free mice that received microbiota from the nonresponder. These results suggest that *Prevotella* mediate the beneficial effects of dietary fiber on glucose metabolism, potentially by promoting increased glycogen storage. Although the beneficial effects of dietary fiber intake on glucose metabolism and prevention of T2DM have been demonstrated in many studies, these effects may depend on the abundance of *Prevotella* in each individual.

Personal and gut microbiome feature accurately predict postprandial glycemic responses

Finally, we introduce epoch-making studies demonstrating the utility of personalized diet utilizing machine-learning algorithms and gut microbiome features to prevent postprandial hyperglycemia. Zeevi et al. continuously monitored week-long glucose levels using subcutaneous glucose sensors and measured responses to 46,898 real-life meals and 5107 standardized meals to comprehensively characterize postprandial glycemic responses (PPGRs) in 800 individuals (Zeevi et al., 2015). Interestingly, high interpersonal variability in the PPGRs to identical meals was observed, suggesting that universal dietary recommendations may have limited utility in preventing postprandial hyperglycemia. Furthermore, the authors devised a machine-learning algorithm (a model based on gradient-boosting regression) that integrated blood parameters, dietary habits, anthropometrics, physical activity, and gut microbiota.

This algorithm accurately predicted personalized PPGRs to real-life meals (Pearson correlation coefficient of predicted and measured PPGRs = 0.68), and it was validated in an independent cohort consisting of 100 individuals (Pearson correlation coefficient of predicted and measured PPGRs = 0.70). Finally, the authors performed a two-arm blinded randomized controlled dietary intervention based on this algorithm. Profiling of PPGR, clinical data, and gut microbiome was also performed in 26 participants as conducted in an 800-person cohort, which were inputted into the algorithm predicting PPGRs for each person. Then, 12 of these participants consumed a diet composed of the meals predicted by the algorithm to have low PPGRs ("good" diet) or high PPGRs ("bad" diet) based on their measured PPGRs and all meals during the profiling week (predictor-based arm). Conversely, 14 participants consumed "good" or "bad" diets judged by a clinical dietitian and a researcher (expert-based arm). In the predictor-based arm, 10 out of the 12 participants had significantly higher PPGRs in the "bad" diet than that in the "good" diet. The accuracy of the predictor-based arm was superior to that of the expert-based arm in which 8 out of the 14 participants had significantly higher PPGRs in the "bad" diet than that in the "good" diet. Importantly, the abundance of gut microbiota including *Roseburia inulinivorans*, *Eubacterium eligens*, and *Bacteroides vulgatus* increased following the "good" diet and decreased following the "bad" diet. Previous studies have also shown that patients with T2DM had decreased abundance of these bacteria. Therefore, interpersonal variability in the gut microbiota response to identical meals may partly contribute to that in PPGRs.

Furthermore, Korem et al. also predicted the personal glycemic responses to traditionally made sourdough-leavened whole-grain bread or industrially made white bread (Korem et al., 2017). Surprisingly, 10 out of the 20 subjects had higher glycemic responses to sourdough bread, although the published glycemic indices for similar bread types were 70 and 54 for white bread and sourdough bread, respectively (Foster-Powell et al., 2002). They employed a stochastic gradient-boosting regression and successfully and accurately classified the glycemic response-inducing bread (sourdough or white bread) for each individual using only microbiome data.

Taken together, the authors demonstrated that PPGRs of different individuals to the same food vary greatly, which may be associated with gut microbiome features and can be predicted by machine-learning algorithms. These findings suggest that a personalized diet utilizing gut microbiome data may be useful for glycemic control, thereby contributing to the prevention of T2DM.

Summary and perspectives

As reviewed in this chapter, the genetic architecture of T2DM is being addressed by large-scale genetic association analyses, and several T2DM-associated variants have been shown to interact with dietary intake to modulate T2DM risk. Furthermore, recent advances in gut microbiome research have revealed that the human gut microbiome plays important roles in the development of T2DM and interpersonal variability in glycemic responses to food. These findings realize the existence of considerable heterogeneity in the pathogenesis of T2DM and the heterogeneous dietary effects on T2DM risk and glycemic response. A deeper

understanding of these relationships may contribute to the development of effective prevention strategies utilizing genomic information. However, it remains unknown whether these findings can be universally replicated across various ethnic populations. Because dietary habit and other environment factors are quite different among ethnic groups and interact with each other to influence T2DM risk, population-specific interactions of these factors with genetic factors or gut microbiome may exist. Further nutrigenomic studies consisting of various ethnic populations with large sample sizes are expected to provide additional evidence and the underlying mechanisms to achieve personalized nutrition for the prevention of hyperglycemia and T2DM in the near future.

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CHAPTER

21

Epigenetics and fructose metabolism: A new mechanism of fructose effects

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OUTLINE

SUMMARY POINTS

- The chapter describes the mechanism of the adverse effect of fructose intake.
- It has been suggested the association between the increment of fructose consumption and the incidence of several diseases such as insulin resistance, diabetes, and obesity.
- Fructose-mediated attenuated gene expression may be mediated by alterations of DNA methylation status.

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- The Developmental Origins of Health and Disease Hypothesis indicates that maternal nutrition in pregnancy has a significant impact on offspring disease risk later in life.
- Maternal fructose intake appears to impact metabolic function and brain function.
- Maternal fructose-induced epigenetic alternation is also observed.

Key facts

- Fructose is a dietary monosaccharide present naturally in fruits and vegetables.
- The consumption of fructose has increased gradually in the industrial countries.
- Chronic fructose consumption causes several diseases such as obesity and diabetes.

Fructose consumption

The three major monosaccharides are glucose, galactose, and fructose, whereas the three major disaccharides are sucrose (glucose-fructose), lactose (glucose-galactose), and maltose (glucose-glucose). Fructose is a dietary monosaccharide present naturally in fruits and vegetables, either as free fructose or a part of the disaccharide sucrose, and as a free monosaccharide in honey. In addition, it is present in refined sugars such as granulated sugars (e.g., white crystalline table sugar). The chemical reaction for synthesizing fructose from glucose was developed in the 1960s (Douard and Ferraris, 2008), and this method led to a gradual increase in the manufacture and consumption of high-fructose corn syrup (HFCS) (Fig. 1). Fructose is the sweetest saccharide of all natural sugars, which may be why HFCS is used extensively in the formulation of food and beverage products (Douard and Ferraris, 2008).

The drastic increase in the consumption of sweetened soft drinks may be one factor for an increase in the incidence of obesity (Pereira, 2014). The sugar intake of the general population has increased considerably in the past 40 years. Soft drinks, which were developed about a century ago, are a significant source of energy and additional sugars. In most popular sugar-sweetened beverages, fructose accounted for >55% of the sugar content (Ventura et al., 2011). In the mid-18th century, the average American consumed approximately 1.8 kg sugar/year. This value increased drastically to 9.1 kg sugar/year in the mid-19th century and further to 54.4 kg/year by the end of the 20th century. The sugar consumption is expected to continue increasing in the coming decade. Another study showed that the consumption of soft drinks increased from 38 L/person/year to >190 L/person/year between 1950 and 2000 (Elliott et al., 2002), which amounts to 500 mL of soft drinks/person/day. In addition, according to a study by Bray et al. (Bray et al., 2004), the per capita consumption of HFCS in the United States increased from 0.292 kg/person/per year in 1970, when HFCS was introduced into the marketplace, to 33.4 kg/person/year in 2000, indicating a >100-fold increase. Considering that

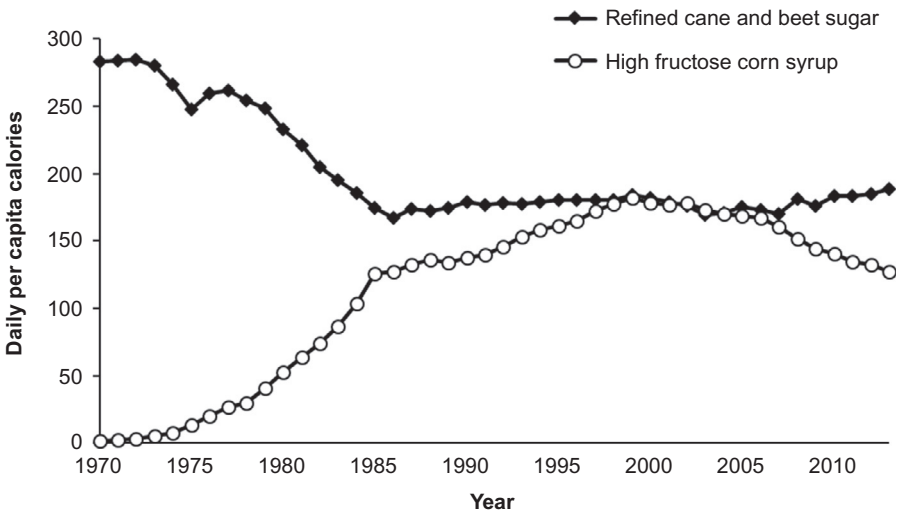

FIG. 1 Daily per capita calories of high-fructose corn syrup in the United States (data source is the US Department of Agriculture—Economic Research Service).

many types of foods have high natural fructose content and people ingest such foods constitutively, the actual fructose consumption in the population might be higher than predicted on the basis of common assumptions regarding HFCS composition. However, despite these limitations, the most recent data available (1977–2004) on the levels of fructose consumption in the population indicate that infants and children have the highest fructose consumption (normalized to body weight).

Fructose metabolism

Fructose is mainly imported into cells by the glucose transporter (GLUT) 5 (Hannou et al., 2018), which is expressed in the small intestine and has a high affinity for fructose ($K_m = 6$ mM). Barone et al. reported that GLUT5 is essential for the absorption of fructose, but the lack of GLUT5 did not cause fructose-induced hypertension (Barone et al., 2009). Fructose import through this transporter is regulated by thioredoxin-interacting protein, which interacts with GLUT5 and alters its transport activity (Dotimas et al., 2016). In addition, carbohydrate response element-binding protein (ChREBP), a transcriptional factor for thioredoxin-interacting protein, regulates intestinal GLUT5 expression (Hannou et al., 2018).

Another glucose transporter—GLUT2—contributes to fructose transport but, unlike GLUT5, has a low affinity for fructose ($K_m = 11$ mM), suggesting that GLUT2 is not the main transporter in intestinal fructose transport (Hannou et al., 2018). However, in contrast to GLUT5, GLUT2 is highly expressed in the liver and may contribute to hepatic fructose intake (Karim et al., 2012). Another glucose transporter, GLUT8, has recently emerged as a contributor to hepatic fructose intake (DeBosch et al., 2013).

In human cells, fructose and glucose are differentially metabolized, although glucose metabolism through glycolysis uses many of the enzymes and intermediates involved in fructolysis (Hannou et al., 2018; Tappy and Le, 2010). Although fructose has the same molecular formula as glucose, the former contains a ketone group in its structure, and the latter contains an aldehyde group. As such the metabolism and biological processes of these two sugars are quite different. For example, the rate-limiting step of glycolysis is mediated by phosphofructokinase (PFK); however, fructose metabolites are metabolized to triose phosphate without the involvement of PFK.

The first step in fructose metabolism is the phosphorylation of the monosaccharide by the enzyme fructokinase in the liver, which catalyzes its conversion into fructose 1-phosphate (F1P) (Tappy and Le, 2010). Because glucokinase has a higher affinity for glucose, phosphorylation of fructose to fructose 6-phosphate is inhibited by glucose. Therefore, almost all fructose is metabolized in the liver via the F1P pathway (Tappy and Le, 2010) (Fig. 2). F1P is converted by aldolase B into glyceraldehyde and dihydroxyacetone phosphate. Subsequently, dihydroxyacetone kinase-2 phosphorylates glyceraldehyde, leading to the synthesis of glyceraldehyde 3-phoshate. The products generated from this fructolysis reaction, that is, glyceraldehyde 3-phospate and dihydroxyacetone phosphate, are substrates for glycogen synthesis or lipogenesis. As mentioned earlier, the production of glyceraldehyde 3-phospate and dihydroxyacetone phosphate from fructose is not regulated by PFK, unlike the case for glucose. Instead, ingested fructose stimulates the production of triose phosphate, enhancing metabolic processes such as lipogenesis.

Intermediate metabolites in the triose phosphate synthesis pathway may interact with elements of glucose metabolism. Brown et al. (1997) demonstrated that the glucokinase regulatory protein interacts with glucokinase and reduces its activity (Brown et al., 1997). In contrast, F1P decreases the glucokinase regulatory protein-glucokinase interaction, leading

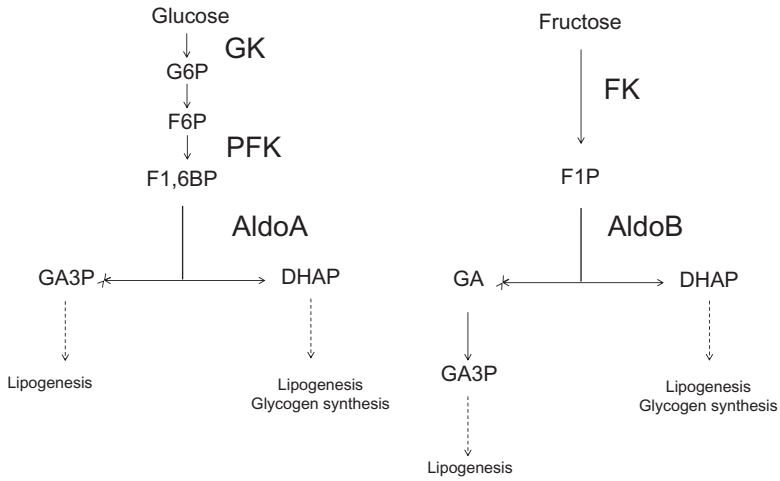

FIG. 2 Metabolism of fructose (*left*) and glucose (*right*). Fructose is metabolized by FK. Fructose is converted to GA and DHAP by Aldo B. These metabolites are used for lipogenesis and ultimately for glycogen synthesis. It should be noted that there is no negative-feedback system for fructokinase activity.

to the upregulation of glucose metabolism. Furthermore, F1P increases glycogen synthesis by inhibiting glycogen phosphorylase. An early study by Niewoehner et al. (1984) reported that expression of hepatic F1P is upregulated at a concentration of approximately 1 mM (10 times higher than the normal values) immediately after fructose injection (10 min), and this upregulation continues for a few hours after stimulation (Niewoehner et al., 1984). Therefore, fructose injection may cause rapid and sustainable effects on hepatic glucose metabolism. Collectively, fructose has a larger role than glucose in the process of lipogenesis. Fructose consumption typically leads to an increase in the plasma triglyceride levels and favors triglyceride synthesis, particularly in the presence of glucose. Thus the liver is the primary site of fructose extraction and metabolism, where the rates of extraction approach 50%–70% of the total fructose delivery to the liver.

Adverse effects of fructose

The worldwide increase in fructose consumption may be associated with the incidence of several diseases such as insulin resistance, diabetes, hypertension, and obesity (Goran et al., 2013; Herman and Samuel, 2016; Tappy and Le, 2010) (Fig. 3). In a study by Maersk, overweight subjects ($n=47$) were randomly given four different drinks (1 L/day): regular cola, isocaloric semiskimmed milk, aspartame-sweetened diet cola, and water (Maersk et al., 2012). Daily intake of regular cola for 6 months increased ectopic fat accumulation and lipid levels compared with that of milk, diet cola, or water. Similarly, another study showed that fructose consumption (25% of the energy requirement) for 10 weeks increased visceral adipose volume in subjects (Stanhope and Havel, 2009); in addition, such fructose consumption induced impaired insulin sensitivity and glucose intolerance. Schwarz and colleagues compared the effects of short-term feeding of a fructose-high diet and an isocaloric diet in which

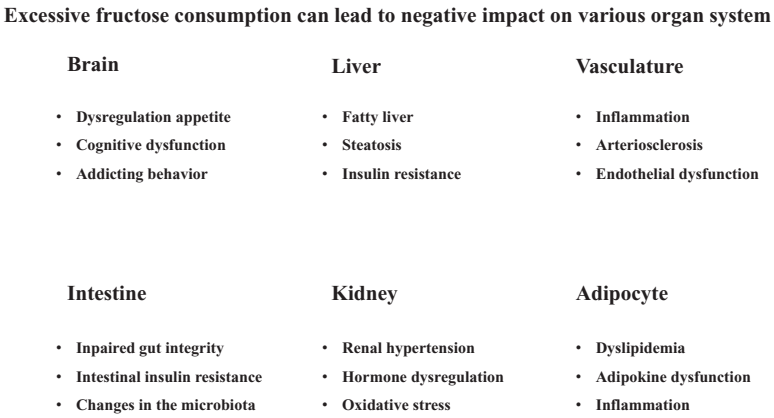

FIG. 3 Adverse effects of fructose consumption extend throughout the body. In addition to fructose-induced metabolic dysregulation such as insulin resistance, excessive fructose consumption can lead to negative impact on various organ systems (Hannou et al., 2018; Tain et al., 2016; Tain et al., 2014).

the complex carbohydrate was substituted for fructose (Schwarz et al., 2015) and reported that only high-fructose consumption stimulates lipogenesis and attenuates the suppression of hepatic glucose production mediated by insulin (Schwarz et al., 2015). Importantly, a moderate amount of fructose intake attenuated insulin sensitivity, but showed no significant change in the glucose consumption in healthy men.

In addition to the effects mentioned earlier, fructose consumption causes hyperuricemia (Goran et al., 2013; Herman and Samuel, 2016; Tappy and Le, 2010). Because there is no negative-feedback system in the reaction catalyzed by fructokinase, which converts fructose to F1P, this reaction wastes cellular ATP, resulting in the activation of enzymes involved in AMP degradation and uric acid formation (Carran et al., 2016; Tappy and Le, 2010). A randomized controlled trial demonstrated that fructose metabolism upregulated plasma uric acid production (Carran et al., 2016). Uric acid is known to decrease nitric oxide production by inhibiting nitric oxide synthase (Park et al., 2013). Such inhibition attenuates the vascular effect of insulin, which may be a cause of fructose-induced insulin resistance (Nakagawa et al., 2006). Moreover, nitric oxide plays an important role in the insulin-mediated increase of blood flow and nutrition circulation (Steinberg et al., 1994; Tappy and Le, 2010). In humans, uric acid, which is a metabolite of fructose degradation, activates the transcription of fructokinase that is mediated by the transcriptional factor ChREBP (Iizuka et al., 2004). As such, uric acid induces adipocyte dysfunction, causing metabolic syndrome. Additionally, an increase in the expression of transcription factors and enzymes involved in lipogenesis, including sterol regulatory element-binding protein 1c (SREBP1c), has been observed after fructose consumption (Herman and Samuel, 2016).

ChREBP enhances the expression of liver enzymes involved in lipogenesis. ChREBP is phosphorylated when the intracellular carbohydrate level is low (Xu et al., 2013) and dephosphorylated when the intracellular carbohydrate level is high. This dephosphorylation leads to transcriptional activation of key lipogenesis enzymes such as fatty acid synthase. Iizuka et al. demonstrated that ChREBP deficiency in mice reduces lipogenesis induced by a fructose-containing diet (Iizuka et al., 2004). Like ChREBP, SREBP1c plays an important role in lipogenesis, is considered a major protein in the regulation of lipid metabolism, and stimulates the transcriptional activation of lipogenesis-related enzymes. SREBP1c is inactivated under fasting conditions and activated by insulin stimulation after feeding (Xu et al., 2013). Following insulin signaling, activated SREBP1c binds to specific DNA sequences called sterol regulatory elements and upregulates fatty acid synthesis. Liver-specific insulin-receptor knockout mice did not show the upregulation of hepatic triglycerides mediated through a high-fructose diet (HFD), suggesting the physiological importance of SREBP1c in HFDs (Haas et al., 2012).

In a review article on fructose-induced insulin resistance, Herman et al. reported that fructose-mediated lipogenesis increases the hepatic diacylglycerol content, which activates protein kinase C (Herman and Samuel, 2016). This protein kinase C, in turn, suppresses the activation of insulin-receptor kinase and decreases the activation of downstream kinases, which limits the ability of insulin to suppress gluconeogenesis. Nagai et al. reported that the addition of the peroxisome proliferator-activated receptor-α (PPARα) ligand—fibrate—improved the fructose-induced impairment in insulin sensitivity, suggesting that fructose consumption may alter PPARα signaling (Nagai et al., 2002). Moreover, fibrate reduced

mRNA expression of carnitine palmitoyltransferase 1A (CPT1A) (Nagai et al., 2002). As CPT1A expression is regulated by PPARα, a reduction in its receptor expression may lead to decreased CPT1A levels. These phenomena may be associated with attenuated β-oxidation activity and contribute to lipid accumulation. PPARα also plays important roles in regulating insulin sensitivity. Consistent with the results of Nagai et al., in most experimental studies, the activation of PPARα in rodents improved insulin sensitivity (Guerre-Millo et al., 2000; Moller and Berger, 2003; Ye et al., 2001). Together, these studies indicate that the unfavorable effect of fructose consumption may be at least partially mediated by the alternation of PPARα signaling.

The mechanism underlying fructose-induced obesity has been well studied. As fructose is the sweetest among all naturally occurring sugars, its sweetness stimulates food palatability, which in turn induces feeding and overeating via dopamine-mediated signaling (Avena, 2007; Davis, 1973). In addition, fructose consumption causes leptin resistance, which increases the food intake and causes obesity. Teff et al. (2009) reported that dietary fructose reduces circulating insulin and leptin levels, attenuates postprandial suppression of ghrelin, and increases triglyceride levels (Teff et al., 2009). As such, fructose induces disruption of the hypothalamic signals. Compared with consumption of glucose, that of fructose results in a distinct pattern of regional cerebral blood flow, indicating that fructose has a unique impact on brain function and may cause obesity (Page et al., 2013).

The brain uses sugars as the main source of energy, and the regulation of sugar metabolism is critical for brain physiology. Glucose and fructose, two of the most important monosaccharides, have a roughly equal number of calories but are metabolized differently. The differences in their metabolism may explain their different effects on the neuronal pathways. The recent increase in fructose consumption has sparked interest on this topic, and several recent studies on nutrient-related changes in memory and cognition have revealed an association between fructose consumption and cognitive impairment.

The mechanisms of the adverse effects of fructose consumption have been mainly studied in animal models. Rodent studies have shown that fructose intake may lead to insulin resistance in the brain, causing diminished cognitive function. Specifically, consumption of fructose for 6 weeks reduced phosphorylation of the insulin receptor, leading to impaired insulin signaling in hamsters fed 60% fructose solid food and rats fed 15% fructose liquid (Agrawal and Gomez-Pinilla, 2012). Brain insulin resistance is associated with memory impairment in rats, as indicated by the increased latency times in the Barnes maze test (Agrawal and Gomez-Pinilla, 2012). Additional evidence to corroborate the harmful effect of fructose on cognitive function was provided by Agrawal et al., who showed that 6 weeks of consumption of 15% fructose solution diminished phosphorylation of cAMP response element-binding (CREB) and synapsin I and reduced synaptophysin levels. Moreover, fructose may affect neurogenesis in the hippocampus and nucleus tractus solitarius (van der Borght et al., 2011). Using 5′-bromo-2′-deoxyuridine, van der Borght et al. (2011) evaluated the amount of newborn neurons under different dietary conditions and found that 4 weeks of fructose and sucrose consumption (23% solutions) significantly reduced hippocampal neurogenesis, whereas glucose consumption did not have such an effect. Further, Rafati et al. demonstrated that the nucleus tractus solitarius showed neuronal loss after fructose consumption (10% solution) for 6 weeks (Rafati et al., 2013). To understand the effects of fructose on the central and

peripheral neuronal pathways and link the molecular and behavioral fructose-induced changes, the molecular mechanisms underlying these processes should be investigated; in particular, the lack of studies on the epigenetic effect of fructose should be addressed.

Epigenetics and fructose

Epigenetics can be defined as the heritable states of gene expression resulting from changes in the chromatin structure without alterations in the DNA sequence, including DNA methylation, histone modifications, and chromatin remodeling. Thus far, studies on epigenetics mainly aimed to clarify the mechanisms of aging and cancer. However, lately, epigenetics has been studied in various diseases such as metabolic syndromes (obesity, insulin resistance, and diabetes), inflammation, and autoimmune diseases. Since epigenetic information may be altered by environmental factors and can, in turn, alter gene expression, many researchers are now focusing on epigenetics. Further, alterations in epigenetic information can be inherited and sustained after cellular proliferation; therefore, a change in the epigenetic information may result in changes in cellular function and phenotype. Thus, epigenetics can provide new information on etiological factors in environmental diseases, embryonic development, and aging.

DNA methylation adds a cytosine to the CpG dinucleotide with methyl groups, and this reaction is catalyzed by DNA methyltransferase (DNMT). Generally, DNMT regulates gene expression by altering the chromatin structure. The activity of DNMT is influenced by nutrients and bioactive food components, which can change the DNA methylation level and result in altered gene expression. Among nutrients, folate (a water-soluble B vitamin)-induced DNA methylation has been extensively studied. In an animal study, dietary folate was found to stimulate DNA methylation at the *p16* promoter region (Keyes et al., 2007). Animal studies have also demonstrated that dietary folate affects the DNA methylation status during the postweaning period, which might influence disease susceptibility. Moreover, a low-folate diet provided from the postweaning period to puberty has been reported to increase genomic DNA methylation in rat liver, which persisted in adulthood (Kotsopoulos et al., 2008). Collectively, nutrients such as folate play important roles in maintaining epigenetic conditions.

Inadequate nutrient intake may cause inappropriate epigenetic changes and lead to metabolic syndrome. For example, Young Kim et al. demonstrated that administration of a high-fat diet for 20 weeks reduced adiponectin mRNA levels in mice adipocytes (Kim et al., 2015). Transcriptional repression is mediated by the upregulation of DNA methylation in the promoter regions of the adiponectin gene, which may cause metabolic diseases. In addition, Widiker et al. reported that a high-fat diet decreases methylation of the melanocortin-4 receptor (*Mc4r*) in mice (Widiker et al., 2010). *Mc4r* plays important roles in body-weight regulation. In high-fat-diet-fed mice, DNA methylation levels of *Mc4r* were low, which lead to increasing of *Mc4r* expression. On the basis of these findings, we hypothesized that fructose intake causes epigenetic alternation. We previously found that melatonin improves fructose-induced metabolic syndrome (Kitagawa et al., 2012). Daily intraperitoneal administration of melatonin (1 or 10 mg/kg body weight) attenuated these changes at 6 weeks of fructose feeding more effectively (Kitagawa et al., 2012). In an oral glucose-tolerance test, high-fructose-fed rats showed a higher serum insulin-response curve and a normal serum glucose-response curve as

compared with the corresponding animals that received the control diet. In addition, administration of a HFD for 4 or 6 weeks caused insulin resistance. Daily administration of melatonin ameliorated the insulin resistance and high serum insulin-response curve in the oral glucose-tolerance test. These results indicate that melatonin improves metabolic syndrome induced by high-fructose intake in rats. Given the potential roles of melatonin in regulating DNA methyltransferase activity (Korkmaz and Reiter, 2008), the positive effect of melatonin on fructose-induced disease may be mediated by epigenetic alternations. Another study investigating the effect of fructose consumption on epigenetic regulation showed that after feeding rats 20% fructose solution for 14 weeks, the serum triglyceride and total cholesterol levels increased, indicating the induction of fructose-mediated metabolic syndrome (Ohashi et al., 2015). Moreover, total DNA methylation was upregulated, suggesting epigenetic regulation by intake of fructose solution. On the basis of these data, we examined the association between fructose-induced metabolic syndromes and DNA methylation levels. We selected four genes involved in lipid metabolism, as previously reported: *CPT1A*, *PPAR*α, *LDLR*, and *MTTP*. Of these, hepatic mRNA expressions for *PPAR*α and *CPT1A* were decreased. The transcription of *PPAR*α is regulated epigenetically (Gluckman et al., 2007; Lillycrop et al., 2005). Administration of fructose solution for 14 weeks resulted in twofold upregulation of DNA methylation in the promoter regions of *PPAR*α (from −20 to −138 bp upstream from the transcription site). Similarly, fructose intake caused hypermethylation of the *CPT1A* promoter. Considering that *CPT1A* is a target for *PPARα*, reduced expression of this gene may lead to subsequently decreased levels of *CPT1A*. In general, DNA methylation decreases the accessibility of transcriptional factors. Therefore, reduced expression of *PPARα* and accessibility of the receptor to the promoter may cause transcriptional repression of *CPT1A*, leading to hepatic lipid accumulation. Contrary to these results, Chang et al. (2010) did not observe any hypermethylation of *CPT1A* in rats fed a high-fat diet. The discrepancy in the previously mentioned results suggests that HFD and high-fat diets may induce metabolic syndrome through different mechanisms (Chang et al., 2010).

Collectively, we showed that fructose consumption increases DNA methylation in the promoter regions of *PPARα* and *CPT1A*, which may be associated with reduced mRNA levels of these genes and lead to the downregulation of β-oxidation activity. *PPARα* is a master regulator of fatty acid oxidation, and its reduction may lead to the development of metabolic syndrome. Conversely, *PPARα* agonists have been shown to ameliorate metabolic syndrome in rats. Consistent with our present findings, a HFD has been shown to reduce *PPARα* expression in rat liver. However, although *PPARα* is known to play a central role in fructose-induced metabolic syndrome (Nagai et al., 2002), the mechanism for the reduction of *PPARα* expression by fructose has not been elucidated yet. Our present findings suggest that hypermethylation of the *PPARα* promoter suppresses its gene expression via fructose-mediated signals.

The Developmental Origins of Health and Disease (DOHaD) hypothesis

The DOHaD hypothesis, first proposed by David Barker, states a possible explanation for the influence of early-life exposures on later health (Osmond et al., 1993). As observed in a

Dutch famine study, individuals who experienced malnutrition during gestation have increased rates of obesity and metabolic syndrome, indicating the important role of maternal health in the physiological status of the offspring (Narain et al., 2016; Ogden et al., 2011). In addition to the maternal malnutrition, maternal overnutrition and mental stress may have adverse effects on the offspring. In animal studies, offspring exposed to poor nutritional status and stress exhibited decreased cognitive function and insulin resistance after maturation (Lemaire et al., 2000; Tozuka et al., 2010). The molecular mechanisms underlying the DOHaD principle are currently under investigation, and epigenetics plays a potential key role in this principle (Painter et al., 2006).

Generally, the DOHaD principle may be explained by various epigenetic mechanisms, including histone modification, miRNA expression, and DNA methylation. For example, it is well known that DNA methyltransferase methylates cytosine bases, resulting in repressed gene expression to regulate cellular differentiation and development. Heijman et al. reported the possibility that early-life environmental conditions cause epigenetic changes in humans that persist throughout life (Heijmans et al., 2008). This is consistent with the findings of animal studies, wherein rats exposed to a poor nutritional status during gestation and lactation underwent epigenetic modifications and developed predispositions for several diseases such as diabetes (Laker et al., 2014; Park et al., 2013).

The DOHaD hypothesis applies to not only metabolic diseases but also mental disorders. In the Dutch cohort study from which the DOHaD concept was first developed, the incidence of schizophrenia approximately doubled in fetuses from malnourished populations (Susser et al., 1998). Furthermore, early-life stress decreases adult hippocampal neurogenesis and results in increased vulnerability to neuropsychiatric disorders (Lajud and Torner, 2015). As such, maternal health may affect the physiology of the offspring.

DOHaD hypothesis from the perspective of maternal fructose consumption

Due to the increase in fructose consumption, pregnant women currently consume more fructose than they did several decades ago, raising concerns about the potential transgenerational effect of fructose. The effect of maternal fructose consumption has mainly been examined using animal models. When dams were fed a fructose-rich diet during pregnancy and lactation, their offspring showed a lower birth weight than normal offspring, suggesting a negative effect of maternal fructose consumption on fetal development (Jen et al., 1991). In addition, excess maternal fructose consumption during pregnancy and lactation causes glucose intolerance and fatty liver in the rat offspring (Zou et al., 2012); thus the effect of maternal fructose consumption can be explained by the DOHaD hypothesis.

Although excess fructose can lead to adverse systemic effects, majority of the studies have only examined its effects on metabolic disease. For example, Vickers et al. reported that offspring from mothers fed a fructose diet during lactation showed increased body weight and food intake and decreased insulin sensitivity (Vickers et al., 2011). In addition, Tain et al. suggested that maternal fructose intake causes transcriptome changes and programmed hypertension in the offspring in later life (Tain et al., 2016). Ching et al. demonstrated that maternal fructose consumption during gestation and lactation increases serum levels of

triglycerides, free fatty acids, and insulin in adult offspring (Ching et al., 2011). The mechanism underlying this phenomenon is explained by maternal fructose-induced expression of CPT1a and acetyl-coenzyme A carboxylase-beta. Similarly, Saad et al. reported that maternal fructose consumption induces dysregulated metabolic conditions such as obesity, hypertension, and insulin resistance in the adult offspring (Saad et al., 2016). Additional evidence showed that male offspring delivered from dams fed with a fructose diet during gestation had hyperglycemia, hypertriglyceridemia, and hyperleptinemia (Alzamendi et al., 2016). Tain et al. found that maternal HFD caused increases in blood pressure in 12-week-old offspring and melatonin therapy blunted the fructose-induced programmed hypertension and increased the nitric oxide level in the kidneys (Tain et al., 2016; Tain et al., 2014). Collectively, these data indicate that maternal fructose consumption induces metabolic dysfunction. More recently, maternal fructose has been found to cause systemic adverse effects in the offspring.

Transgenerational effect of fructose

The hippocampus plays important roles in memory and learning. Stress during early life, such as environmental stress and malnutrition, causes persistent cognitive dysfunction. Interestingly, some studies demonstrated that maternal fructose consumption impairs hippocampal function in the offspring (Wu et al., 2016; Yamazaki et al., 2018). Wu et al. studied adult female offspring rats from mothers fed a 60% HFD during pregnancy and lactation and found that the female offspring showed impaired hippocampal function and low expression of brain-derived neurotrophic factor (BDNF), which could be explained by the upregulation of histone deacetylase 4 (Wu et al., 2016). Yamazaki et al. studied fructose-induced impairment of hippocampal function in further detail and showed that maternal fructose-induced reduction of BDNF expression is mediated by DNA methylation (Yamazaki et al., 2018). Such reduced BDNF expression may decrease neurogenesis and impair hippocampal function. Interestingly, these fructose-induced effects are not observed with maternal glucose consumption (Yamazaki et al., 2018). Specifically, offspring from dams fed glucose showed normal hippocampal function, suggesting that fructose has a unique transgenerational biological activity.

Hippocampal neurogenesis is known to be attenuated by maternal fructose and is involved in differentiation of stem cells. Therefore, the adverse effects of maternal fructose should be considered at the stem cell level. The homeostasis of the hippocampus is maintained by the resident stem cells, and the maternal fructose-mediated reduction in hippocampal neurogenesis might reflect impaired stem cell function. Since stem cell division actively occurs in the hippocampus, the negative effect of fructose on neurogenesis may appear as impaired cognitive function.

A novel hypothesis of carcinogenesis, the “fetal cell carcinogenesis” hypothesis, was established on the basis of molecular evidence of carcinogenesis by Takano (Takano, 2007, 2014; Takano and Amino, 2005). According to this hypothesis, cancer cells are derived directly from the remnants of fetal cells instead of well-differentiated somatic cells by dedifferentiation. This theory can help interpret the DOHaD principle (transgenerational effect) from a new viewpoint. Stem cells asymmetrically self-renew and differentiate into progeny cells

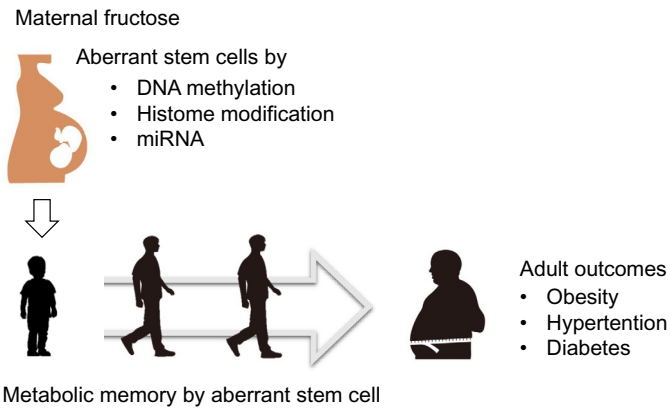

FIG. 4 Putative mechanism for transgenerational effect of fructose. When fetus is exposed to fructose via maternal circulation, afferent stem cell may be generated by fructose-induced epigenetical alternation. Aberrant stem cell might have metabolic memory and cause various diseases such as obesity and hypertension.

to develop or maintain tissues in the normal condition, but some stem cells lose their function due to probable aberrant epigenetic modification, resulting in the loss of tissue integrity and physiological function (Liu and Rando, 2011). Execution of the epigenomic program that influences the quality of the stem cell progeny is modulated by environmental factors during development. Excess maternal fructose, as an environmental factor, can contribute to the changes in stem cell progeny, resulting in altered tissue function of the offspring, fetal development, and child health later in life. We believe that maternal fructose consumption may induce epigenetic alteration of stem cells/fetal cells, and this epigenetic change appears to be a novel mechanism of fructose-induced disease pathology (Fig. 4).

Concluding remarks

Herein, we analyzed the effects of fructose consumption from the viewpoint of epigenetics. Chronic fructose consumption results in alterations in DNA methylation in both nuclear DNA and mtDNA. In addition, excess maternal fructose consumption may induce DNA methylation, which may, in turn, cause metabolic disease susceptibility. Fructose-induced epigenetic changes appear to be a novel mechanism of disease pathology and may induce epigenetic alterations in stem cells/fetal cells, resulting in adverse effects during fetal development and child health later in life.

Glossary

High-fructose corn syrup (HFCS) HFCS is a fructose-glucose liquid sweetener that's found in a wide range of processed foods, from ketchup and cereals to crackers and salad dressings.

Insulin resistance (IR) IR is a syndrome in which a given concentration of insulin produces a less than expected biological effect.

Developmental Origins of Health and Disease (DOHaD) DOHaD is provided new concept into the early origins of health and disease from a lifecycle perspective.

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CHAPTER

22

Alterations of galactose metabolism caused by deficit of galactose-1-phosphate uridylyltransferase activity: An overview of galactosemia type I

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OUTLINE

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SUMMARY POINTS

- This chapter focuses on galactosemia type I, which is caused by a deficiency of the galactose-1-phosphate uridylyltransferase enzyme (GALT).
- Galactose (Gal) is metabolized to glucose-1-phosphate through the Leloir pathway for glycolysis, this pathway maintains the pools of UDP-sugars for the biosynthesis of glycoconjugates.
- Galactosemic patients show accumulation of Gal, Gal-1-P, galactitol, and galactonate and decrease levels of UDP-hexoses, all are responsible of the observed phenotype.
- Even when patients undergo strict restriction of Gal lifelong, some develop mild to severe long-term complications, with a pathophysiology that is still not fully understood.
- Gal and Glc are the principal source of energy in infants, and Gal also has an important structural role.
- Prenatal alterations and endogenous Gal production probably have an important role in the pathophysiology.
- Human *GALT* gene comprises 11 exons that encoded for a polypeptide of 379 amino acids.
- Galactosemia type I patients harbor variations along the whole sequence of the *GALT* gene, most variations are of the missense type and are commonly present in compound heterozygous state.
- The p.Q188R and p.K285N galactosemic alleles showed a high frequency in Caucasians and are associated to null blood GALT activity and a severe clinical phenotype.
- The substitution p.S135L is common in Africans and is associated to a mild phenotype albeit having less than 1% residual enzymatic GALT activity.
- The Duarte variant has a worldwide distribution, and combination with a null-allele causes a milder phenotype referred to as DG.
- Galactosemia type I is considered an aggregation disease, and molecular chaperones might be used as a treatment.
- Some genotypes are related to atypical phenotypes, and Gal-restriction diet in these cases is necessary only during the first year of life.

Galactosemia type I

Galactosemia type I is an inborn error of galactose (Gal) metabolism caused by a deficiency of the galactose-1-phosphate uridylyltransferase enzyme (GALT, EC 2.7.7.12) (OMIM ID: 230400). This disease includes three variants: classic (CG), clinical variant, and biochemical variant galactosemia, based on GALT enzyme activity in red blood cells (RBCs), the levels of intermediates from the Gal metabolism, and the likelihood to show acute and chronic long-term complications (Berry, 2000).

GALT is the second enzyme in the Leloir pathway (Leloir, 1951) (Fig. 1), which includes (1) galactokinase (GALK, EC 2.7.1.6), (2) GALT, and (3) UDP-galactose-4′-epimerase (GALE, EC 5.1.3.2). Mutations in any of these three enzymes produce different types of

galactosemias (Novelli and Reichardt, 2000). GALT deficiency is inherited in an autosomal recessive pattern.

Based on screening programs, galactosemia occurs in approximately 1:30,000 to 1:70,000 Caucasian newborns. However, galactosemia incidence is higher in some countries, like Ireland and Greece (1:23,000), or in regions with high levels of inbreeding (Southern Iran 1:6000). Also, it is increased among itinerants (1:700) and in the Irish traveler community (an endogamous group of commercial/industrial nomads) (1:480). Conversely, populations of African and Asian descent showed lower galactosemia incidence (Institute of Health Economics, 2016; Murphy et al., 1999).

A toxic build-up of intermediates from the metabolism of Gal is produced in galactosemic neonates following Gal intake, mainly from breast milk. In few days, various symptoms appeared such as lethargy, poor feeding, jaundice, and hepatomegaly (Table 1). Neonatal acute toxicity may progress toward the development of *Escherichia coli* septicemia in the baby's second week of life, coagulopathy, vitreous hemorrhage, cataracts, encephalopathy, and in severe cases death (Levy et al., 1996; McCandless, 2009). The pathology of GALT deficiency is directly related to the type of galactosemia variant and to the residual enzymatic activity (Demirbas et al., 2018). The galactosemic patients principally show accumulation of Gal, galactose-1-phosphate (Gal-1-P), galactitol, galactonate, and decreased levels of UDP-hexoses with dysglycosylation; all of these alterations contribute to the pathology of the disease (McCorvie et al., 2016).

As we will describe later, the treatment prevents acute symptoms, is life saving for the neonate, and is relatively easy to accomplish. However, even when the patients undergo strict and lifelong restriction of Gal in their diet, some develop mild to severe long-term complications, including developmental delay, language impairment, and motor abnormality, and hypergonadotropic hypogonadism (infertility in females) with low levels of anti-Müllerian hormone, regardless of genotype or age at the beginning of treatment (Table 1) (Berry, 2000; Schweitzer-Krantz, 2003). The pathophysiology of these complications is still not fully understood, but patients primarily show cognitive and gonadal impairment (Coss et al., 2013; Timson, 2016). A recent report showed that a strict galactose restriction has been associated with a less favorable outcome, according to observational data derived from 15 countries that include 509 patients registered in the GalNet database. These patients were diagnosed with CG and variant galactosemia on the basis of a residual GALT activity of $\leq$10% and/or GALT pathogenic disease-causing mutations. Perhaps, a minimun amount of exogenous dietary galactose is required for regular glycosylation reactions even in galactosemia patients (Rubio-Gozalbo et al., 2019).

Metabolic pathway

D-Gal is found in bacteria, plants, and animals, as α and β anomeric forms, being the βD isomer the most common configuration. It is found either free or bound in complex carbohydrates, glycoproteins, and glycolipids (Coelho et al., 2015a). Both this natural aldohexose and glucose (Glc) are the main sources of energy in infants (around 40%), since they are the components of the lactose disaccharide, that is also involved in brain development (Coelho et al., 2015a). This disaccharide is contained in most animal milks and dairy foods and in some plant

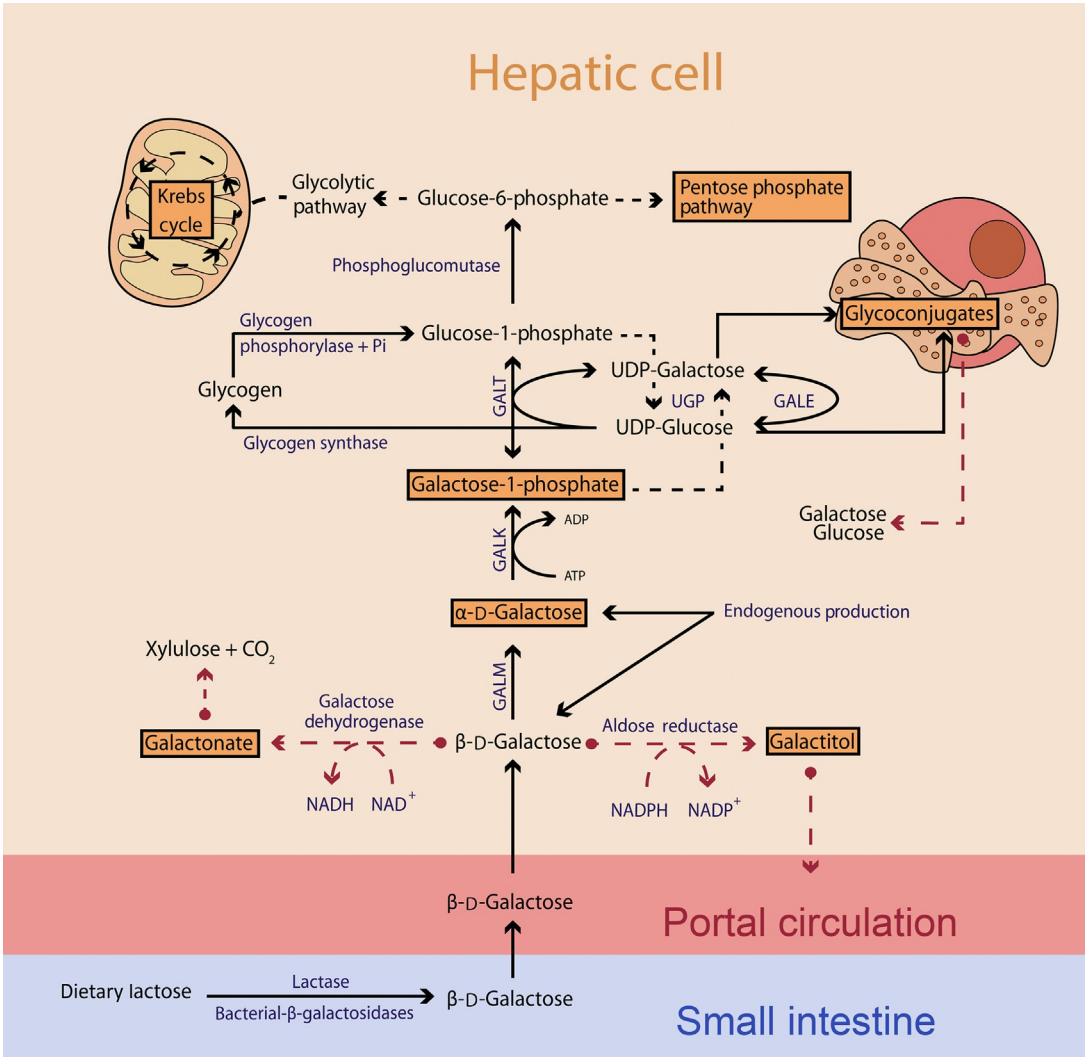

FIG. 1 Gal metabolism and Leloir pathway. Lactose derivatives from the diet are broken down into Glc and Gal, which reach the liver via portal circulation. In the hepatocyte, βD-Gal from dietary and endogenous sources is converted into the αD isomer by GALM. Then, αD-Gal is metabolized to Glc through the Leloir pathway for glycolysis. First, αD-Gal is phosphorylated to Gal-1-phosphate, a reaction that is catalyzed by GALK. Subsequently, GALT catalyzes the transfer of a UMP group from UDP-Glc to Gal yielding UDP-Gal and Glc-1-P. This pathway ends when GALE converts UDP-Gal to UDP-Glc. The last two reactions are reversible and may supply UDP-Gal, when ingestion of Gal in the diet is inadequate. The UDP-Glc and UDP-Gal that are required are generated from Glc-1-P and Gal-1-P, respectively, by the action of UDP-Glc pyrophosphorylase (UGP). These UDP-hexoses have a role in the biosynthesis of glycoconjugates. Glc-1-P is converted into Glc-6-phosphate by phosphoglucomutase. Glc-6-phosphate might either enter the pentose phosphate pathway or be converted into Glc by the Glc-6-phosphatase and then be transported in the blood or get into the glycolytic pathway. Endogenous Gal is produced from UDP-Gal and glycoconjugates. When the Leloir pathway is blocked due to alterations of the GALT enzyme, Gal and Gal-1-P are accumulated. As a consequence, alternative routes for Gal disposal are activated, and galactitol and galactonate are produced by the action of aldose reductase and Gal dehydrogenase, respectively.

TABLE 1 Neonatal and long-term symptoms in patients with CG

Neonatal symptoms after milk introduction	Long-term complications
Failure to thrive	Cataracts
Poor feeding	Ovarian failure
Vomiting	Hypergonadotropic hypogonadism or POI in females
Diarrhea	Growth disturbance
Bleeding diathesis	Reduced bone mineral density
Jaundice	Learning problems
Hepatomegaly	Cognitive deficits
Splenomegaly	Verbal dyspraxia
Ascites	Delayed speech development
Hypoglycemia	Neurological disorders
Hypotonia	Ataxia/tremor/dystonia
Lethargy	Cerebellar disorders
Bulging anterior fontanel	Spatial orientation and visual perception difficulties
Renal tubule dysfunction	Anxiety disorders
E. coli sepsis	Depression
Pseudotumor cerebri	Attention deficit hyperactivity disorder
Death	Autism spectrum disorder

Note: symptoms underline are also observed in untreated patients with clinical variants of galactosemia, however long-term complications are not present (Rubio-Gozalbo et al., 2019; Sarafoglou et al., 2017).

products such as fruits, legumes, nuts, grains, tubers, and vegetables. Human milk contains 55–70 g/L of lactose and 5–8 g/L of about 100 different oligosaccharide complexes that contain Gal (Kunz et al., 2000), which warrants that Gal levels do not become limiting during neonatal development.

Lactose is hydrolyzed in the small bowel by intestinal lactase or bacterial β-galactosidases, which cleave the β-1,4-glycosidic linkage between Gal and Glc (Newburg, 2013). Gal is absorbed through the mucosal lining into the apical side of enterocytes by the sodium-dependent glucose active cotransporter 1, aka sodium-glucose-linked transporter 1 (SGLT1), and is secreted from these cells at their basolateral membrane via facilitated diffusion through the glucose transporter 2 (GLUT2). Then, Gal is transported to the surrounding capillaries toward the portal circulation, and finally, it is transferred to the liver where Gal is removed from the blood (blood concentration < 1 mmol/L) (Wright et al., 2003). In the hepatocyte, Gal is converted to UDP-Glc through the Leloir pathway (Fig. 1). This pathway has a role in maintaining pools of UDP-sugars for the biosynthesis of glycoproteins and glycolipids. Ultimately, Gal conjugated with Glc produces milk sugar by the action of the prolactin hormone in the human mammary gland.

Pathogenesis of galactosemia

The most important mechanism implicated in the pathogenesis of galactosemia is the toxic build-up of Gal and its intermediates (galactitol, galactonate, and Gal-1-P), together with deficiency of metabolites as a consequence of blocked pathways. These molecules cause permanent damage to various tissues and organs (Fig. 1). Long-term complications in patients under dietary treatment are probably caused by products of the endogenously synthesized Gal, which even in small amounts are toxic metabolites that accumulate and exert chronic harmful effects in cells, and/or may reveal dysfunctions produced during intrauterine life (Coelho et al., 2017). De novo synthesis of Gal has been shown in healthy and galactosemic subjects at levels of between 0.48 and 1.71 mg/kg/h, independently of the presence of short-term exogenous Gal (Berry et al., 2004; Schadewaldt et al., 2014). Gal-1-P levels in RBCs usually are >10 mg/dL in the newborn period (normal value: <1 mg/dL) (Berry, 2000). Concentrations of Gal-1-P, Gal, galactitol, and residual enzymatic activity in untreated and treated patients and controls are showed in Table 2. High levels of *follicle-stimulating hormone* (FSH), luteinizing hormone (in females), metabolic acidosis with aminoaciduria, altered liver functions tests, coagulopathy, hypoglycemia, direct hyperbilirubinemia, hypoalbuminemia, albuminuria, and glycosuria have also been revealed in CG patients (Sarafoglou et al., 2017).

Gal in excess is reduced to galactitol by aldehyde reductase and is oxidized to galactonate by galactose dehydrogenase (Cuatrecasas and Segal, 1966) (Fig. 1). Galactonate can be metabolized to xylulose and CO_2 or being excreted in urine. However, galactitol is not further metabolized and accumulates in blood and various tissues due to its inability to be transported across the plasma membrane and excreted by urine (Berry et al., 2001; Palmieri et al., 1999). This highly osmotically active compound attracts water into the lens and brain producing cataracts and cerebral edema, respectively, by a mechanism that is not totally understood (Dvornik et al., 1973). In the acute phase the osmotic stress might be the main contributor to cataract formation, while oxidative stress caused by activation of the unfolded protein response (UPR) is responsible for the progression of this pathology (Viggiano et al., 2018). Also, galactitol has been reported to be an inhibitor of mammalian mutarotase, resulting in an increase of unmetabolized Gal (Keston, 1963). Interestingly, a galactose-free diet has been shown to contribute to cataract disappearing in 54.5% of patients that displayed this clinical sign during their neonatal period (Rubio-Gozalbo et al., 2019).

Reduced GALT activity produces a shortage of UDP-hexoses leading to the disruption of glycosylation in the posttranslational modification of proteins and lipids (Ng et al., 1989; Coss et al., 2014b). Galactosemia has been classified as a secondary disorder of glycosylation with defects in the assembly and processing of glycans (Coman et al., 2010; Maratha et al., 2016). Besides, Gal metabolism intermediates could produce competitive inhibition of glycosyltransferases (Lai et al., 2003). UDP-Gal and UDP-Glc are important precursors in the synthesis of glycoconjugates, such as gangliosides, galactocerebrosides, sphingolipids, mucopolysaccharides, and membrane glycoproteins (Coss et al., 2014a; Lebea and Pretorius, 2005; Maratha et al., 2016). Glycoproteins are essential in many biological systems such as cell-cell signaling and adhesion, migration, protease resistance, mechanisms of defense, antigenicity, coagulation, immunity, and fertility. Indeed, pediatric and adult patients that are homozygous for p.Q188R have higher level of agalactosylated structures than normal controls. These alterations are independent of Gal levels and seem to be present ever since the

TABLE 2 Classification, metabolites concentration, and enzymatic activities in galactosemia type I

Classification	Gal-1-P in RBCs (mg/dL)	Gal in RBCs (mg/dL)	Galactitol in urine (mmol/mol of creatine)	Residual enzymatic activity in RBCs (%)	Frequency	Comments
Classic galactosemia (CG)[a,c–f]	43–272	90–360	397–743 (<1 y) 215–382 (1–6 y) 125–274 (>6 y)	0–1	1:40,000–1:60,000[b]	Mainly homozygous or compound heterozygous for null mutations (e.g., p.Q188R or gene deletion)
Biochemical variant or Duarte galactosemia (DG)[a–c]	14.7–33.8	NR	54–172	15–35	1:4000	Requires no treatment
Clinical variant galactosemia[c,e–i]	0.1–1.3	20–100	20–100	1–10	1:14,400–1:20,000[j]	Individuals homozygous for the p.S135L variant have no enzymatic activity in RBCs (~0%) and a residual enzymatic activity of about 10% in the liver
Normal levels[a,e]	<1	0–4.3	8–107 (<1 y) 7–74 (1–6 y) 2–15 (>6 y)	–	–	

[a] *Pasquali et al. (2018).*
[b] *Frequency estimated in US newborns.*
[c] *Lai et al. (1996).*
[d] *Berry et al. (2000).*
[e] *Yager et al. (2006).*
[f] *Landt et al. (1997).*
[g] *Welling et al. (2017b).*
[h] *Henderson et al. (2002).*
[i] *Manga et al. (1999).*
[j] *Frequency of galactosemia associated to the p.S135L variant in African origin populations, other variants had been described just in one case or family (Manga et al., 1999; Henderson et al., 2002).*
NR, *not reported;* RBCs, *red blood cells;* y, *years.*

prenatal stage, which may explain long-term complications in patients with positive screening that have been early treated (Coss et al., 2014a). On the other hand, proteins associated with CDG-I, such as alpha-1,2-mannosyltransferase (ALG9) and the inflammatory gene annexin A1 (ANXA1) showed changes in their cellular localization in an independent Gal intoxication manner, when control cells and cells from CG patients were compared, suggesting fundamental dysregulation in CG (Coss et al., 2014a).

Several hypotheses have been proposed to rationalize acute and long-term complications caused by impaired GALT function, including enzymatic inhibition, osmotic, endoplasmic reticulum, and oxidative stress; epigenetic modification; and activation of the UPR (Jumbo-Lucioni et al., 2013; Balakrishnan et al., 2016; De-Souza et al., 2014). Likewise, the D-Glc starvation that is observed in galactosemia and is related to glycogenolysis defects

due to inhibition of glycogen phosphorylase and phosphoglucomutase by Gal-1-P is known to activate the UPR, a mechanism that has been implicated in the progression of neurodegenerative diseases (Scheper and Hoozemans, 2015). Moreover, UPR activation impacts on the transport and targeting of membrane receptors (Staubach et al., 2017). Several studies have found that Gal intoxication, through epigenetic modifications, can induce the dysregulation of genes involved in pathways that include N- and O-glycan biosynthesis, inositol, leptin metabolism, and inflammatory pathways in prenatal and neonatal periods (Maratha et al., 2016; Coss et al., 2014b; Colhoun et al., 2018).

Gal plays an important function in the formation of myelin during fetal life until childhood. The myelin sheath is composed in a 70%–80% of lipids, being galactocerebrosides, cholesterol, and phosphoglycerides the major components. van Erven et al. (2017a) contrasted galactosemic patients that were treated since they were 11 years old on average with comparable controls and detected abnormalities on occipital regions linked to visual-spatial capacities and working memory. Various galactosylated glycans form the foundation of the extracellular synaptomatrix of synaptic cleft and perisynaptic space and directly modulate transsynaptic pathways during synaptogenesis (Dani and Broadie, 2012). These results were correlated to clinical neurocognitive phenotypes in galactosemia. Dysglycosylation both in the prenatal period and in treated galactosemic adults appears to affect long-term neurological development (Coss et al., 2014b; Maratha et al., 2016). By working with GALT-deficient cell models, Staubach et al. (2017) found that dysglycosylation of at least some glycosylated membrane proteins, rather than down-regulation at the transcriptional level, is the reason for the decrease in membrane expression of certain glycoproteins. However, downregulation of the phosphoinositide 3 kinase/protein kinase B signaling pathway has been shown, associated to a loss of Purkinje cells in the cerebella of both treated and untreated galactosemic patients and with the premature loss of primordial ovarian follicles in young mutant mice (Balakrishnan et al., 2017).

Hypergonadotropic hypogonadism causing severe primary ovarian insufficiency (POI) and in turn premature menopause has been reported in almost all females with galactosemia (79.7%); however, males only have a higher-than-expected prevalence of cryptorchidism and low semen volumes without reproductive problems (Gubbels et al., 2013; Rubio-Gozalbo et al., 2019). Chronic exposition (prenatal, perinatal, or postnatal) to Gal-1-P and galactitol has been hypothesized to directly damage the ovaries; yet the pathophysiological mechanism is still unknown. In some cases, ovarian histology has shown the presence of severely decreased numbers of normal primordial follicles with the absence of intermediate and Graafian follicles, which suggested a maturation arrest. The epigenetic origin of POI in galactosemia should be explored (Rubio-Gozalbo et al., 2010). Colhoun et al. (2018) have found a significant dysregulation of specific genes that are key in N-glycan synthesis, inflammation, and leptin signaling in adult CG patients suffering of POI and/or infertility. Moreover, *FSH* is a glycoprotein that is qualitatively abnormal in CG with an apparently reduced terminal galactosylation. This gonadotropin has been found elevated from infants to the onset of puberty in CG patients. Females with CG require a continuous evaluation of their gonadotropin levels and initiation of estrogen and progestin support. Frederick et al. (2018) showed that a level of anti-Müllerian hormone of $\geq$0.04 ng/mL can be used as a biomarker to predict a good ovarian function in girls and women with CG. As it has been seen in women with GC (van Erven et al., 2017b), Tang et al. (2014) have also found a reduced fertility rather than a complete infertility using a homozygous

GalT gene-trapped mice model. Meyer et al. (1992) reduced the ovarian galactitol accumulation and the toxic effects on rat oocytes by inhibiting the aldose reductase enzyme, which confirmed that galactitol damages the ovarian cells.

Other galactosemia clinical signs are growth delay and low bone mineral density in many patients (Berry, 2000). However, van Erven et al. (2017c) by using metaanalysis concluded that CG patients have a lower bone mineral density than the general population, but the values were within two standard deviations of the reference mean of zero. They also found vitamin D levels in the low reference range and serum calcium levels within the reference range. Meanwhile, Rubio-Gozalbo et al. (2019) reported a diminished bone mineral density in 26.5% of 287 Caucasian patients even after following a diet.

Galactosemia has been suggested as a multisystem disorder affecting numerous organs and pathways (Coss et al., 2014b) in which understanding the role of glycosylation in the development of these complications is essential.

GALT: Gene and protein

The human *GALT* gene is located at the short arm of chromosome 9 (9p13.3) (Shih et al., 1984). The gene is composed by 11 exons and spans over 4.3 kb of genomic DNA (NM 000155.3) with a coding region of 1295 pb in length that encode for a protein of 379 amino acids (Reichardt and Berg, 1988) (Fig. 2). The promoter region of the *GALT* gene exhibited characteristics of housekeeping genes, lacking a TATA box and possessing three GC-rich specific protein 1 (Sp1) sites: an activator protein 1 (AP-1) site, a CAAT box, and two enhancer motifs (E-box) (Leslie et al., 1992; Elsas et al., 2001). Regarding the codifying sequence, exons 6 and 10 are the most evolutionary conserved regions with 100% identity compared with the mouse *GALT* gene sequence and >70% identity with respect to homologous genes in bacteria and yeast (Leslie et al., 1992; Coelho et al., 2014a).

The active GALT protein is a homodimer with an estimated molecular mass of 88 kDa (Fridovich-Keil and Walter, 2008). The amino acid sequence of each subunit has 39%, 46%, and 87% of similarity with respect to the yeast, *E. coli*, and mouse counterparts, respectively (Leslie et al., 1992). Its quaternary three-dimensional structure has been recently elucidated by x-ray crystallography at 1.9-Å resolution, finding one active site and a zinc ion–binding site per monomer (McCorvie et al., 2016) (Fig. 3).

Protein catalysis comprises a ping-pong mechanism, where a residue of histidine at position 186 is uridylylated after the hydrolysis of the UDP-Glc substrate, releasing Glc-1-P. Then, Gal-1-P attacks the covalently uridylylated intermediate, forming the final product of the reaction, UDP-Gal, and restoring the lateral chain of His186 for a new cycle of catalysis (Thoden et al., 1997). The catalytic site has a similar structure as the histidine triad superfamily of proteins (HPH, H184–186) and is highly conserved between uridylyltransferase proteins (Brenner, 2002). The *E. coli* GALT enzyme (galT) also functions as a homodimer of 80 kDa (348 amino acids) (Wedekind et al., 1995). The 3-D structure is also similar between the bacterial and the human proteins containing five α-helices and a small three-stranded β-sheet, both flanking a core of nine-stranded β-sheets (Wedekind et al., 1995; McCorvie et al., 2016) (Fig. 3). Likewise, uridylylation of GALT causes global conformational changes in

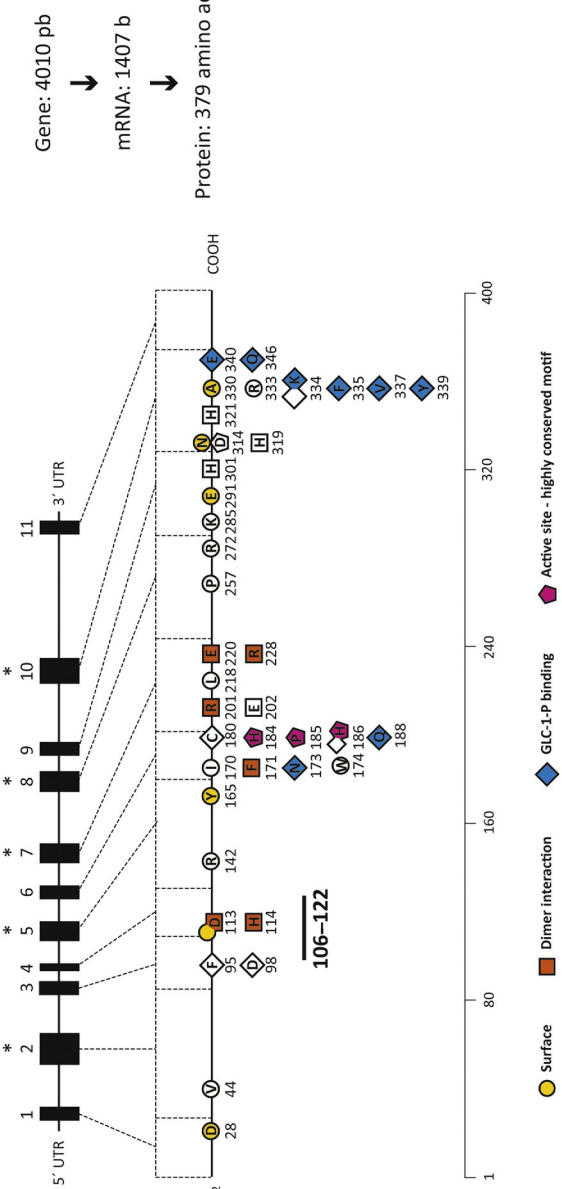

FIG. 2 Structure of the human *GALT* gene and its product. (A) Organization of the *GALT* gene. The 11 exons are illustrated as boxes interspaced by introns *(lines)*. The length of each box is proportional to the codifying sequence extension. Exons indicated with *asterisks* hold ≈ 60% of missense galactosemic variants reported as "pathogenic." (B) Human GALT protein displaying the main amino acid residues that are implicated in the function of the enzyme (*circle, square, diamond, and pentagons)*. In addition, the position of the most common Duarte polymorphism, p.N314D, is indicated (*circle and pentagon with double letters)*. Amino acids in the dimerization loop are showed in *bold letters*.

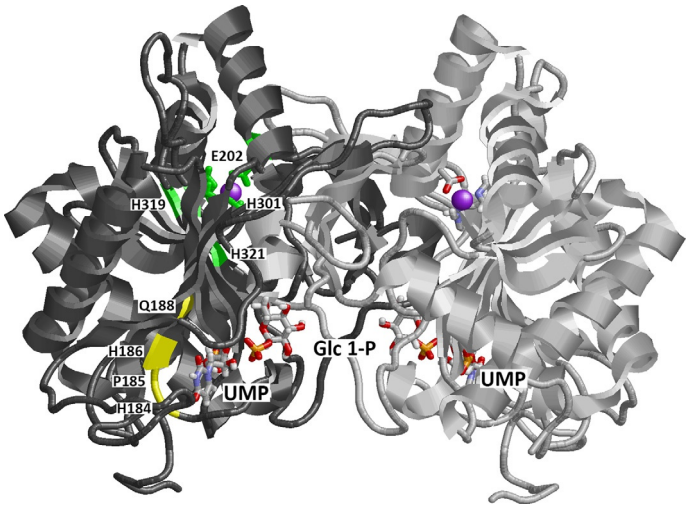

FIG. 3 X-ray crystal structure of human GALT (PDB code 5IN3). Each monomer in the homodimer is highlighted with different tones of grey. The ligands, UMP and Glc-1-P, are represented by sticks. Residues that either bind to the Zn ion (balls) or participate in the active site are indicated. The image was elaborated by using the RasMol software, version 2.7.5.2 (Bernstein, 2000).

the protein, decreasing its flexibility (Henderson et al., 2000; McCorvie et al., 2016). The Zn^{2+}-binding site is constituted by residues Q202, H301, H319, and H321 and is capable of binding other divalent cations with less affinity, such as Co^{2+} and Cu^{2+}. Metal ion binding helps stabilizing the UMP-phospho-H186 intermediate and confers structural stability to the homodimer, according to thermal denaturation and native proteolysis resistance experiments (McCorvie et al., 2016).

Mutation spectrum in the *GALT* gene

Since the first report of mutations on the *GALT* gene (Reichardt et al., 1991), allelic heterogeneity was observed as a hallmark for the galactosemia type I disease. Around 400 mutations have been described on the human *GALT* gene and comprise single-nucleotide variants (SNV), deletions, insertions, insertions/deletions, and whole gene deletion (http://www.arup.utah.edu/database/GALT/GALT_display.php, last accessed July 2019) (Calderon et al., 2007). Most of the SNVs reported are missense changes (~50%), which affect the protein folding (117), the protein dimerization (25), the substrate-binding site (12), the metal ion–binding site (3), or both the substrate binding and the folding of the protein (1) (McCorvie et al., 2016). Other missense variations do not produce any effect on the protein activity and are considered as polymorphisms (Chhay et al., 2008; McCorvie et al., 2016). However, a few synonymous variants have been shown to affect the splicing, causing a decrease in enzymatic activity (Boutron et al., 2012; Ohlsson et al., 2019).

Generally, galactosemic alleles have low frequency, and only few have high frequency with clear ethnic distribution, such as p.Q188R (c.563A>G, exon 6) and p.K285N (c.855G>T, exon 9) in Caucasians and p.S135L (c.404C>T, exon 10) in Africans (Tyfield, 2000). The p.Q188R substitution affects a crucial interaction of the UDP-Gal substrate and decreases enzyme flexibility. The p.K285N and p.S135L variations remove H-bond interactions

with structurally adjacent amino acids, disturbing the appropriate folding of the protein. Blood enzymatic activity in patients that are homozygous for these mutations is less than 1% of the normal values (Table 2) (Reichardt et al., 1991; Lai et al., 1996; Greber-Platzer et al., 1997; Coss et al., 2014a; Coelho et al., 2014a; Viggiano et al., 2015). Although p.Q188R and p.K285N homozygotes have a severe clinical phenotype, p.S135L homozygotes display mild clinical outcomes (Lai et al., 1996; Tyfield et al., 1999; Tyfield, 2000). The p.Q188R amino acid substitution is predominantly found in Caucasians from Europe (45%–94%), according to its origin from an ancestral central/eastern European patient around 20,000 years ago (Flanagan et al., 2010). It has different allele frequencies between populations, with highest values in Ireland (93.6%) (Murphy et al., 1999) and Great Britain (77%) (Tyfield et al., 1999), followed by Portugal (57%) and Spain (50%) (Gort et al., 2006). This nucleotide substitution was also detected in 60%–70% of Caucasians from the United States (Elsas et al., 1995; Yang et al., 2002), 40%–58% of Hispanics with Mexican ancestries (Yang et al., 2002; Velázquez-Aragón et al., 2008), 22% of Brazilians (Garcia et al., 2016), and 5.5% of Indians (Singh et al., 2012), but it has not been reported in Chinese and Japanese individuals (Suzuki et al., 2001).

The nucleotide change p.K285N is a more recent mutation (~12,500 years ago), which has been found in individuals from Central and Eastern Europe, with a suggested Slavic origin due to the high allele frequency (~30%) in subjects of Czech, Slovak, Polish, and Austrian origin (Greber-Platzer et al., 1997; Kozák et al., 1999; Lukac-Bajalo et al., 2007; Suzuki et al., 2001).

Similarly, the p.S135L mutation is prevalent in African descent patients, with a frequency of 91% in South Africans and 48% in African Americans (Lai et al., 1996). This galactosemic allele has also been detected in Mexican and Brazilian patients with a frequency of 5.3% and 12%, respectively (Velázquez-Aragón et al., 2008; Garcia et al., 2016).

There are additional DNA changes found exclusively in certain populations like c.252-2A>G (intron 2), p.F171S (c.512T>C, exon 6), and p.L195P (c.584T>C, exon 7), which have been reported in affected subjects from Mexico, Africa, and Spain, respectively (Yang et al., 2002; Velázquez-Aragón et al., 2008; Gort et al., 2006).

In vitro expression analysis

Different in vitro expression systems have been used to evaluate missense DNA variants from galactosemic patients. Mammalian heterologous expression system such as 293 cells (Ko et al., 2010) and monkey kidney COS cells have been used to demonstrate the deleterious effects of amino acid substitutions found in galactosemic patients (Reichardt and Berg, 1988). Also, a yeast strain lacking the Gal7 locus ($gal7^{-/-}$), which is homologous to the human *GALT* gene, has been useful as a model to test metabolic consequences of null GALT activity, because it displays low level of UDP-Gal and Gal toxicity with the accumulation of Gal-1-P (Riehman et al., 2001). A similar phenotype is observed when the gal7-strain of yeast expresses mutant alleles from galactosemic patients lacking detectable GALT blood activity (p.F171S, p.Q188R, p.R231H, p.R259W, p.K285N, and p.R333W) (Fridovich-Keil and Jinks-Robertson, 1993; Fridovich-Keil et al., 1995; Wells and Fridovich-Keil, 1997; Riehman et al., 2001). Heterologous expression of human wild-type and mutant GALT proteins has also been performed in a bacterial system (Lai et al., 1996; Coelho et al., 2014b). In addition, bioinformatic tools have been

used to predict the effect of missense variants on GALT enzyme. Computational models of human GALT mutant proteins have been generated (Lai et al., 1996; Marabotti and Facchiano, 2005), and a web tool to visualize missense *GALT* mutations in the protein three-dimensional structure has been designed, which included the possibility of modeling DNA variants in both homozygous and heterozygous forms (d'Acierno et al., 2018).

Most missense *GALT* mutations affect protein stability due to misfolding and cause reduced or absence of catalytic activity. This pathophysiological mechanism of aggregation and degradation of mutant polypeptides suggests that molecular chaperones might be employed as a promising treatment strategy for CG (Coelho et al., 2017). Recently, it has been shown that arginine stabilizes GALT, including the p.Q188R and p.K285N variant forms, supporting the idea that small molecules may enhance the stability and activity of this protein (Coelho et al., 2015b).

Several missense mutations that are located on the protein surface have unknown effects (p.D28N/H/Y, p.R67C, p.D113N, p.Y165H, p.G291K/V, p.A330V, and p.D314N). These changes might affect either residues involved in posttranslational regulation of in vivo GALT activity or residues implicated in interactions with other proteins. An intracellular localization study of the homologous GALT protein from yeast (Galt7) suggested a possible association with galactokinase and UDP-galactose 4-epimerase (Christacos et al., 2000). Further experimental studies have to be performed to determine whether the human GALT protein is capable of associating with other enzymes from the Leloir pathway and form a multienzymatic complex (McCorvie and Timson, 2011).

Los Angeles (D1) and Duarte (D2) polymorphism variants

The term Duarte allele has been used to identify two different alleles encoding for two isoforms of the GALT protein. The first described, D1 or Los Angeles variant (LA), corresponds to two polymorphisms in linkage disequilibrium, the synonymous variant p.L218L (c.652C > T, exon 7) and the nonsynonymous variant p.N314D (c.940A > G, exon 10). Protein expressed in blood samples from individuals that are homozygous for the D1 variant exhibited an increased enzymatic activity (110%–130%) compared with the wild-type enzyme (Podskarbi et al., 1996). Harboring one or two copies of the D1 allele is not associated with a clinical outcome (Langley et al., 1997; Carney et al., 2009).

The second variant detected, D2 or Duarte variant, conserves the same p.N314D amino acid substitution involved in linkage disequilibrium with four noncoding additional variations: a 4-bp deletion in the 5′ untranslated region that corresponds to 116–119 bases upstream from the first methionine codon (c.-119_116delGTCA) and three intron polymorphisms, c.378-27G > C (intron 4), c.508-24G > A (intron 5), and c.507 + 62G > A (intron 5) (Podskarbi et al., 1996; Kozák et al., 1999). The D2 allele is an ancient DNA transition (15,000–477,000 years ago), which is common in different ethnic groups, with a panethnic allele frequency of 8%–11% worldwide (Elsas et al., 2001; Suzuki et al., 2001; Flanagan et al., 2010). However, the D2 allele has a low frequency between Asian and African individuals (Lukac-Bajalo et al., 2002; Pyhtila et al., 2014).

D2 homozygotes have approximately 50% of the normal GALT blood activity, while D2 heterozygotes possessed about 75% of the expected enzymatic activity (Yang et al., 2002).

Since the 4-pb deletion included in the D2 genotype (c.-119_116delGTCA) has low *GALT* promoter activity in liver and ovarian cells in vitro (Elsas et al., 2001), it has been suggested to be responsible for the lower activity of this variant (Carney et al., 2009). Specifically, this deletion affects the spacing between recognition sites for trans-activating factors (E-boxes) and a positive regulatory site (AP-1), diminishing in turn the transcription of the *GALT* gene (Carney et al., 2009).

Genotype-phenotype correlation

The D2 variant is commonly found in compound heterozygosity with alleles that cause a very low to absence of GALT function. This clinical form is referred to as Duarte galactosemia (DG) (Table 2) to differentiate it from the severe phenotypic form of the disease, designated as CG. Patients with DG have around 25% of the regular GALT enzymatic activity in RBCs and mild to none clinical manifestations (Hughes et al., 2009; Milánkovics et al., 2010). Usually, DG patient are diagnosed during neonatal screening (Pyhtila et al., 2014), displaying normal values of Gal and related metabolites during biochemical follow-ups through their life, but might manifest long-term complications, mainly neurological and speech defects, independently of dietary treatments (Ficicioglu et al., 2008; Powell et al., 2009; Lynch et al., 2015).

Contrastingly, CG patients harbor two null-type mutations in compound heterozygosity or homozygosity, which are mainly associated to the absence of blood GALT activity, higher levels of Gal-1-P, and a severe clinical outcome even if diagnosis and treatment were carried out and started since the neonatal stage (Hughes et al., 2009; Milánkovics et al., 2010). Particularly, more severe phenotypes are mainly associated with the combination of the alterations p.Q188R, p.K285N, and/or p.L195P in homozygous or heterozygous forms. For example, patients harboring the p.Q188R mutation are more susceptible to display speech problems and ovarian failure (Guerrero et al., 2000; Kaufman et al., 1994; Robertson et al., 2000).

Molecular analysis of the *GALT* gene helps to distinguish between variants, allowing accurate decision-making about the patient long-life treatment and prognosis (Pyhtila et al., 2014). For example, some cases of transient galactosemia could be explained by the presence of *GALT* or *GALE* mutations in a heterozygous state (Coelho et al., 2017). However, several patients that were positive for CG following newborn screening showed tolerance to Gal during their development, even though they have undetectable blood GALT activity. Different factors have been proposed to explain phenotype heterogeneity and mild phenotypes in patients harboring severe mutations.

- Individual oxidative Gal capacity: Measurements of $^{13}CO_2$ in the expired air following administration of oral or intravenous [1-^{13}C]Gal tracer have been used to evaluate whole-body oxidative capacity in galactosemic patients with different genotypes (homoallelic and heteroallelic). This protocol has been suggested as a tool for classifying severe and mild metabolic phenotypes (Berry et al., 1995, 2000). Patients harboring either two p.Q188R alleles or one p.Q188R allele in combination with another severe variant (p.L195P, p.E308K, p.V151A, p.M142K, and p.Q344K) had a residual 6% and 10% oxidation capacity when compared with normal subjects after 1 and 5 h of Gal load, respectively (Berry et al., 2000). In contrast, homoallelic patients for the p.S135L mutation, which is associated with a mild phenotype, had almost a normal oxidative metabolic rate (Lai et al., 1996; Berry et al., 2000).

This phenomenon has been explained by differences on tissue-expression patterns of the *GALT* gene or variations on the degradation rate of misfolding and/or inactive proteins (Lai et al., 1996).

- A low production of potentially dangerous Gal metabolites, like galactitol (Segal, 2006): This idea is supported by the absence of the galactosemic phenotype in GALT knockout mice in which galactitol was not accumulated due to differential expression of the enzyme aldose reductase (Leslie et al., 1996; Ning et al., 2000, 2001) (Fig. 1).
- Heterodimer interactions: The p.Q188R variant appears to act as a partial dominant negative in combination with other galactosemic alleles (Elsevier and Fridovich-Keil, 1996; Wang et al., 1998). For example, subunit interactions between human wild-type and mutant GALT polypeptides, such as Q188R and R333W, have been demonstrated in which the generated heterodimers possessed 20%–30% residual enzymatic activity with respect to the wild-type homodimer (Elsevier et al., 1996). Wang et al. (1998) found that residual GALT activity of mutant heterodimers varies depending on the combination of alleles. These results help to explain the heterogeneous clinical outcomes seen in galactosemic individuals, given that most of them are compound heterozygotes.
- Atypical galactosemias: It has been postulated that for some genotypes, Gal restriction is only required during the neonatal period. Two patients that harbored *GALT* mutations in both alleles have been reported to have low GALT activity in blood but normal levels of total Gal and Gal-1-P after Gal tolerance tests (Liu et al., 2015; Cocanougher et al., 2015). In both cases, nutritional treatment was withdrawn at the age of 1 year, and clinical follow-ups indicated the lack of manifestations related to galactosemia.
- Additional factors: Intervention of unknown susceptibility and/or modifier genes has been suggested because several adult patients that were homozygous for the p.Q188R mutation with a deficit in GALT erythrocyte activity and elevation of Gal oxidation metabolites (Gal-1-P, galactitol) have shown mild clinical phenotypes without a dietary Gal restriction (Lee et al., 2003; Panis et al., 2006; Krabbi et al., 2011). An in vivo Gal turnover study performed in compound heterozygote patients for several *GALT* alleles (p.Q188R, p.K285N, p.L195P, p.H319Q, p.R148W, p.A320T, p.V151A, p.R259W, p.D28Y, p.Q39P, p.F117C, p.S297P, and p.N97del) has confirmed a decrease in endogenous Gal production with age, irrespectively of genotype or erythrocyte residual activity (Schadewaldt et al., 2014).

In addition, an auxiliary hepatic pathway used to synthesize UDP-Glc has been observed in patients that were homozygous for the p.Q188R mutation (Segal, 2006). Therefore abnormal alternative pathways and epigenetic factors combined with personal differences may play an important role in this disease (Segal, 2001), and similar mechanisms as the epimutation effect described for other metabolic diseases might be uncovered in the future (Guéant et al., 2018).

Treatment in classic galactosemia

Implementation of treatment on a child with clinical suspicion of CG must be done as soon as possible eliminating animal milks and dairy products from the diet, given that they are rich in lactose and Gal as they contained between 2000 and 2500 mg of Gal/100 mL. Treatment is

recommended even before the diagnosis is confirmed, because the enzymatic activity of GALT is not altered by nutritional restriction. Watching a GALT activity of 10% or lower in RBCs and/or the presence of pathological variants in both alleles (inclusive with the p.S135L mutation) is enough to begin treatment. There is no scientific evidence showing that individuals with GALT enzymatic activities of 10%–15% are required to be treated (Welling et al., 2017a).

As an initial measure, it is recommended to immediately suspend breastfeeding, since human milk holds between 6% and 8% lactose, and the intake of any infant formula containing animal proteins. Replacements using either casein hydrolyzates based on soy or elemental formulas containing mixtures of L-amino acids are suggested. Dietary treatments are based on the formulas of milk from isolated soy protein (<1 mg of Gal/100 mL) or powdered whole soy hydrolysates (<5 mg of Gal/100 mL). A greater benefit has been seen when these soy-based formulas were supplemented with medium-chain fatty acids, especially in cases of certain hepatic alterations (Welling et al., 2017a).

Cow's milk casein hydrolysates containing traces of residual lactose (<10 mg of lactose/100 mL) can also be used. However, hydrolysates prepared from buttermilk or whey contained a higher amount of lactose, and as such, their use is not recommended. Lactose-free milk is also contraindicated in CG given that the original lactose is just hydrolyzed to Gal and Glc, and considerable amounts of Gal remained in the final product (Kim et al., 2007). The GC diet reverses the acute clinical symptoms, corrects jaundice, and normalizes the renal and hepatic functions.

The first clinical guide for monitoring patients with CG was published in 2016 (Welling et al., 2017a). Experts reached a consensus, and on the basis of all scientific evidences, they recommended the consumption of any kind of fruits, vegetables, and legumes (Table 3). In most of these foods, the amount of free Gal is less than 50 mg per portion. A report has demonstrated that an adult consuming a regular diet ingested less than 54 mg of Gal per day, which is negligible when compared with the amount of Gal that is endogenously synthesized (Van Calcar et al., 2014a). It has been estimated that a newborn synthesized more than 24.8 mg of Gal/kg/day, and Gal synthesis decreases with age to 8.4 mg of Gal/kg/day. A study by Huidekoper et al. (2005) using patients with CG together with control subjects concluded that the rate of appearance of Gal is not influenced by the intake of exogenous Gal, at least under short-term conditions.

It is acceptable to consume unfermented soy products, hard chesses with <25 mg of Gal/100 g, and foods containing sodium or calcium caseinates as additives. Some hard cheeses, such as Gouda, Emmental, Parmesan, and Gruyere, do not contain Gal due to their fermentation process, being excellent sources of elemental calcium. According to the latest clinical guide, experts recommend the consumption of small amounts of Gal contained in mature cheeses and caseinates, given that some studies found some association between rigorous restriction of nondairy Gal and deleterious outcomes (Frederick et al., 2017; Rubio-Gozalbo et al., 2019). Although the Gal content in fermented soy products is high, its consumption is eventually allowed but in small amounts (Welling et al., 2017a). A study demonstrated that there is a low input of free Gal in chickpea (24.6 mg/100 g) and black beans (5.3 mg/100 g) and it was undetected in pinto or red beans (Van Calcar et al., 2014b).

Lifelong dietary restriction of Gal is required since Gal is transformed to galactitol, and the risk of developing cataracts and/or renal damage is possible. The monitoring of treatment is made by measuring erythrocyte Gal-1-P concentration and urinary galactitol (Berry et al., 2000).

TABLE 3 Allowed and forbidden foods in CG

Allowed foods in CG	Forbidden foods in CG
Milks: Soy protein–based infant formulas (Isomil 1, Enfamil ProSobee, Pregestimil, and Nestlé Nan soya), powdered or liquid soy milk, cream substitutes lacking dairy products, only soy protein products	Breast milk, all milks from animal origin (whole, evaporated, condensed, powdered, liquid, pasteurized, skim, and semiskim). All dairy products including whey solids, casein, powdered milk protein, powdered milk solids, hydrolyzed whey protein, hydrolyzed casein protein, lactose, lactalbumin, buttermilk
Nonfermented soybean products (soy milk, tofu, textured soy protein or soy meat, hydrolyzed vegetable protein, soy protein concentrate, soy flour, and meat analogs), soybean sprouts, soy oil, and tofu	Lactose-free milk and its subproducts
Elemental infant formulas made of L-amino acids	Fermented products include tempeh, natto, miso, and shoyu (Japanese soy sauce)
Meat and fish: Cow, chicken, turkey, fish, lamb, pig, etc.	Breaded meats; canned fish are containing animal milk protein hydrolyzate; viscera; and entrails (liver, kidneys, pancreas, heart, and guts)
Cheeses and others: Jarlsberg, Emmental, Swiss, Gruyere, Tilsiter, mature Parmesan, hard cheddar, etc.	All soft cheeses, fresh cheese, pudding, custard, cured cheeses, cottage cheese, sour cream All preparations carried out with these cheeses
Eggs prepared without any of the forbidden foods	Omelets, soufflés prepared with milk
Cereals: Barley, wheat, oats, rye. Bread and homemade cookies prepared without milk or dairy products, sweet corn, rice, noodles, pasta, spaghetti, Mexican tortillas, popcorn without butter	Bakery products containing milk, instant cream, French toast prepared with milk
Fruits/juices: All	Those prepared with milk or dairy derivatives
Vegetables: All	Those prepared with milk or dairy derivatives, for example, French fries containing lactose as an additive
Seeds: All	Soy sauce fermented and tofu fermented
Snacks: Popcorn, soda cracker, salty cracker, pretzel; without milk or dairy products	All snacks containing dairy products
Sugars: Gummies, fruit jams, candies, maple syrup, NutraSweet, saccharine, sucralose, stevia, sugar cane, sugar beet	Artificial sweeteners are containing lactose, toffee or milk caramels
Fats: Bacon, milk-free margarines, mayonnaise, vegetable oils	Butter, cream, sour cream, all kind of powdered, thick or liquid creams made using animal milk
Desserts: Jelly, water ice creams/ice pops of permitted fruits, desserts prepared with soy milk, pudding and flans without milk	Desserts containing milk or dairy products, such as cakes, cookies, commercial pies, custards, ice cream, or pudding or flans prepared with milk
Beverages: Carbonated beverages, fruit juices (apple, pear, orange, etc.), tea, coffee, beer, wine, soy-based formulas	Beverages are containing milk (whole, evaporated, or condensed), hot chocolate, powdered juices containing lactose
Miscellaneous: Colored candies, pure spices, vegetable gummies, natural and artificial flavorings, all gums including carrageenans. Sodium and calcium caseinate	Chocolate with milk, mocha, medicines, vitamins, minerals, spices, or diet foods containing lactose as an excipient or milk Tagatose

The level of Gal-1-P in blood should be determined as a diagnostic tool in newborn babies up to an age of 9 months old and, then, once a year or when the amount of Gal is increased in the diet. There is no evidence of correlation between serial controls of Gal in blood and urine and a better long-term result.

A decrease in bone mineralization in galactosemic children due to ovarian shortage has been described, even after treatment (Schweitzer-Krantz, 2003). Although normal concentrations of calcium and vitamin D were determined in blood, this decline in bone mineralization might be due to an intrinsic defect in the galactosylation of collagen in the bone matrix (Potter et al., 2008). An annual evaluation of calcium and vitamin D intake is recommended, as well as a determination of both the 25-hydroxyvitamin D level in blood and the bone mineral density from the prepubertal stage on, in order to measure bone health. Based on these parameters the specific requirements for both nutrients, calcium and vitamin D, will be established according to the recommendations for the general population.

It is crucial to consider that lactose is used as an excipient in different medicines (drugs) and products, such as calcium lactobionate or neocalglucon, which are common calcium supplements (Hughes et al., 2009; Lebea and Pretorius, 2005). Parental education is directed toward a careful reading of all food and medicine labels; it is advised not to use prepared foodstuffs that contain milk solids, whey hydrolyzates, canned and preserved foods containing additives or preservatives, and food products of unknown composition, given that they may contain Gal.

A Gal-restricted diet contributes to approximately 40–60 mg/day of Gal, but the exact amount is unknown that should be ingested to avoid toxicity. It has not been identified neither the Gal endogenous synthesis rate in each patient nor the bioavailability of Gal from foodstuffs. It has been observed that the production of endogenous Gal diminishes with age, and it has been suggested that a higher tolerance to the Gal dietary consumption occurs in the adolescent and adult stages. These observations indicate that the tolerance to dietary Gal might increase when patients grow up, suggesting a reconsideration of long-life scheme therapy in classic galactosemia (Schadewaldt et al., 2014).

A free-Gal diet in a woman that is homozygous for *GALT* mutations is beneficial for the fetus during gestation, given that Gal and galactitol may cross the placenta, and galactitol could cause damage to the fetus crystalline (Holton, 1995; Schadewaldt et al., 2009; Elsas and Acosta, 1994).

In the DG form the diet should be absolutely normal, since all studies in treatment-free patients with this variant showed no clinical impairment when compared with individuals without DG. Requirements for proteins, calories, and liquids are based on the recommended dietary allowance for the normal population (Welling et al., 2017a). It is recommended in children following a Gal-restricted diet to carry out a Gal tolerance test at about 1 year old and to measure Gal-1-P levels in RBCs. If the Gal-1-P level is normal (<1.0 mg/dL), the restrictions of Gal in the diet can be suspended, but the diet must be maintained if it is above that value.

Glossary

ALG9 catalyzes two steps in the lipid-linked precursor oligosaccharide (LLO) in the N-linked pathway of glycosylation.

Animal proteins proteins of animal origin considered with a high biological value.

Animal milks milk from any mammal.

ANXA1 annexin A1 is a membrane-localized phospholipid-binding glycoprotein. It is involved in the cell-to-cell binding or to the extracellular matrix and has antiinflammatory activity. It is involved as a neuroprotective agent in the central nervous system, and it is a potential marker of bovine oocyte maturation.

AP-1 site nucleotide sequence (TGA(C/G)TCA) found in many promoters, which bind leucine zipper-type transcription factors like activator protein-1.

Bleeding diathesis an increased, genetic or acquired, susceptibility to bleeding due to a coagulation defect.

Caseinates soluble salts from casein, protein fraction of cow's milk.

Casein hydrolysates the hydrolyzed protein separated into its constituent parts: amino acids. Hydrolyzed proteins usually come from animal or plant sources.

Classic galactosemia it is the most severe form of presentation of the galactosemia disease.

Dairy products includes foods such as milk and its processed derivatives.

Duarte variant variant type of presentation of galactosemia.

D vitamin it is an essential liposoluble vitamin for maintaining the body mineral balance. D3 vitamin (cholecalciferol) is the most active form in humans, and it can be synthesized in the skin by exposing to ultraviolet B radiation.

E-box specific DNA sequence from eukaryotic promoters, which function like a transcriptional enhancer element.

Elemental formulas are milk formulas that contain all the amino acids in free form and have been supplemented with vitamins and minerals.

Encephalopathy is a general term describing a disease that affects the function or structure of the brain. It can present a very broad spectrum of symptoms that range from mild (memory loss or subtle personality changes) to severe (dementia, seizures, coma, or death), sometimes accompanied by physical manifestations.

Enzymatic activity mount of enzyme that transforms 1 mol of substrate per second.

Epigenetic refers to heritable changes in gene expression or phenotype, but do not involve changes in the actual DNA sequence itself but rather modifications in histones that comprise the chromatin and DNA methylation as well as an ever-expanding array of other epigenetic processes.

Epimutation (or epigenetic variant) a heritage change in the chemical structure of DNA that does not change the DNA coding sequence. It is associated with gain or loss of DNA methylation or other heritable modifications of chromatin. These changes may occur with age and exposure to environmental factors, such as diet, exercise, drugs, and chemicals.

Exon coding sections of an RNA transcript or the DNA encoding it that are translated into protein.

Extracellular synaptomatrix portion of the extracellular matrix that lies within the perisynaptic space.

Fermented soy products include tempeh, natto, miso, and shoyu (soy sauce), originally from Japan, which are highly nutritious.

Galactose monosaccharide formed by six carbon atoms or hexose, which is converted into glucose in the liver as an energy supply.

Galactitol a product obtained from the reduction of galactose.

Galactose-1-phosphate uridylyltransferase is an enzyme that catalyzes the second reaction of the Leloir pathway in the galactose metabolism.

Gene a region of deoxyribonucleic acid (DNA) coding either for the messenger RNA encoding the amino acid sequence in a polypeptide chain or for a functional RNA molecule.

Glycosylation is a very common modification where sugar is added to protein or lipids. This process initiates in the ER, and then a mature glycan is accomplished in the GA.

Hard chesses or mature cheeses are those that in their manufacturing process require more time and special care to obtain a unique product, which is why they do not contain galactose.

Hypogonadism the body's sex glands, testes in men and ovaries in woman, produce little or no hormones.

Hypergonadotropic hypogonadism is a condition where individuals show decreased activity of the gonads and retardation of sexual development, associated with high levels of gonadotropins.

Isolated soy protein storage protein contained in discrete particles called protein bodies, which are estimated to contain at least 75%–80% of the total protein of soy.

Jaundice yellowing pigmentation of the skin and mucous membranes caused by an increase in bilirubin in the blood as a result of certain disorders in liver, red blood cells, gallbladder, or pancreas.

Lactose is a disaccharide compound by glucose and galactose, present in milk. It is found in a proportion between 4% and 5% in the milk of mammal females.

Lactobionate calcium salt used as a calcium supplement.

Linkage disequilibrium where alleles (DNA markers) occur together more often than can be accounted for by chance because of their physical proximity on a chromosome.

Medium-chain fatty acids are esters of medium-chain fatty acids (between 8 and 12 carbon atoms) and glycerol.

Missense mutation a genetic alteration in which a single base pair substitution alters the genetic code in a way that produces an amino acid that is different from the usual amino acid at that position. Some missense variants (or mutations) will alter the function of the protein.

mRNA is a subtype of RNA, which carries a portion of the DNA code to other parts of the cell for processing. mRNA is created during the transcription process, where a single strand of DNA is decoded by RNA polymerase, and mRNA is synthesized.

Mutation a change in the usual DNA sequence at a particular gene locus. Although the term often has a negative connotation, mutations (including polymorphisms) can be harmful, beneficial, or neutral in their effect on cell function. The term variant is sometimes used as a synonym for the term mutation.

Neocalglucon is a drug used to prevent or treat low blood calcium levels in people who do not get enough calcium from their diets.

Perisynaptic space the extracellular region immediately adjacent to a synapse.

Phosphoinositide 3 kinase/protein kinase B signaling pathway is an intracellular signal transduction pathway, where phosphatidylinositol 3-kinase and protein kinase B are involved, resulting in the promotion of metabolism, proliferation, cell survival, growth, and angiogenesis.

Polymorphism a common mutation. "Common" is typically defined as an allele frequency of at least 1%. All genes occur in pairs, except when X and Y chromosomes are paired in males; thus a polymorphism with an allele frequency of 1% would be found in about 2% of the population, with most carriers having one copy of the polymorphism and one copy of the normal allele.

Single-nucleotide variants DNA sequence variations that occur when a single nucleotide (adenine, thymine, cytosine, or guanine) in the genome sequence is altered, usually present in at least 1% of the population. Also called SNP.

Sp1 site DNA sequence to which bind a zinc finger transcription factor, specific protein 1, with the consensus sequence 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′.

Soy is a species of the legume family (Fabaceae), cultivated for its seeds.

Synaptic cleft the narrow, fluid-filled space between the plasma membranes of the presynaptic and postsynaptic neurons or between a neuron and a muscle or gland cell across which neurotransmitters diffuse to convey a nerve impulse from one neuron to another or to promote contraction or secretion.

Unfermented soy products includes soybeans, bean sprouts (dragon teeth), soy milk, soybean meal, soybean oil, and tofu.

Unfolded Protein Response is an integrated intracellular signaling pathway that transmits information about the protein folding status in the ER lumen to the cytoplasm and the nucleus, provide adaptive responses for survival. If the protein folding is not corrected, cells undergo apoptosis.

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CHAPTER

23

Glucose and connections with *OLR1* and *IL17A* genes

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OUTLINE

SUMMARY POINTS

- This chapter focuses on the roles of the oxidized LDL receptor (OLR1) and interleukin 17A (IL17A) in diseases related with glucose metabolism.
- Oxidized LDL (ox-LDL) has a critical role in the development of vascular diseases.
- OLR1 is a scavenger receptor and has a proinflammatory role in arterial walls, and its expression is induced by inflammation and oxidative stress.
- OLR1 gene has been implicated in obesity, lipid metabolism, and type 2 diabetes.
- Recently, OLR1 mRNA expression was reported to be increased in type 2 diabetes and positively correlated with the levels of fasting glucose and triglycerides.
- IL17A is a proinflammatory cytokine and has various roles in glucose metabolism disorders.

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- Recently, it has been shown that high glucose induces lymphocyte activation and proinflammatory cytokines including IL17A.
- Besides, in a recent study, LDL oxidation was reported to upregulate IL17 receptors in human primary aortic cells.
- So that ox-LDL triggers the proinflammatory pathways via IL17 receptors.
- Therefore it is necessary to make further research on OLR1 and IL17A genes that will shed light on the molecular pathogenesis of glucose metabolism disorders.
- The accumulated knowledge of OLR1 and IL17A genes, ox-LDL, and their complex relationship with each other can contribute to new drug development efforts.

Key facts of oxidized low-density lipoprotein and interleukins

- Oxidized low-density lipoprotein (ox-LDL) was first found in atherosclerotic lesions of human and rabbits.
- Lectin-like ox-LDL receptor 1 (OLR1) is the receptor of ox-LDL and has a role in ox-LDL uptake by the arterial walls.
- Atherosclerosis is a cardiovascular disease, and it is characterized by inflammation, and plaques are build up within the vessel walls in this disease forming the atherosclerotic lesions.
- Inflammation is a response of immune cells and blood vessels when the tissues are stimulated by bacteria, injured cells, trauma, heat, toxins, etc. Chronic inflammation may lead to some vascular diseases like diabetes and atherosclerosis.
- LDL molecule is oxidized because of inflammation, and besides, when proteins or lipids are exposed to sugars, they become glycated.
- The accumulation of ox-LDL and glycated end products increases the inflammation and makes a basis for some diseases including diabetes and atherosclerosis.
- Interleukins (ILs) were first observed to be secreted from the white blood cells.
- Interleukins are secreted protein molecules made by the immune system and have many functions such as the development of T and B lymphocytes and other blood cells.
- Interleukins are composed of four major groups with different structural features.
- Interleukin 17 (IL17) is a proinflammatory cytokine produced by T helper 17 cells and increases the expression of inflammatory cytokines.
- Both ox-LDL receptor and IL17 are observed to have roles in glucose metabolism and related disorders.

Introduction

Lectin-like oxidized low-density lipoprotein receptor-1 (OLR1) is one of the scavenger receptors for the ligand oxidized LDL (ox-LDL) and has a major role in ox-LDL uptake by the arterial wall cells. Once ox-LDL is taken by the arterial cells, oxidative stress (OS) and inflammation signaling pathways are triggered. When OLR1 is activated, native LDL cholesterol particles are further oxidized.

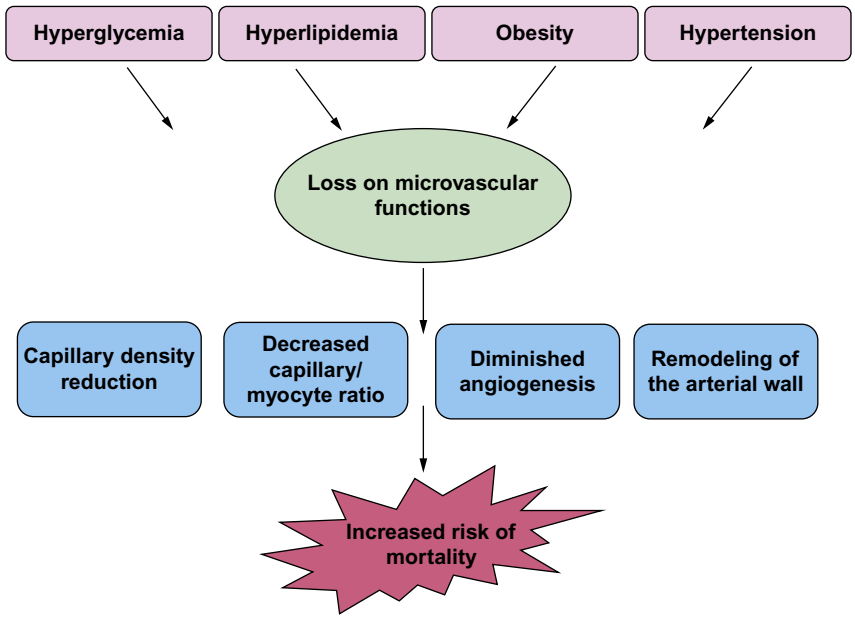

FIG. 1 Association of metabolic diseases with vascular anomalies. *Modified from Lubrano, V., Balzan, S., 2016. Roles of LOX-1 in microvascular dysfunction. Microvasc. Res. 105, 132–140.*

Chronic diseases such as hypertension, type 2 diabetes, and atherosclerotic arterial diseases display microvascular complications caused by inflammation and OS. These microcirculatory dysfunctions develop as a result of the interactions between LDL lipoproteins and the endothelium. Oxidized LDL and advanced glycosylation end products (AGEs) increase the expression of *OLR1*, which upregulate inflammatory cell aggregation and adhesion in the endothelium (Ge et al., 2005; Shiu et al., 2012). LDL cholesterol is modified by OS that play a major role in hypertension, blood glucose regulation, and atherogenesis (Lubrano and Balzan, 2016) (Fig. 1). OS also cause endothelial cell apoptosis through endothelial nitric oxide synthase (eNOS) synthesis (Edirisinghe et al., 2008). Antiapoptotic protein, for example, beclin-2, is decreased, and proapoptotic pathways such as caspase-3 and caspase-9 are activated by OLR1-ox-LDL interaction.

IL17A is a proinflammatory cytokine expressed from Th17 cells and other cell types in the immune system. *IL17A* gene variants and its overexpression were reported to be associated with various vascular diseases (Sumarac-Dumanovic et al., 2009; Eljaafari et al., 2015; Arisawa et al., 2012; Kawaguchi et al., 2004; Espinoza et al., 2011). Carrying various *IL17A* variants and having defective IL17 signaling were associated with the disorders in glucose metabolism and related pathologies (Shin et al., 2009; Zúñiga et al., 2010; Harley et al., 2014; Nakamura et al., 2017).

Oxidized low-density lipoprotein receptor 1 (OLR1)

Oxidized low-density lipoprotein cholesterol (ox-LDL) has a critical role in the development of atherosclerosis. Oxidized LDL receptor 1 (OLR1), also known as lectin-like oxidized LDL receptor 1 (LOX-1), has a proinflammatory function in atherogenesis (Pothineni et al., 2017). OLR1 is a scavenger receptor for ox-LDL in the arterial walls induced by inflammation and OS pathways. OS causes peroxidation of lipids in the arteries, and thus aldehyde products are accumulated such as malondialdehyde.

OLR1, a 50 kDA of transmembrane glycoprotein, is a member of C-type lectin family and first identified in bovine aortic endothelial cells. OLR1 has been shown for the task of binding, internalization, and degradation of ox-LDL (Sawamura et al., 1997). Afterwards, it has been identified in other cell types, such as macrophages, platelets, smooth muscle cells (SMCs), endothelial cells, fibroblasts, and cardiomyocytes (Mehta et al., 2006). The primary expression in healthy conditions is low in various types of cells (Kataoka et al., 1999). However, its expression is stimulated by proinflammatory and prooxidative signals (Kume et al., 1998) and various metabolic pathologies such as hypertension (Nagase et al., 1997), atherosclerosis (Kume and Kita, 2001; Mitra et al., 2011), and cigarette smoking (Xu et al., 2013).

When induced with inflammation, OS, and shear stress, *OLR1* is overexpressed in the cells of atherosclerotic lesions (Kuge et al., 2008; Mehta et al., 2007). A significant reduction of inflammation and smaller atherosclerotic lesions were observed in *OLR1* knockout mice (Mehta et al., 2007).

The binding of ox-LDL with OLR1 raises the levels of intracellular ROS and superoxide anion, which interact with nitric oxide (NO). This reaction leads to diminished levels of NO. After that, peroxynitrite is produced, and increased free radical levels and hypoxia exist (Zeya et al., 2016). OLR1 and ox-LDL interaction activates arginase II. This activation leads to lower NOS levels in endothelial cells, which trigger endothelial dysfunction, the first sign of atherogenesis (Ryoo et al., 2011).

Recent studies demonstrated the relationship between ROS formation by OLR1 and mitochondrial DNA damage that increases the expression of NLR family pyrin domain containing 3 (NLRP3) inflammasome. NLRP3 inflammasome activation leads to the inflammatory cytokines like interleukins (Ding et al., 2014).

OLR1 also has a role in ox-LDL-induced VSMC proliferation and neointima remodeling. In a study, it has been shown that anti-LOX-1 antibody repressed OS, VSMC proliferation, and injury of the intima in response to inflammation (Hinagata et al., 2006). Cell culture studies also showed the association of ox-LDL and OLR1. In a study performed in human umbilical vein endothelial cells (HUVEC), ox-LDL stimulation activates OLR1 expression and increases the expression of adhesion molecules, tissue factors, inflammatory proteins, and also remodeling proteins such as MMP-2 and MMP-9. These remodeling proteins also stimulate angiogenesis (Sugimoto et al., 2009). Another study demonstrated the role of rapamycin, an antiproliferative drug, in atherosclerosis development. Rapamycin suppresses *OLR1* mRNA expression in HUVEC cells and reduces ox-LDL uptake since it inhibits NF-κB activation (Zhou et al., 2016a).

In a study the effects of the bariatric surgery on whole blood mRNA levels were investigated in obese subjects with type 2 diabetes. They reported that 204 transcripts of 200 unique genes were significantly changed after the surgery. One of them is *OLR1* gene previously

implicated in obesity, lipid metabolism, and type 2 diabetes and was significantly downregulated by 37% after the surgery (Berisha et al., 2011). In another study, serum OLR1 levels were increased in patients with type 2 diabetes than in controls (Tan et al., 2008).

OLR1 gene has various variants leading to genetic susceptibility to several vascular diseases including myocardial ischemia, atherosclerosis, and coronary artery disease. However, since these diseases are polygenic and multifactorial, studies have nonconsistent findings about disease genetic susceptibility.

OLR1 gene has six exons and five introns. The SNP identified on exon 4, G501C, is a missense mutation with substitution of lysine (K) to asparagine (N) (K167N) at the 167th amino acid location. This SNP may affect the receptor's ligand binding potential, since the amino acid residue 167 is placed on the C-terminal domain of OLR1.

Several studies reported an association between increased susceptibility with MI (Tatsuguchi et al., 2003), carotid intima-media thickness (Predazzi et al., 2012), and *OLR1* G501C variants. In a study of metaanalysis, CC genotype of SNP G501C has been reported to have a significant association with ischemic stroke (Au et al., 2015). Another study also demonstrated the relationship between 501C allele, serum OLR1 levels, and left ventricular hypertrophy in patients with essential hypertension (Xu et al., 2014). On the other hand, it has been shown that C (asparagine) allele has a lower affinity for binding and internalizing ox-LDL than G (Lysine) allele and plays a role in diminishing atherogenesis (Biocca et al., 2009). Other known SNPs are located on intron 4, intron 5, and 3′ untranslated region (UTR). The six SNPs located on these regions are in linkage disequilibrium (LD) that means they have close association with each other and cosegregate during meiosis (Pothineni et al., 2017) (Fig. 2).

A study demonstrated a strong relationship between the SNP 3′UTR C188T within the LD block on *OLR1* gene and MI (Mango et al., 2003). In this LD block, SNPs make the regulation of alternatively spliced variant of *OLR1* mRNA. Exon 5 is alternatively spliced, and so, *OLR1*

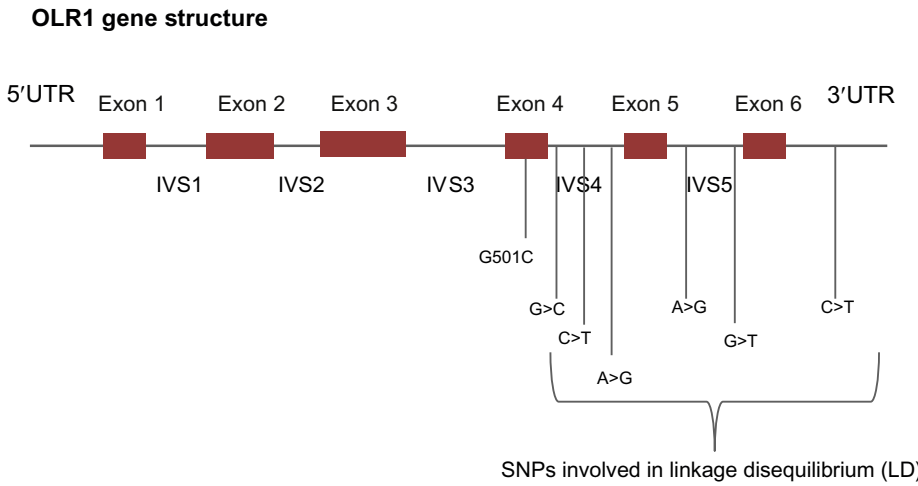

FIG. 2 *OLR1* gene structure and located SNPs. *Modified from Pothineni, N.V.K., Karathanasis, S.K., Ding, Z., Arulandu, A., Varughese, K.I., Mehta, J.L., 2017. LOX-1 in atherosclerosis and myocardial ischemia: biology, genetics, and modulation. J. Am. Coll. Cardiol. 69 (22), 2759–2768.*

mRNA is modulated. As a result, OLR1 isoform is produced by the exclusion of exon 5 and does not have C-terminus lectin-like domain involved in ligand binding. This form of OLR1 was termed as LOXIN. LOXIN heterooligomers form nonfunctional receptors that prevent OLR1 from binding and responding to ox-LDL (Biocca et al., 2008). So, LOXIN has a dominant-negative role to suppress proatherogenic formations (Pothineni et al., 2017). 3′UTR C188T SNP was reported to be correlated with decreased *LOXIN* mRNA levels and mature protein (Mango et al., 2003). Another study showed a relation between C188T variation and atherosclerotic cerebral infarction (Guo et al., 2017). A metaanalysis also showed that C188T variation was related with increased risk of MI (Feng et al., 2015).

In recent years, noncoding RNAs have also been shown to play a role in endothelial cell function and atherosclerosis. Ox-LDL has been shown to affect the expression levels of some miRNAs. For instance, *OLR1* upregulated microRNA-365, however, downregulated let-7g. The regulation of these microRNAs involves in the reduction of inflammation and apoptosis and inhibits senescence in endothelial cells (Araldi and Suárez, 2016).

Interleukin 17A (IL17A)

IL17 is a proinflammatory cytokine produced by a novel T helper subset, Th17 cells, and has six isoforms from A to F (Tesmer et al., 2008). IL17A is the first described IL17 family member. IL17A and F have 50% homology. IL17 isoforms are secreted as homodimers and also as heterodimers like IL17A/F (Onishi and Gaffen, 2010; Reynolds et al., 2010). IL17A was reported to be more potent than IL17F. The heterodimer form, IL17A/F, has intermediate activity (Gaffen, 2009). IL17B, IL17C, and IL17D are also members of proinflammatory cytokines. However, their roles are not thoroughly known (Reynolds et al., 2010). IL17R was identified as a new cytokine receptor (Yao et al., 1995) and has been investigated that it has five subunits. All receptor subunits have single transmembrane domain (Gaffen, 2011).

IL17A is a broadly studied cytokine belonging IL17 protein family. IL17 family members have a conserved C-terminal segment and contain a cysteine-knot fold structure (Weaver et al., 2007). IL17A has a significant role in host defense and tissue inflammation. IL17A and IL17F are close relatives with each other, and they are the ligands of the same receptor complex, IL17R. IL17R has IL17RA and IL17RC subunits (Fig. 3). Recent years, molecular targeting strategies are being under development for drug discovery using IL17A and IL17A producing cells for the inflammatory and autoimmune diseases (Chen and Kolls, 2017).

IL17A is glycoprotein and can either form a homodimer or heterodimer with IL17F. IL17A activates NF-κB and mitogen-activated protein kinases. IL17A can induce the expression of IL-6 and cyclooxygenase-2 and also nitric oxide (NO) production. When IL17A expression is upregulated, some chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and multiple sclerosis can be developed (https://www.ncbi.nlm.nih.gov/gene/3605 (Accessed 12.10.18)).

IL17A gene consists of three exons and spans 4252 base pairs (Fig. 4). IL17A protein consists of 155 amino acids. It has a 23 amino acid signal peptide and 132 amino acid mature peptides. IL17A is a secreted protein, and IL17A homodimer is 35 kD (Kolls and Lindén, 2004; http://atlasgeneticsoncology.org/Genes/IL17AID40945ch6p12.html (Accessed 12.10.18)).

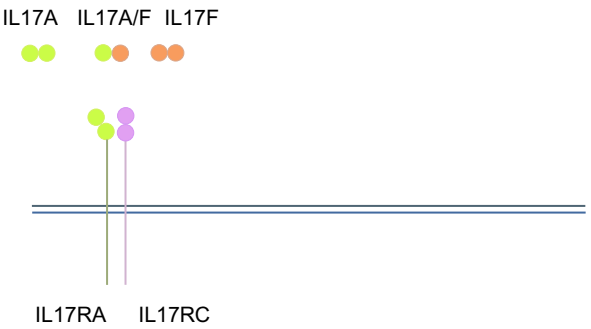

FIG. 3 Structure of IL17R. *Modified from Beringer, A., Noack, M., Miossec, P., 2016. IL-17 in chronic inflammation: from discovery to targeting. Trends Mol. Med. 22 (3), 230–241.*

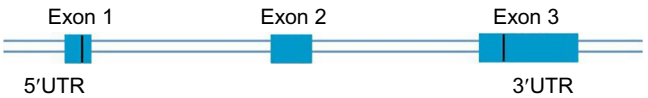

FIG. 4 *IL17A* gene structure. *Modified from http://atlasgeneticsoncology.org.*

Glucose and its association with OLR1 and IL17A

Hyperglycemia induces ROS that promotes atherogenic activities in the vessel wall. Thus high blood glucose triggers vasculopathies. Type 2 diabetes is a type of a vascular disease and is one of the factors that trigger atherogenesis. Diabetes mellitus is characterized by high blood glucose levels because of insulin nonsecretion or sensitivity leading to carbohydrate, lipid, and protein metabolism defects (Chen et al., 2015).

OLR1 is expressed in various cell types. OLR1 receptor not only does bind to ox-LDL but also can bind to different ligands. Advanced glycation end products (AGEs) are the ligands of OLR1 as well (Jono et al., 2002) involved in endothelial cell dysfunction and monocyte adhesion (Li and Mehta, 2000; Hayashida et al., 2002). Besides, 15-lipoxygenase-modified HDL (Pirillo et al., 2012) and glycoxidized LDL (Shiu et al., 2009) are other reported ligands for OLR1. *OLR1* expression does stimulated not only by ox-LDL but also by inflammatory cytokines, OS, vasoconstrictive peptides, and shear stress (Mehta et al., 2006; Morawietz et al., 2002). Li et al. demonstrated that high glucose induced human monocyte adhesion to endothelium via OLR1-dependent signals. Thus high glucose triggered *OLR1* expression in endothelial cells, which stimulated elevated OS and NF-κB, PKC, MAPK transcription (Li et al., 2003). Recently, Zhou et al. explored the effects of the relationship between high glucose levels on *OLR1* expression and ox-LDL–induced apoptosis in human renal proximal tubular epithelial cells. They reported that high glucose levels upregulated *OLR1* expression leading to elevated ox-LDL binding to the renal epithelial cells. Furthermore, they concluded that high glucose concentration increased *OLR1* promoter activity and ox-LDL-induced apoptosis in renal epithelial cells (Zhou et al., 2016b).

On the other hand, SNPs carried on *OLR1* receptor gene may cause various disease susceptibilities. A study demonstrated a positive relation between *OLR1* intron 4, GG genotype

of IVS4-14 A > G substitution and microalbuminuria, and fasting glycemia. They reported an association between the SNP IVS4-14 A > G and higher type 2 diabetes frequency, disrupted glucose metabolism, elevated insulin level, elevated microalbuminuria, fasting serum glucose, HOMA index, and also increased lipid peroxidation in patients with metabolic syndrome (Palmieri et al., 2013). In another study performed by Arslan et al., *OLR1* and *IL17A* mRNA levels were upregulated in femoropopliteal artery disease patients than in healthy controls. And also, increased plasma levels of IL4, IL6, IL10, IL22, IL31, and IL33 were reported. Besides, *OLR1* gene expression was positively correlated with the levels of fasting glucose and triglycerides regarding its effect on glucose metabolism (Arslan et al., 2017).

Musso et al. studied the functional effects of *OLR1* SNP IVS4-14 A > G in nonalcoholic steatohepatitis and reported the association of *OLR1* polymorphism with liver disease severity and predispose to CVD. In their study, G allele carriers demonstrated more severe liver disease during oral fat load test, lower total antioxidant status, higher resistin and lower adiponectin levels, and also postprandial small triglyceride-rich lipoprotein accumulation. In addition, G allele was independently related with insulin resistance, impaired β-cell function, and incretin effect in the course of OGGT (Musso et al., 2011). Rasouli et al. investigated the relationship between scavenger receptors in adipose tissue and insulin resistance in nondiabetic subjects and in vitro. They reported that adipose tissue scavenger receptors—scavenger receptor-A (SRA), CD36, and OLR1—were strongly associated with insulin resistance. They also demonstrated that pioglitazone, a drug with antidiabetic properties, and also adiponectin regulate *SRA* and *OLR1* gene expression (Rasouli et al., 2009).

Lu et al. reported that high glucose could increase the expression of scavenger receptors SR-A, CD36, and OLR1 in dendritic cells (DCs) responsible for ox-LDL uptake. Also, high glucose induced proinflammatory cytokines in human DCs causing DC maturation. They supported the hypothesis that hyperglycemia-induced DC activation and ox-LDL uptake worsen atherosclerosis (Lu et al., 2013).

Several studies have demonstrated the relationship between IL17A and glucose metabolism disorders. High glucose-induced inflammatory gene profiling was studied in lymphocytes and showed that 64 proinflammatory genes including *IL17A* and *IL-6* were significantly upregulated through essential signaling pathways causing lymphocyte activation (Kumar et al., 2014). IL17A levels were also reported to be increased in peripheral blood and adipose tissue in obese patients (Sumarac-Dumanovic et al., 2009; Eljaafari et al., 2015).

Various cytokine levels were reported to be increased in type 2 diabetes and obesity (Donath and Shoelson, 2011; Lafontan, 2014). These disease conditions are associated with chronic inflammation. In a study, it has been reported that IL17 produced by Th17 cells has been involved in glucose metabolism and diabetes pathogenesis through triggering low-grade inflammation (Singh et al., 2013). *IL17* gene has some variants that regulate *IL17* mRNA expression and IL17 synthesis (Arisawa et al., 2012; Kawaguchi et al., 2004; Espinoza et al., 2011). In a study, *IL17F* rs76780 variants were associated with posttransplant diabetes (Romanowski et al., 2015). Besides, Treg, Th1, and Th17 cells have significant roles in fatty liver disease formation (Vonghia et al., 2013). Th17 cells secrete miscellaneous cytokines like IL17, IL-21, IL-22, and TNF-α, which induce tissue inflammation (Ouyang et al., 2008). In a study, it has been reported that IL17A aggravated diabetic retinopathy-like pathology in rodents partly by damaging the function of retinal Müller cells, and it was suggested that blocking IL17A may be a useful therapeutic approach for diabetic retinopathy (Qiu et al.,

2017). Besides, in vivo studies performed in IL17 signaling defective mice, elevated adiposity, and hepatic steatosis were reported (Zúñiga et al., 2010; Harley et al., 2014). Furthermore a case study reported that IL17A blockade caused weight gain with fatty liver and worse getting diabetes (Nakamura et al., 2017). Another study suggested that IL17A inhibited adipocyte differentiation (Shin et al., 2009).

Concluding remarks

In recent years, new therapeutic strategies are being developed alongside pharmacological therapies to prevent microvascular damage formed by atherosclerosis and type 2 diabetes. OLR1 is a scavenger receptor and plays a major role in the formation of endothelial dysfunction and progression of vascular diseases. So the regulation of OLR1 production and the prevention of its interaction with ox-LDL are among the new treatment strategies of vascular diseases. For instance, using a neutralizing antibody, anti-LOX-1, diminished the area of infarction, brain edema, and apoptosis (Akamatsu et al., 2014). Several drugs have been shown to inhibit *OLR1* expression and activity in the vasculature including statins, antihypertensive drugs, antiinflammatory agents, antioxidants, and antihyperglycemic drugs (Lubrano and Balzan, 2016). On the other hand, genetic studies showed that an allelic variant lacking of the functional domain of the receptor expressed in exon 5 of the gene reduces the plasma membrane localization and ox-LDL ligand binding. This variant has been shown to inhibit atherogenesis induced by *OLR1* overexpression in an in vivo model (White et al., 2011). When *OLR1* and its variant were coexpressed, they interact with each other and form a multimeric protein complex that leads to diminish surface OLR1 receptors and disrupt ox-LDL binding and uptake (Biocca et al., 2008). Thus OLR1 is a promising strategic target for the molecular treatment of atherosclerosis and its vascular complications. To date, no clinical evidence for using antibodies against OLR1 has been shown. *OLR1* expression and signaling are affected by ACE inhibitors, AT1 antagonists, and calcium channel blockers. Naturally occurring plant compounds also affect OLR1 with their antioxidant properties. Healthy nutritional diet using these plant compounds having antioxidant features can ameliorate damaged endothelial layer (Hofmann et al., 2017). IL17A has also significant roles as a proinflammatory cytokine in glucose metabolism. Nevertheless, LDL oxidation was reported to upregulate IL17 receptors in human primary aortic cells (Mai et al., 2016). Thus ox-LDL triggers the proinflammatory pathways via IL17 receptors. In future, further approaches should be developed in drug discovery investigations regarding the relationship between ox-LDL, OLR1, and IL17.

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Further reading

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Index

Note: Page numbers followed by *f* indicate figures and *t* indicate tables.

D

E

F

G

H

I

J

K

Q

R

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