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Byung Pal Yu *Editor*

Nutrition, Exercise and Epigenetics: Ageing Interventions

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Byung Pal Yu
Editor

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Editor
Byung Pal Yu
Physiology
University of Texas Health Science Center
San Antonio, TX
USA

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Preface

Achieving healthy longevity is an innate desire of humans and the ultimate goal of aging research endeavors. Aging intervention, popularly called "anti-aging" refers to slowing down the progress of aging and the accompanying disease processes. Many modern antiaging studies have attempted to uncover clues into the underlying mechanisms of aging or a means by which to manipulate genes and gene regulation of experimental organisms in effort to modulate the aging process.

The past several decades' work has made clear that searches for any genetic or gene manipulation or for aging genes, in particular, have produced disappointing results. This failure is neither unexpected nor surprising in view of our limited understanding of the precise functional genomic involvement in aging processes.

Investigations of various other means of aging interventions, like dietary supplements, antioxidants, hormones, and pharmacologic agents, have also produced only limited and discouraging outcomes. In most cases, the efficacy of these interventions was shown mainly in disease incidence, not necessarily on the aging process itself. As we all are aware by now, the most effective aging intervention requires both the retardation of the aging process and the suppression of accompanying diseases, as has been proven by epigenetic calorie restriction (CR) and physical exercise. One intriguing aspect yet to be answered about these two paradigms is their similar efficiencies, despite their vastly different modus operandi. Discernible answers are likely to come from epigenetic analysis showing age-related modifications to histone, chromatin, and chromosomes, all which are the targets of differentially modifying calorie restriction or by physical exercise.

The major thrust of this book is to expose epigenetic modifications of the aging process that can be attributed to two well-established antiaging modifiers, CR, and physical exercise. At present, no other book covering similar topics is available as a resource book. The majority of the book's 11 chapters discusses how age-related epigenetic imprints such as DNA methylation and histone acetylation are modified by these two interventions. Chapter topics were selected to provide the reader not only insightful mechanistic clues into the ability of CR and exercise to exert beneficial effects in specific pathophyological systems, but also to offer information

on salient aging research topics, including nutritional epigenetics, chronic inflammation, CR mimetics, and nonhuman primate CR studies.

For the completion of this book, I want express my personal thanks to all the chapter contributors who spent substantial effort and their valuable time to make this publication possible. I am also thankful to Dr. Suresh Rattan, Editor-in-Chief, *Healthy Ageing and Longevity* Book series, who invited me to be the editor of this book. Finally, my thanks go out to Ms. Corinne Price who helped me with her excellent editorial assistance.

One remarkable possibility for the future of epigenetic aging intervention is that modified histone imprints could become inheritable by passing onto following generations through the transgenerational inheritance process. Advancement of our knowledge on transgenerational epigenetic inheritance raises hope for new opportunities in achieving a healthy aging status for future generations without further interventions.

Byung Pal Yu

Contents

Chapter 1
Nutritional Epigenetics and Aging

Kyong Chol Kim and Sang-Woon Choi

Abstract Epigenetics refers to an inheritable but reversible phenomenon that changes gene expression without altering the underlying DNA sequence. Thus, it is a change in phenotype without a change in genotype. The field of epigenetics is quickly growing especially because environmental and lifestyle factors can epigenetically interact with genes and determine an individual's susceptibility to disease. Interestingly, aging is associated with substantial changes in epigenetic phenomena. Aging induces global DNA hypomethylation and gene-specific DNA hypermethylation due to the altered expression of DNA methyltransferases (*DNMTs*). Histone acetylation can also be changed by age associated imbalance of histone acetyltransferases (HATs) and histone deacetylases (HDACs). It is also known that the profile of microRNA expression changes with age. However, it is not yet clear whether these epigenetic changes are genetically preprogrammed or just randomly acquired due to various environmental and lifestyle factors. Whatever the answer is, it is clear that epigenetic alterations caused by aging may provide a milieu that can develop age-associated diseases such as cancer, cardiovascular diseases, neurocognitive diseases and metabolic diseases. Nutrition is one of the most important environmental factors that can modify epigenetic phenomena. Therefore, one might speculate that nutrition may delay the age-associated epigenetic change and possibly reverse the aberrant epigenetic phenomena that can cause age-associated diseases. Indeed, many nutrients and bioactive food components, which can affect one-carbon metabolism that can regulate methylation of DNA and histone or directly inhibit epigenetic modifying enzymes, are showing promising results in delaying the aging process and preventing age-associated diseases through epigenetic mechanisms.

K.C. Kim · S.-W. Choi (✉)
Chaum Life Center, CHA University School of Medicine, 442, Dosan-daero, Gangnam-gu, Seoul 135-948, Korea
e-mail: sang.choi@cha.ac.kr

K.C. Kim
e-mail: joyks71@chamc.co.kr

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1.1 Introduction

Epigenetics is defined as an inheritable phenomenon that affects gene expression without altering DNA base pairs. Epigenetic phenomena include DNA methylation, histone modifications, chromatin remodeling and possibly microRNAs (Fig. 1.1). DNA methylation, the phenomenon that has been the most widely studied, is the addition of a methyl group (CH_3) to the carbon 5′ position of cytosine by DNMTs. Because DNA methylation is clustered at the CpG islands of gene promoters, it can affect transcriptional activity, which may in turn cause aberrant gene expression. Histone modification, another common epigenetic phenomenon, is the modification of histone tails, such as acetylation, methylation, phosphorylation, ubiquitination, and biotinylation. Recently, microRNAs have also become considered an epigenetic phenomenon because of their effect on gene expression without altering DNA base sequence. MicroRNAs are non-coding RNAs that are around 20–24 nucleotide long and differ from messenger RNAs (mRNAs) that directly mediate the transcription of DNA into proteins.

During our lifetime, epigenetic phenomena alter the gene expression pattern under environmental influences, and may have an effect on the development of age-associated diseases. Since nutrients, bioactive food components and diet can influence epigenetic machineries, epigenetics is considered to be an important mechanism that can explain the role of nutrition in the aging process as well as the development of age-associated diseases. Indeed, many published studies have investigated whether individual nutrients and bioactive food components influence aging through epigenetic phenomena such as DNA methylation and various types

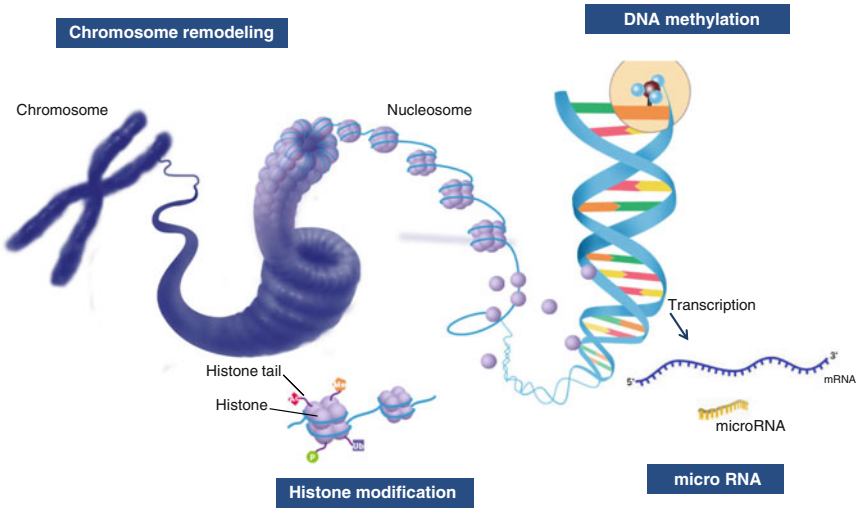

Fig. 1.1 Schematic view of epigenetic phenomena that include DNA methylation, histone modifications, chromatin remodeling and microRNA

of histone modifications. In this chapter, we address the fundamental epigenetic mechanisms by which nutrition and aging can influence long-term health. We also describe whether proper nutrition may delay the aging process and prevent age-associated diseases.

1.2 Individual Epigenetic Phenomena

1.2.1 DNA Methylation

DNA methylation is a major epigenetic phenomenon where a methyl group is added to the 5′-position of cytosine at the cytosine-guanine dinucleotide (CpG) residues, which then can interfere with the binding of transcription factors and RNA polymerases. CpG dinucleotides are clustered in the promoter region of human genes, called "CpG islands". These CpG islands are usually unmethylated in the germline and in most somatic tissues, allowing transcription factors to easily bind to the transcription sites. Methylation of the promoter CpG islands leads to the binding of methyl-CpG binding proteins (MBDs) and transcription repressors including HDACs. This results in the formation of a compact nucleosome structure that blocks the initiation of transcription.

In general, hypermethylation of the CpG islands represses a gene, while hypomethylation activates it. However, these actions are gene- and cell-specific. Depending on the gene and cell, the opposite effect may occur. In such a case, hypermethylation may up-regulate transcription and hypomethylation may down-regulate gene expression. DNMTs catalyze the addition of methyl groups to DNA. There are three members of the DNMT family: DNMT1, DNMT3a, and DNMT3b. DNMT1 is a maintenance DNMT that copies the methylation pattern of the original DNA strand onto the daughter strands after DNA replication and also has the ability to repair DNA methylation. DNMT3a and DNMT3b are known as de novo DNMTs because they can newly methylate cytosine. DNMT3b is required during early embryonic development, whereas DNMT3a is required for normal cellular differentiation. DNMTs catalyze the methylation reaction using the methyl donor cofactor S-adenosylmethionine. After donating the methyl group, S-adenosylmethionine turns into S-adenosylhomocysteine, which is an inhibitor of DNMTs. Compared to gene-specific DNA methylation, global hypomethylation may induce genomic instability, which may increase cancer risk.

Recently, 5-hydroxymethylcytosine was discovered as an intermediate form of an active demethylation process, in which the methyl group from 5-methylcytosineis removed, returning the methylated cytosine to its unmodified form. However, 5-hydroxymethylcytosine plays a different regulatory role in gene expression compared to 5-methylcytosine. It appears that the affinity of MBDs to 5-hydroxymethylcytosine is much less than that of 5-methylcytosine.

1.2.2 Histone Modifications

In eukaryotic cells, DNA is packaged into highly compact structures by supporting proteins called histones. Approximately 147 base pairs of DNA tightly wrap around an octamer of histones. This octamer unit comprises two sets of four core histones (H2A, H2B, H3, and H4) and forms a nucleosome, the fundamental unit of chromatin. Each nucleosome is connected with linker DNA and linker histone (H1). The compactness of the nucleosome determines whether it allows transcription factors to easily bind to DNA or blocks their binding. Each histone protein contains a structured core and an N-terminal tail domain, on which various posttranslational modifications can occur such as acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, and biotinylation. These modifications control gene expression by altering the ionic charge of the histone tail, modifying chromatin compactness, or by functioning as a binding platform for other proteins.

Histone acetylation is one of the most common histone modifications and occurs on lysine residues of the histone tails. The acetylation of histone tails alters the local packaging of DNA, leading to the loosening of the compact DNA structure. This in turn alters the level of gene expressions. Histone acetylation is a reversible process that is balanced by two different enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, HATs are transcriptional activators that neutralize the positive charge of one or more lysines on histone tails by transferring acetyl groups onto the lysine residues. In contrast, HDACs are transcriptional repressors that restore the positive charge on the histone tail by catalyzing the removal of acetyl groups from the lysine residues.

Histone methylation can occur on both lysine and arginine residues of histone tails. Methylation is controlled by enzymes, such as histone methyltransferases (HMTs) and histone demethylases (HDMs). HMTs use the unique methyl donor cofactor, S-adenosylmethionine, and are inhibited by S-adenosylhomocysteine. Histone ubiquitination, which regulates initiation and elongation of transcription, occurs on the C-terminal tails of two histones, H2A and H2B, especially at lysine 119 and lysine120. Histone phosphorylation commonly occurs on serine10 and 28 of histone H3, serine 1 of histone H4 and serine 1 of histone H2A. Poly-ADP ribosylation of histones is catalyzed by poly-ADP ribose polymerase (PARP). Histone biotinylation attaches a molecule of biotin, a water soluble B vitamin, to lysine residues through enzymes, biotinidase and holocarboxylase synthase.

1.2.3 MicroRNA

MicroRNAs are small non-coding single stranded RNA of approximately 18–25 nucleotides in length that play a critical role in cellular activities, including cell development, differentiation, proliferation and apoptosis. Ever since the first microRNA was discovered in 1993 in *Caenorhabditis elegans*, about 2,800 human

RNAs have been registered in the microRNA database (miRBase). In contrast to protein-coding RNAs, microRNAs, which represent a large portion of human transcripts and was previously considered as junk RNA, have no apparent protein product and instead play gene regulatory roles in higher eukaryotic organisms. Overall, microRNAs are known to regulate about 30 % of gene expression. They inhibit gene expression by directly binding to the 3′UTR target sequence of the mRNA or by assembling the RNA-induced silencing complex (RISC), a template for capturing the target mRNA and thereby facilitating mRNA degradation. A single microRNA may target multiple mRNAs: at the same time, a single mRNA may also be affected by multiple microRNAs. MicroRNA may be transcribed from their own gene, but certain microRNAs are encoded within the intron of protein coding genes and are transcribed along with the primary transcript. These primary transcripts are called primicroRNAs, and become processed into mature microRNAs.

Similar to that of mRNAs, the expression of microRNAs can also be regulated by other epigenetic phenomena. DNA hypermethylation of the CpG islands is a common mechanism by which the aberrant expression of microRNA occurs in human cancers. Histone modifications also control the expression of microRNA. Both trimethylation of histone H3 lysine 4 and dimethylation of histone H3 lysine 79 activate microRNA expression, whereas trimethylation of histone H3 lysine 27 represses microRNA expression in colorectal cancer. MicroRNAs themselves may also control these epigenetic phenomena. It controls DNA methylation and histone modifications by altering epigenetic modifiers, such as DNMTs, HATs and HDACs.

1.3 Epigenetic Changes with Age

Epigenetic phenomena have been regarded as a key mechanism underlying the progression of aging and development of age-associated diseases. With age, the total level of 5-methylcytosine in DNA gradually decreases, which leads to global hypomethylation in most vertebrate tissues and paradoxical hypermethylation in promoter regions in a gene-specific and tissue-specific manner. Several mechanisms have been suggested to explain the changes in global DNA methylation during aging: (1) a progressive decrease in the activity of DNMT1 may lead to global hypomethylation, and increased expression of *DNMT3a* and *3b* de novo methyltransferases may lead to promoter hypermethylation; (2) a decline of sex hormones during aging may also reduce global DNA methylation; (3) hyperhomocysteinemia, a condition associated with aging, may contribute to global hypomethylation because concurrent increase in the cellular S-adenosylhomocysteine inhibits the activity of DNMTs; and (4) altered dietary patterns may contributes to global DNA hypomethylation by changing one-carbon metabolism that controls S-adenosylmethionine and S-adenosylhomocysteine levels.

With age, the fine balance of enzyme activities between HATs and HDACs becomes impaired. The aberrant histone acetylation by aging can alter the gene expression profile and may increase the development of age-associated disease. It is worth noting that increased histone acetylation extends the lifespan of yeasts, which indicates that maintaining the optimal chromatin structure is critical for slowing down the aging process. It is also known that loss of certain HMTs and HDMs alters lifespan in invertebrates. In fact, histone hypomethylation may cause many aging phenotypes, such as insulin resistance. Another example of an age-associated imbalance between HATs and HDACs is the change in the levels of sirtuins (class III HDACs) with aging. The sirtuin (SIRT) family, which has 7 members (SIRT1-7) in mammals, is known to promote longevity in many organisms. Nicotinamide adenine dinucleotide (NAD+) is a cofactor of these enzymes. In yeast there is an age-associated decrease in the level of silent information regulator 2, whose homolog in mammals is SIRT1, and is accompanied by an increase in acetylation at histone H4 lysine 16, leading to transcriptional repression of genes.

MicroRNAs have recently emerged as important regulators of the aging process and cellular senescence. Bohem and Slack reported that microRNA of lin-4 regulates the lifespan of *C. elegans*. They found that reducing the activity of lin-4 shortened the lifespan of *C. elegans* and accelerated tissue aging, whereas over-expressing lin-4 or enhancing the activity of lin-14 extended the lifespan [1]. By comparing the tissue-specific expression of various microRNAs in young and aged mice, microRNAs that are either up- or down-regulated with aging were identified. In livers of aged rat miR-29a, miR-29c, miR-195 and miR-497 were up-regulated, whereas miR-301a, miR-148b-3p, miR-7a, miR-93, miR-106b, miR-185, miR-450a, miR-539 and miR-301b were down-regulated. It seems that these age-dependent changes in microRNA levels may play an important role in aging by regulating the progression of the cell cycle in liver senescence.

1.4 Nutritional Influence on Epigenetic Phenomena: Focused on Cancer

1.4.1 The Effect of Nutrients on DNA Methylation

1.4.1.1 One-Carbon Metabolism and DNA Methylation

Epigenetic phenomena can be modified by nutrients because certain nutrients act as methyl donors in one-carbon metabolism that produce S-adenosylmethionine, the universal methyl donor. In addition, some bioactive food components can directly inhibit enzymes that mediate DNA methylation and histone modifications (Table 1.1). In one-carbon metabolism folate, vitamin B-12, B-6, and B-2 act as coenzymes and methionine, choline, betaine, and serine are methyl donors (Fig. 1.2). Folate accepts a methyl group from serine, and then donates the methyl group to

Table 1.1 Nutrients and bioactive food components that may affect DNA methylation

Category	Nutrient	Epigenetic mechanism	Reference
One carbon nutrient	Folate	Methyl acceptor and donor	[2]
	Vitamin B-12	Coenzyme for MS	[4]
	Vitamin B-6	Coenzyme for SHMT and CBS	[6]
	Vitamin B-2	Coenzyme for MTHFR	[6]
	Methionine	Precursor of SAM	[7]
	Choline	Precursor of betaine, methyl donor	[5]
	Betaine	Homocysteine remethylation by BHMT	[5]
	Serine	Methyl donor to folate by SHMT	[6]
Micronutrient	Retinoic acid	Increases GNMT activity	[8]
	Zinc	Cofactor for DNMTs, MAT and BHMT	[9]
	Vitamin D	Passive demethylation, unknown mechanism	[10]
Bioactive food component	Genistein	Inhibition of DNMTs	[11, 12]
	Tea polyphenol	Inhibition of DNMTs	[14, 15]
	Raspberry	Inhibition of DNMT1, DNMT3B	[16]
	Sulforaphane	Inhibition of DNMT1, DNMT3B	[17, 18]

BHMT betaine homocysteine methyltransferase, *CBS* cystathionine ß-synthase, *GNMT* glysine *N*-methyltransferase, *MS* methionine synthase, *MTHFR* methylenetetrahydrofolate reductase, *MAT* methionine adenosyltransferase, *SHMT* serine hydroxymethyltransferase, *DNMT* DNA methyltransferase

homocysteine, which is then converted to methionine. Methionine, which is derived from either diet or homocysteine remethylation, is utilized to synthesize S-adenosylmethionine. After donating a methyl group to methylation reactions, Sadenosylmethionine is converted to S-adenosylhomocysteine, which is further converted to homocysteine. Importantly, S-adenosylhomocysteine has a high affinity for DNMTs and thereby acts as an inhibitor for S-adenosylmethionine dependent methyltransferases, including DNMTs and HMTs. An alternative mechanism for the regeneration of methionine is folate independent homocysteine remethylation by betaine, which is directly supplied from diet or converted from choline. Through the transsulfuration pathway, homocysteine can be converted to cystathionine in an irreversible reaction catalyzed by the vitamin B-6 dependent enzyme, cystathionine-ß-synthase (CBS). Cystathionine is further hydrolyzed to form cysteine through an enzymatic reaction that also requires vitamin B-6 as a cofactor. Because many metabolites in one-carbon metabolism can be derived from our diet, deficiency or supplementation of these nutrients may have the potential to alter DNA methylation by changing the availability of S-adenosylmethionine and S-adenosylhomocysteine. As a reciprocal reaction, one carbon metabolism also controls the biochemical pathways for DNA synthesis. 5,10-methylenetetrahydrofolate, an intracellular coenzymatic form of folate, is required for the conversion of deoxyuridylate to

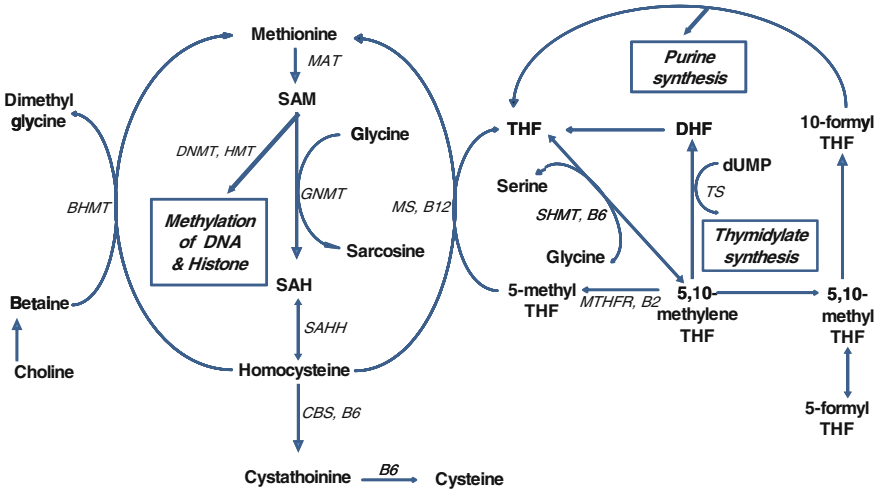

Fig. 1.2 One-carbon metabolism that regulates methylation of DNA and histone. *THF* tetrahydrofolate; *DNMT* DNA methyltransferase; *HMT* histone methyltransferase; *MTHFR* methylenetetrahydrofolate reductase; *MS* methionine synthase; *SHMT* serine hydroxymethyltransferase; *GNMT* glycine N-methyltransferase; *CBS* cystathionine-ß-synthase; *MAT* methionine adenosyltransferase; *SAHH* S-adenosylhomocysteine hydrolase; *BHMT* betaine homocysteine methyltransferase; *TS* thymidylate synthase; *SAM* S-adenosylmethionine; *SAH* Sadenosylhomocysteine; *DHF* dihydrofolate; *dUMP* deoxyuridine monophosphate

thymidylate and can be oxidized to 10-formyltetrahydrofolate for purine synthesis (Fig. 1.2). Thus, folate depletion may involve in DNA damage and repair that may also increase the risk of cancer by accelerating mutagenesis.

Folate, a water-soluble B vitamin, has been extensively studied for its association with DNA methylation. Animal studies have shown that reduced folate intake leads to global DNA hypomethylation, especially when aging is involved. On the other hand, dietary folate intake increases both global and *p16* promoter methylation levels in the colon mucosa of aged mice [2]. Folate metabolism is closely associated with fetal development and growth, and maternal folate deficiency can cause fetal neural tube defect. Based on the animal studies that demonstrated the importance of methyl availability during cranial neural tube closure, it has been hypothesized that inhibiting methyl transfer or reducing folate intake can increase the risk of human neural tube defects by reducing DNAmethylation. This hypothesis is supported by Waterland and Jirtle who demonstrated that the dietary methyl supplementation of *a/a* dams with extra folic acid, vitamin B-12, choline, and betaine altered the phenotype of their *Avy/a* offspring by increasing DNA methylation at each of the seven *Avy* pseudoexon 1A (*PS1A*) CpG sites [3]. It is also reported that periconceptional folic acid supplementation was associated with changes in methylation at the differentially methylated region of insulin like growth factor 2 (*IGF2*) in children between 12 and 18 months of age. *IGF2* is an imprinting

gene in which the methylated allele at the differentially methylated region is repressed.

Vitamin B-12 serves as a cofactor for methionine synthase that catalyzes the remethylation of homocysteine to form methionine. Deficiency of vitamin B-12 is known to decrease DNA methylation in the rodent colon [4]. Choline, a water-soluble essential nutrient, is also known to alter DNA methylation after being converted to betaine. Deficiency of choline can change DNA methylation independently or in conjunction with deficiency of other methyl donors. Indeed the availability of choline is essential for fetal neurogenesis, such as hippocampal development, and memory function throughout an organism's lifetime. In a transgenerational study, maternal choline deficiency during fetal development altered global and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains [5]. Similarly, prolonged intake of a diet deficient in choline, methionine, folate, and vitamin B-12 rapidly induced profound global hepatic DNA hypomethylation and subsequently developed liver cancer, suggesting the importance of DNA methylation status and methyl supply in cancer development [6]. However, only a few studies have demonstrated whether individual deficiency of methyl donor nutrients actually changes DNA methylation, indicating that single nutrient deficiency might not be strong enough to alter DNA methylation compared with the multiple deficiencies of methyl donor nutrients [7].

1.4.1.2 Other Micronutrients and DNA Methylation

Glycine *N*-methyltransferase (GNMT), which catalyzes the reaction from S-adenosylmethionine to S-adenosylhomocysteine, is an essential one-carbon metabolism enzyme for the optimal balance of methyl groups. *Gnmt* deficient mice can spontaneously develop primary liver cancer. In rats which were fed vitamin A and its derivatives, 13-cis- and all-trans-retinoic acids, for 10 days the expression of hepatic *Gnmt* was up-regulated with hypomethylation of hepatic DNA [8]. Zinc functions as a coenzyme for methionine adenosyltransferase (MAT), which catalyzes the conversion of methionine to S-adenosylmethionine. Wallwork and Duerre demonstrated that a deficiency of zinc reduced utilization of methyl groups from S-adenosylmethionine in rodent liver, resulting in global hypomethylation of both DNA and histone [9].

Nowadays the role of vitamin D in regulating gene expression is extremely important because vitamin D has a variety of biological functions other than calcium regulation. Even though it has been already suggested that vitamin D interacts with the epigenome on multiple levels, the actual mechanisms behind the effect of vitamin D on DNA methylation need to be clarified. A human study demonstrated that the intake of vitamin D was strongly associated with reduced methylation of dickkopf1 (*DKK1*) and wingless-type MMTV integration site family, member 5A (*WNT5A*) genes in colorectal cancer patients. It is also reported that 1,25-D3 treatment of the triple negative breast cancer cell line MDA-MB-231 resulted in the reduction of DNA methylation in the *E-cadherin* promoter [10].

1.4.1.3 Bioactive Food Components and DNA Methylation DNA Methylation

Genistein, a phytoestrogen found in soybean, is known to affect DNA methylation by inhibiting the DNMTs in various cancer cell lines. In mice, consumption of a genistein-rich diet was positively correlated with changes in gene-specific DNA methylation in their prostates. In KYSE 510 cells, a human esophageal squamous cell carcinoma cell line, genistein reversed DNA hypermethylation by inhibiting DNMTs, and activated repressed retinoic acid receptor beta (*RARβ*), *p16* and O-6methylguanine DNA methyltransferase (*MGMT*) genes in a dose-dependent manner [11]. In another study, treatment with genistein induced demethylation in the glutathione S-transferase pi 1 (*GSTP1*) and EPH receptor B2 (*EPHB2*) promoter regions, which resulted in the increment of their protein expression [12]. In vivo genistein treatment reduced the neuroblastoma tumor volume in a nude mice model. Genistein decreased the expression of DNMT3b which in turn reduced the hypermethylation level of chromodomain helicase DNA binding protein 5 (*CHD5*) and enhanced the expression of *CHD5* [13].

Among tea components, the polyphenol (–)-epigallocatechin-3-gallate (EGCG) is the most potent DNMT inhibitor. In a cultured cell study, Fang et al. demonstrated that EGCG inhibited DNMTs and activated epigenetically silenced genes [14]. Furthermore, each of the tea polyphenols (catechin, epicatechin, and EGCG) and bioflavonoids (quercetin, fisetin, and myricetin) inhibited DNA methylation in a concentration-dependent manner. In an in vivo study carried out by Mittal et al, the topical application of EGCG in a hydrophilic cream resulted in a significant inhibition of UVBinduced global DNA hypomethylation pattern in a SKH-1 hairless mouse model [15].

Black raspberry is well-known to have potent anti-cancer properties. In colorectal cancer patients, oral administration of black raspberry powder decreased promoter methylation of tumor suppressor genes, such as secreted frizzled-related protein 2 (*SFRP2*), *SFRP5*, and Wnt inhibitory factor 1 (*WIF1*). Black raspberry-derived anthocyanins induced hypomethylation of tumor suppressor genes through the inhibition of DNMT1 and DNMT3B in colon cancer cells [16]. Sulforaphane is an isothiocyanate derived from cruciferous vegetables, which demonstrated potent anti-proliferative effects in prostate cancer cells. Hsu et al. showed that sulforaphane also significantly decreased the expression of DNMTs, especially DNMT1 and DNMT3b. Additionally, sulforaphane significantly decreased methylation in the cyclin D2 promoter regions containing c-Myc and multiple Sp1 binding sites [17]. A recent study analyzed genome-wide promoter DNA methylation after the treatment of sulforaphane in normal prostate epithelial cells and prostate cancer cells, finding that sulforaphane induced widespread changes in promoter methylation patterns [18] (Fig. 1.3).

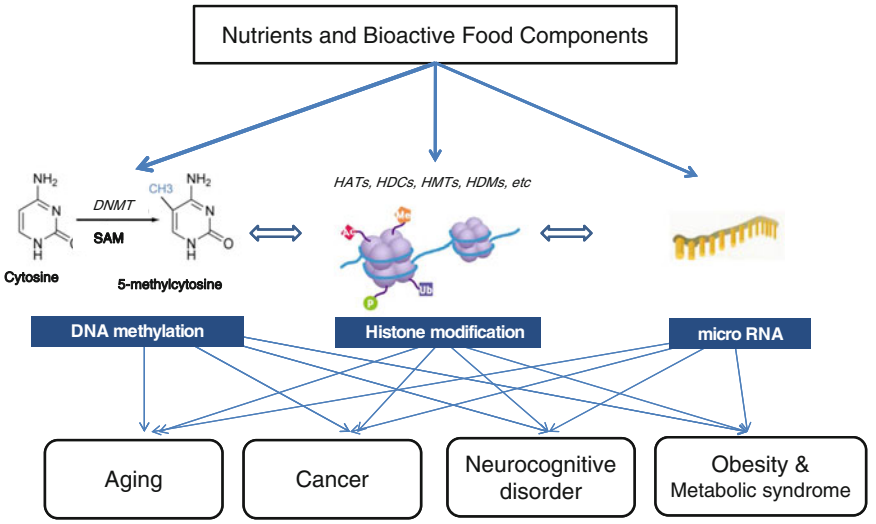

Fig. 1.3 Epigenetic effects of nutrients and bioactive food components on aging and the development of cancer, neurocognitive disorders, obesity and metabolic syndrome. *DNMT* DNA methyltransferase; *SAM* S-adenosylmethionine; *HATs* histone acetyltransferases; *HDACs* histone deacetylases; *HMTs* histone methyltransferases; *HDMs* histone demethylases

1.4.2 The Effect of Nutrients on Histone Modifications

1.4.2.1 One-Carbon Metabolism and Histone Methylation

Histone methylation is an epigenetic mark that occurs on lysines and arginines. Histone methylation of lysine is catalyzed by the histone methyltransferases (HMTs), whereas histone methylation of arginine is performed by a family of proteins called arginine methyltransferases (PRMTs). Both HMTs and PRMTs use S-adenosylmethionine as methyl donor and are inhibited by S-adenosylhomocysteine, similar to DNMTs. Histone methylation can induce either transcriptional activation or repression site specifically.

A combined dietary deficiency of methionine, choline, folate and vitamin B-12 in rats, which is known to develop primary liver cancer, also alters histone modifications, especially trimethylation of histone H4 lysine 20 and histone H3 lysine 9 as well as acetylation of histone H3 lysine 9 and histone H4 lysine 16. During tumorigenesis, the methyl deficiency also demonstrated a gradual decrease in the expression of suppressor of variegation 4-20 homolog 2 (*Suv4-20h2*) and retinoblastoma-interacting zinc-finger protein 1 (*Riz1*) histone methyltransferase and an increase in the expression of *Suv39-h1 HMT* and *HAT1* [19]. This study indicates that a methyl-deficient diet is capable of inducing tumor progression, not only by aberrant DNA methylation but also by derangement in the regulation of histone methylation and acetylation.

As described above, maternal choline intake is essential for fetal neurogenesis. In a transgenerational study using C57BL/6 mice, choline deprivation during gestation (12-17 days) altered the methylation pattern of histone H3 in E17 fetal hippocampi. Histone methylation changes occurred in a tissue-specific manner; histone H3 lysine 9 monomethylation was decreased in the ventricular and subventricular zones, whereas histone H3 lysine 9 dimethylation was decreased in the pyramidal layer [20].

1.4.2.2 Bioactive Food Components and Histone Modification

Genistein, a soy-derived bioactive isoflavone, affects tumorigenesis through epigenetic regulations. Genistein can induce promoter DNA hypomethylation and enhance the histone acetyl transferase (HAT) activity of the B-cell translocation gene 3 (*BTG3*), which is usually silenced in prostate cancer. It appears that the effect of genistein on DNA methylation is similar to that of 5'-azadeoxycytidine, a potent DNMT inhibitor that is currently under the phase II clinical trials for prostate cancer treatment [21]. In breast cancer cell lines, soy phytoestrogens, including genistein, daidzein, equol, 17 β-estradiol and suberoylanilidehydroxamic acid, decreased histone trimethylation marks and increased acetylation marks in six selected genes [22]. In colon cancer cell lines, treatment with genistein suppressed the activity of HDAC [23] (Table 1.2).

The polyphenolic compound of curry, a yellow spice called curcumin (diferuloylmethane), has been shown to have antitumor properties through the regulation of DNA methylation and histone acetylation. It is known that curcumin can enhance HAT activity and induce apoptotic cell death through a (PARP)- and caspase 3-mediated manner in neural progenitor cells [24]. In a mouse model, the intraperitoneal injection of curcumin using a nanostructured lipid carrier decreased histone acetylation in the central nervous system [25]. Similarly, a polyphenolic compound of green tea, EGCG can regulate histone acetylation. In methylation-sensitive colon cancer cells, EGCG acted as DNMT and HDAC inhibitors by inhibiting E3 ubiquitin ligase and ubiquitin-like with PHD and ring finger domains 1 (*UHRF1*) [26]. In another study using human colon cancer cells, EGCG decreased HDAC1 activity [23]. In pancreatic adenocarcinoma cells, EGCG inhibited HDAC activity via regulation of the expression of Raf kinase inhibitor protein (*RKIP*) and the invasive metastatic activity [27].

Resveratrol is a compound derived from grape skin that can suppress the cancer cell growth by inhibiting HDAC type III. In human hepatoblastoma cells, resveratrol also inhibited the activity of HDAC I, II and IV enzymes in a dose-dependent manner [28]. Resveratrol is a potent activator of SIRT1 which up-regulates the growth arrest and DNA-damage-inducible gamma (*GADD45G*) gene mediated by HDAC inhibition in malignant lymphoid cells [29]. In an in vitro study, resveratrol caused the activation of SIRT1, which in turn down-regulates the anti-apoptotic protein survivin by reducing the acetylation of histone H3 lysine 9 within its promoter [30]. In prostate cancer cells, resveratrol also increased p53 acetylation

Table 1.2 Nutrients that may affect histone modification and its mechanism

Bioactive food components	Epigenetic mechanism	Reference
Genistein	Increased HAT activity in prostate cancer	[21]
	Demethylation and acetylation of histones in breast cancer cell lines	[22]
	HDAC suppression in colon cancer cell lines	[23]
Curcumin	Enhanced HAT activity in neural progenitor cells	[24]
	HAT induction in the mouse central nervous system	[25]
EGCG	Decreased DNMT3A and HDAC3 activity in colon cancer cells	[26]
	Decreased HDAC1 activity in colon cancer cells	[23]
	Decreased HDAC activity in pancreatic cancer cells	[27]
Resveratrol	Pan-HDAC (class I, II and IV) inhibitor in hepatoblastoma cells	[28]
	Potent activator of SIRT1 that enhances HDAC inhibition	[29]
	Enhanced p53 acetylation and apoptosis in prostate cancer cells	[31]
Butyrate	Decreased HDAC1 and DNMT1 activity in colon cancer cells	[33]

HAT histone acetyltransferase, *HDAC* histone deacetylase, *DNMT* DNA methyltransferase, *SIRT1* sirtuin 1

and subsequent apoptosis by repressing the metastasis-associated protein 1 (*MTA1*) [31] (Table 1.2).

Butyrate is a short-chain fatty acid generated in the colon during the fermentation of dietary fibers by anaerobic bacteria. Butyrate is involved in the enhancement of memory recovery and osteoblast formation as well as reducing obesity and tumorigenesis through the inhibition of HDAC activity [32]. In colon cancer cells, the combination of EGCG and butyrate decreased both HDAC1 and DNMT1 activity [33].

1.4.3 The Effect of Nutrients on MicroRNA

1.4.3.1 Micronutrients and MicroRNA

A growing body of evidence suggests that diet, nutrients and bio-active food components can affect the production of microRNA (Fig. 1.3). Dietary folate has been shown to modulate microRNA expression in various model systems, and this result is in accordance with the chemopreventive effect of folate against cancer. A methyl-deficient diet induced inhibition of microRNA expression: specifically, miR-34a, miR-127, miR-200b, and miR-16a, which are involved in the regulation

of apoptosis, cell proliferation, cell-to-cell connection, and epithelial-mesenchymal transition in rat liver [34]. Kerek et al. demonstrated that methyl donor deficiency induced persistent defects in the brain of rats exposed in utero and in the hippocampal progenitor cell line (H19-7 cell line). The mechanism by which this defect occurred was through the reduction of signal transducer and activator of transcription 3 (*Stat3*) signaling by up-regulation of miR-124, a microRNA that targets *Stat3* signaling [35].

Retinoic acid, an active metabolite of vitamin A, is involved in cellular differentiation and has also been shown to modulate microRNA expression in various cells. In cells derived from acute promyelocytic leukemia, all-trans-retinoic acid treatment induced the up-regulation of miR-15a, miR-15b, miR-16-1, let-7a-3, let-7c, let-7d, miR-223, miR-342 and miR-107, and the down-regulation of miR181b [36]. In neuroblastoma cell lines, all-trans-retinoic acid treatment induced the up-regulation of miR10a/b. The vitamin D receptor complex is composed of 1,25-dihydroxyvitamin D (1,25 $(OH)_2D$) receptor, retinoid X receptor, activating transcription factor, HAT, and the basal transcriptional machinery. It can suppress or induce the expression of microRNAs, either by direct transcriptional regulation, indirect transcriptional regulation through other transcription factors, or by affecting microRNA gene promoters. In a reversible manner, microRNAs may also regulate vitamin D synthesis and metabolism. They may influence themselves through the vitamin D hormone receptor signaling in a dynamic feedback mechanism. 1,25 $(OH)_2D$ was shown to regulate the tumor suppressor microRNAs, miR-100 and miR125b, in primary prostate cells and tumor tissues [37]. 1,25$(OH)_2D$ also up-regulated the expression of miR-627 that suppressed the proliferation of human colorectal cancer cells and the growth of xenograft tumors in mice [38].

1.4.3.2 Bioactive Food Components and MicroRNA

Genistein, a major isoflavonoid isolated from dietary soybean, has been demonstrated to inhibit a variety of cancers both in vitro and in vivo by altering the expression of microRNAs (Table 1.3). The treatment of pancreatic cancer cells with the natural compound genistein led to the up-regulation of miR34a and the down regulation of onco-miR-223, which inhibited cell growth and induced apoptosis [39]. In prostate cancer cells, the level of mRNA and protein expression of ras-related C3 botulinum toxin substrate 1 (*RAC1*), epidermal growth factor receptor (*EGFR*) and E1A binding protein p300 (*EP300*) genes were significantly down-regulated, as was the level of the tumor suppressor miR-574-3p. However, treatment with genistein up-regulated the expression of tumor suppressor miR-574-3p [40]. The level of oncogenic miR-27a was higher in ovarian cancer tissues compared to normal ovarian tissues in humans. A study by Xu et al. showed that the treatment of ovarian cancer cells with genistein inhibited the growth and migration of ovarian cancer cells by the suppression of miR-27a [41].

Table 1.3 Bioactive foods that may regulate microRNA in cancer

Bioactive food components	Epigenetic mechanism	Reference
Genistein	Up-regulation of tumor suppressor miR-34a in pancreatic cancer cells	[49]
	Down-regulation of oncogenic miR-223 in pancreatic cancer cells	[39]
	Up-regulation of tumor suppressor miR-574-3p in prostate cancer cells	[40]
	Down-regulation of oncogenic miR-27a in ovarian cancer cells	[41]
Curcumin	Up-regulation of tumor suppressor miR-203 in bladder cancer cells	[42]
	Modulates miR-19/PTEN/AKT/p53 axis to exhibit its protective effects against BPA-associated breast cancer	[43]
	Up-regulation of tumor suppressor miR-205-5p in murine melanoma	[44]
EGCG	Up-regulation of tumor suppressor miR-210 in lung cancer cells	[45]
	Down-regulation of oncogenic miR (miR-92, miR-93, miR-99a) and up- regulation of tumor suppressor miR (miR-7-1, miR-34a, miR-106b) in human malignant neuroblastoma cells	[46]
	Enhanced efficacy of cisplatin by down-regulating miR-98-5p in NSCLC A549 cells	[47]
Resveratrol	Up-regulation of tumor suppressor miR-200c in lung cancer cells	[50]
	Mediates anti-inflammatory properties and suppresses intestinal tumorigenesis through miRNA modulation	[48]

miR microRNA, *PTEN* phosphatase and tensin homolog, *AKT* v-akt murine thymoma viral oncogene homolog 1, *BPA* bisphenol A, *NSCLC* non-small cell lung cancer

Curcumin (diferuloylmethane) has also been known to have anticancer properties through the regulation of microRNA. Saini et al. showed that curcumin up-regulated the expression of tumor suppressor miR-203 in bladder cancer cell lines [42]. This up-regulation was conveyed by hypomethylation of the miR-203 promoter that consequently increased apoptosis and decreased proliferation in bladder cancer cell lines. In another study using breast cancer cell lines, bisphenol induced the up-regulation of oncogenic miR-19a and miR-19b, while curcumin reversed it [43]. In C57BL/6 mice, curcumin treatment up-regulated the tumor suppressor miR-205-5p, resulting in a significant reduction of skin melanoma [44].

EGCG from green tea has been investigated for its capability of altering gene expression through the regulation of microRNA expression in cancer cells. EGCG in particular caused the up-regulation of the tumor suppressor miR-210 in both human and mouse lung cancer cells [45]. In human malignant neuroblastoma cells, EGCG down-regulated oncogenic microRNAs (miR-92, miR-93, and miR-99a) and

up-regulated tumor suppressor microRNAs (miR-7-1, miR-34a, and miR-106b) [46]. In non-small cell lung cancer cells, EGCG augmented the efficacy of cisplatin, which was mediated by the downregulation of the hsa-miR-98-5p followed by an increase in the expression of *p53*. Thus, the combination of cisplatin, a cancer chemotherapy drug, and EGCG might be an effective therapeutic strategy in the treatment in non-small cell lung cancer [47].

Resveratrol is also known for its anti-tumor activity, which is carried out by anti-inflammatory, antioxidative and epigenetic mechanisms that includes the regulation of microRNA. Mice treated with resveratrol for five weeks demonstrated a decrease in the number and size of colon polyps, as well as a reduction in cell damage and the proliferation of epithelial cells in intestinal mucosa. In addition, two microRNAs with anti-inflammatory effects, miRNA-101b andmiRNA-455, were up-regulated after resveratrol treatment [48].

1.5 Epigenetic Effect of Nutrition on Age-Associated Disorders

1.5.1 Neurocognitive Disorder

1.5.1.1 The Role of Epigenetics in Age-Associated Cognitive Decline

Cognitive functions, such as learning and memory, decline with age. A severe decline in cognitive function is characteristic of Alzheimer's disease, which has now become a major health problem in aged societies. The mechanism of cognitive aging remains unclear, but epigenetic phenomena are being studied for their possible roles in cognitive decline as well as neurogenesis and synaptic plasticity, which contribute to learning and memory.

A few researchers have investigated whether there is an association between DNA methylation and cognitive dysfunction. In fact, DNA methylation changes by the altered activity of DNMTs were shown to affect cognitive decline. Oliveira et al. demonstrated that the level of two enzymes involved in methylation seems to affect cognitive ability. In aged mice, increased Dnmt3a2 expression was associated with restored cognitive function of the hippocampus [51], while reduced Dnmt3a2 activity by small hairpin RNA (shRNA)-mediated knockdown was sufficient to disrupt the memory formation of mice. These results suggest that Dnmt3a2 is an important epigenetic enzyme for the hippo campus dependent memory formation.

Activity-regulated cytoskeleton-associated protein (*ARC*) is involved in memory consolidation and enduring synaptic plasticity in the hippocampus. Penner et al. demonstrated that the transcriptional activity of the *Arc* gene was lower in the hippocampus of aged rats compared with young adult rats. Of note is that the *Arc* gene in aged rats showed aberrant DNA methylation changes. This observation suggests that epigenetic changes during aging and the subsequent transcriptional

repression may cause less efficient memory storage and cognitive function [52]. Brain-derived neurotrophic factor (*BDNF*) is one of the critical genes for learning, memory and neural plasticity. Recent evidence indicates that aberrant DNA methylation of *Bdnf* in the hippocampal neuron cells down-regulates *Bdnf* expression and results in cognitive decline in mouse [53].

Histone modifications seem to play an important role in key cognitive performances such as learning and memory. As of now, histone acetylation shows the strongest link between epigenetic alteration and cognitive decline. Guan et al. demonstrated that a lack of histone acetylation by neuron-specific overexpression of HDAC2 reduced the long-term memory formation and synaptic plasticity. Furthermore, HDAC2-deficient mice, when treated with selective inhibitors of HDAC2, improved their memory function [54]. Peleg et al. observed that deregulation with deacetylation of histone H4 lysine 12 inhibited the expression of a hippocampal learning and memory-associated gene, thereby decreasing memory formation in aged mice. In agreement with those findings, an HDAC inhibitor that can block the deacetylation process was shown to restore histone acetylation and recover age-associated memory decline [55].

Decline in memory function can be improved by environmental conditions. A treadmill exercise program, for example, can increase the levels of histone H4 acetylation and decrease the levels of proinflammatory markers in the hippocampus, thus biochemically reversing the process of age–associated memory decline in rats. *BDNF*, as described above, is one of the genes affected by changes in histone acetylation during learning and synaptic plasticity. Administration of isoflurane, an anesthetic agent, to aged mice was shown to decrease the level of histone acetylation of the *Bdnf* as well as increase inflammation and apoptosis in the hippocampus. Histone acetylation of the *Bdnf* gene suppressed the expression of the gene encoding *Bdnf*-tyrosine kinase receptor B (*TrkB*). When a histone acetylation inhibitor, sodium butyrate, was added, alterations in histone acetylation status were reversed and cognitive impairment was improved [56].

Borrelli et al. summed up the process of neuronal plasticity by posttranslational epigenetic modifications. The epigenetic codes were indexed using the keywords; “writers”, “erasers”, and “readers”. Writers are enzymes, such as HATs, HMTs, and kinases, which add acetyl, methyl or phosphate groups to the histone tail. Erasers include HDACs, HDMs and phosphatases that remove those modifications. Readers are regulatory proteins, such as CREB-binding protein (CBP) and p300, which share unique bromodomain and recognize acetylated or methylated lysines [57]. Recently, Chatterjee et al. demonstrated that intraperitoneally administered activator of Cbp/p300 acetyltransferases passed through the blood-brain barrier and promoted maturation and differentiation of adult neuronal progenitor cells in mice. Interestingly, Cbp/p300 activation significantly extended the memory duration, suggesting that Cbp/p300-mediated histone acetylation can be a target for improving long-term memory [58].

MicroRNAs play an important role in the development of neuronal connectivity and regulate synaptic plasticity and cognitive functions. Almost 50 % of known microRNAs are highly expressed in the adult nervous system such as dendrites,

synapses and distal axon of sympathetic neurons. Schaefer et al. showed that when dicers, which cleave long double stranded RNA (dsRNA) into microRNA, are deficient in the Purkinje cells of the cerebellum, this resulted in a lack of microRNA in mice, followed by Purkinje cell death and development of ataxia [59]. A separate study showed that the deletion of *Dicer1* gene, which encodes dicer, in adult mouse forebrain was associated with a loss of brain-specific miRNAs along with a decrease in cognitive function [60].

In fact, these epigenetic phenomena dynamically interact with each other to regulate cognitive functions. The expression of the *BDNF* gene, a key mediator of the activity-dependent processes in the brain, is regulated by the combination of DNA methylation, histone-modifications and microRNA machineries. An imbalance in any component of this regulatory network can lead to an impairment of cognitive function in learning and memory capabilities. Interestingly, these epigenetic networks can be modified by environmental factors, such as stress exposure, drugs, exercise, toxins and diet.

1.5.1.2 Nutrients that Enhance Cognitive Function Through Epigenetic Mechanism

Although the effects are not strong, numerous studies have suggested that dietary supplementation with certain nutrients may improve cognition (Fig. 1.3). Among them are folate and choline, both of which are involved in one-carbon metabolism and DNA methylation. However, the results of studies on folate and neuro-cognition are not consistent. Low folate status has been associated with a decline in cognitive function but results of folic acid supplementation studies are contradictory. Recently a randomized clinical trial could not demonstrated the beneficial effect of B-vitamins, such as folate, vitamin B-12, and vitamin B-6 on improving the cognition of subjects with mild to moderate Alzheimer's disease [61]. In another study, however, taking B vitamins (folic acid 0.8 mg/d, vitamin B-12 0.5 mg/d, vitamin B-6 20 mg/d) for 24 months decreased the cerebral atrophy in gray matter, which is the main degenerative region in the brain of Alzheimer's disease, thereby slowing down the cognitive decline among subjects with mild cognitive impairment. The effect of B vitamins was more significant in subjects with high levels of homocysteine than those with low levels, which suggests that reducing hyperhomocysteinemia using B vitamin supplementation may ameliorate the neurocognitive decline.

In one-carbon metabolism, choline is oxidized to betaine that is utilized for folate independent remethylation of homocysteine to form methionine, which is catalyzed by betaine homocysteine methyltransferase (BHMT). Not only a precursor of betaine, choline is also an important precursor for the formation of acetylcholine, sphingomyelin and phosphatidylcholine, the latter two being essential components of the neuronal cell membrane. In contrast to folate studies, the cognitive enhancing effects of choline are more consistent. Dietary supplements with choline improved the hippocampal-dependent selective-impairment in long-term memory of female Sprague-Dawley rats aged between 3 and 15 months [62]. In the

Framingham offspring cohort study, a high intake of choline was associated with improvements in verbal and visual memories, whereas a low intake of choline was inversely associated with reduced white matter hyperintensity volume, which is associated with future development of Alzheimer disease [63].

Curcumin has been widely used for the treatment of mild cognitive disorders and the prevention of Alzheimer's disease. It is one of the many bioactive food components that regulate gene expression through the induction of epigenetic changes such as DNA methylation, histone modifications and mircoRNA. However, until now, no study has explained whether the effect of dietary curcumin on cognitive function is conveyed through epigenetic machineries. Resveratrol is also known for its neuroprotective role and slowing down cognitive impairment. Zhao et al. reported that the intraventricular injection of resveratrol for 8-9 months improved long-term memory formation and long-term potentiation, which is a long-lasting increase in synaptic efficacy following high-frequency stimulation of afferent fibers in the hippocampus slices of mice. Resveratrol acts like a Sirt1 agonist by increasing the expression of microRNAs, miR-124 and miR-134, thereby increasing the expression of cAMP responsive element binding protein (*Creb*) and *Bdnf* [64].

1.5.2 Obesity and Metabolic Syndrome

1.5.2.1 The Epigenetic Mechanism Underlying Obesity and Metabolic Syndrome

The prevalence of metabolic syndrome increases with age. Aging and metabolic syndrome are frequently accompanied by the same pathological conditions, such as increased lipoperoxidation, increased free radicals, increased peroxidation of nitric oxide (NO) to toxic species, and altered epigenetic phenomena. In this section, we discuss the role of epigenetics in age-associated obesity and metabolic syndrome and how epigenetic modifiers, especially dietary factors, can slow down metabolic aging (Table 1.4).

DNA methylation of genes, such as proopiomelanocortin (*POMC*), glucokinase (*GCK*), pancreatic and duodenal homeobox 1 (*PDX-1*), and fatty acid binding protein 3 (*FABP3*), has been shown to be associated with metabolic conditions. The anorexigenic leptin pathway in the arcuate nucleus of the brain is important for appetite control. A variant of leptin-responsive *POMC* suppresses this pathway, leading to early-onset monogenetic obesity. In peripheral blood cells two CpG sites in *POMC* are hypermethylated in obese children compared with normal-weight children. The hypermethylation of the third exon of *POMC* interferes with the binding of the transcription enhancer P300 so that transcription of the *POMC* gene is repressed [65]. This data provides evidence that DNA methylation can increase individual susceptibility to obesity. Similarly, a change in the DNA methylation pattern of the promoter of hepatic *Gck* is associated with decreased *Gck* expression and increased susceptibility to hepatic insulin resistance and diabetes in aged rat

Table 1.4 Nutrients that may affect obesity and metabolic syndrome through epigenetic mechanism

Nutrients	Metabolic disorder	Epigenetic mechanism	Reference
Methyl donors			
Folate	Insulin resistance, obesity	DNA methylation	[73, 74]
Vitamin B-12	Insulin resistance, obesity	DNA methylation	[73, 74]
Methionine	Insulin resistance, obesity	DNA methylation	[73, 74]
Choline	Liver steatosis	DNA methylation	[73, 75]
Betaine	Insulin resistance, Liver steatosis	DNA methylation	[73, 75]
Bioactive food components			
Genistein	Obesity	DNA methylation	[76]
Resveratrol	Obesity, liver steatosis	Histone acetylation, SIRT1 activation	[77]
Curcumin	Obesity, insulin resistance	Histone acetylation	[78]
EGCG	Obesity, inflammation	Histone acetylation	[79]

EGCG (–)-epigallocatechin-3-gallate, *SIRT1* sirtuin 1

models [66]. In rats, along with increased DNA methylation, 8 weeks of high-fat diet induced obesity, insulin resistance, type 2 diabetes mellitus, and non-alcoholic steatohepatitis, as well as down-regulated *Gck* and L-type pyruvate kinase (*Lpk*) [67], showing that DNA methylation may regulate glycolytic enzymes in high-fat diet-induced obesity. In human diabetic subjects, DNA methylation of the pancreatic duodenal homeobox 1 (*PDX-1*) gene in pancreatic islets was increased, and the expression of the gene was decreased [68]. The CpG methylation status of a key regulator of lipid homeostasis, fatty acid-binding proteins (*FABP3*), in peripheral white blood cells was associated with abnormal cholesterol, blood glucose, adiponectin, blood pressure and insulin sensitivity. This finding indicates that epigenetic marks in blood can be a risk predictor of metabolic syndrome.

Obesity-associated epigenetic alterations are known to be inherited to the next generation. During embryonic development in mammals, it is thought that the gene expression patterns in various cells and organs are established by epigenetic reprogramming. The fetal origin hypothesis proposes that obesity and type 2 diabetes mellitus can develop when the fetus tries to adapt to the aberrant intrauterine environment, such as malnutrition, stress or drug exposure. Epidemiologic studies have shown that maternal obesity and diabetes, which can lead to small sized newborn babies, are strong markers for the development of metabolic syndrome and other chronic conditions, such as cancer, in their later life. In mice, maternal high-fat diet leads to small-sized pups, which develop a postnatal phenotype that closely resembles the phenotype of the human metabolic syndrome, hepatic steatosis and impaired insulin sensitivity, which can be inherited to two subsequent generations. This abnormal postnatal phenotype in the offspring included an increased expression of the hepatic cell cycle inhibitor, cyclin-dependent kinase inhibitor 1A (*Cdkn1a*). The *Cdkn1a* gene was hypomethylated at specific CpG dinucleotides, suggesting an epigenetic inheritance by the maternal high fat diet [69].

The famous Dutch famine studies support the notion that the epigenetic phenotype acquired by prenatal exposure to an aberrant environment can last until late in life of the offspring and may affect their health conditions. Individuals who were prenatally exposed to the Dutch Hunger Winter in 1944, when people had to survive on a few hundred calories a day for several months, had lower DNA methylation in the differentially methylated regions of insulin-like growth factor 2 (*IGF2*) gene and were more obese than their siblings, who did not experience the famine [70].

Histone modification and microRNAs play key roles in obesity and metabolic conditions. Histone lysine methylation and acetylation are well-known post-translational modifications that regulate metabolic pathways. These histone modifications are postulated to influence the balance between energy storage and expenditure. Apoprotein E (*Apo E*)-deficient mice fed a high-fat diet showed an aberrant histone methylation, especially histone H3 lysine 9 methylation and histone H3 lysine 4 trimethylation on a peroxisome proliferator-activated receptor alpha (*Pparα*) network genes, which was associated with hepatic lipid accumulation. In addition, acetylation of histone has been shown to regulate adipocyte differentiation. SIRT1, a member of the HDAC family III, promotes fat metabolism in adipocytes by repressing *PPARγ*, which means that up-regulating SIRT1 increases lipolysis and thus induces fat loss. SIRT1 also regulates adiponectin gene expression through the forkhead box protein O1 (*FOXO1*)-CCAAT-enhancer-binding proteins (*C/EBPα*) transcriptional complex. Interestingly, during differentiation in 3T3-L1 adipocytes, promoters of adipogenesis genes were selectively hyperacetylated, while expression of lysine deacetylases (*KDAC*) 1, 2 and 5 and overall KDAC enzymatic activity decreased. These observations suggest that the activity of adipogenic transcription factors and that of deacetylases may together be essential for regulating adipocyte differentiation [71].

A body of evidence suggests a role for miRNAs in fat cell development and obesity. Proadipogenic microRNAs, such as miR-103, miR-17/92, and mir-210, are known to accelerate adipocyte differentiation, whereas anti-adipogenic microRNAs such as mir-14, mir-27a,b, miR-448 and miR15a, suppress adipocyte differentiation by blocking signal transduction pathways, such as the mitogenactivated protein kinases (MAPK) pathway [72]. Some miRNAs, such as the miR-17/92 cluster, are involved in adipogenesis by regulating the retinoblastoma (RB)-E2F pathway that controls mitotic entry from clonal expansion to terminal differentiation. MicroRNAs are also involved in glucose metabolism, free fatty acid-induced pancreatic ß-cell dysfunction, lipid metabolism and diabetes complications. The discovery of microRNA and its association with obesity and metabolic syndrome provide a breakthrough in the prevention and treatment of obesity and diabetes mellitus.

1.5.2.2 The Epigenetic Effects of Nutrition on Obesity and Metabolic Syndrome

Evidence indicates that intake of a certain nutrient or bioactive food component may reduce the development of obesity and metabolic syndrome by modulating

epigenetic phenomena. In the *agouti* mouse model, Cooney et al. demonstrated that maternal dietary levels of methyl donor nutrients, such as choline, folic acid, vitamin B-12 and methionine, can determine the DNA methylation status in the A^{vy} allele and subsequently determine the coat color, yellow to black. The coat color may indicate future health and particularly of future development of obesity and insulin resistance [73]. The percentage of mice with an *agouti* coat color (black) increased as the level of methyl supplements added to the diet increased. Two possible mechanisms are proposed to explain the epigenetic effects of maternal nutrients on the offspring phenotype: (1) a decrease in methyl availability may compromise one-carbon metabolism or the activity of Dnmt1, and thus inhibit normal DNA methylation at the A^{vy} allele; and (2) the repression of critical genes may occur during de novo DNA methylation at early fetal development. Moreover, such prenatal insult can result in a permanent defect of epigenetic regulation mediated by DNA methylation, suggesting a possible mechanism that can explain how early epigenetic event influence the development of later-life diseases. In sheep, periconceptional depletion of vitamin B-12, folate and methionine has been associated with a heavier and fatter phenotype, insulin-resistance and elevated blood pressure when the offspring becomes an adult. These phenotypes were accompanied by widespread changes in DNA methylation [74]. Paradoxically, when Wistar rats were fed a 10-fold higher multi-vitamin diet during pregnancy and then weaned to the recommended diet, the offspring were more likely to have obesity and metabolic syndrome. The high multi-vitamin diet decreased the expression of several hypothalamic genes, such as neuropeptide Y (*Npy*), pro-opiomelanocortin (*Pomc*), insulin receptor (*Ir*), leptin receptor (*Lepr*), and *Bdnf* along with increase global DNA methylation, which increased food intake. This obesity induced by prenatal high-vitamin exposure can be prevented by taking a high-vitamin and high-folate diet during the post-weaning period, which may promote hypomethylation in the promoter of the *Pomc* gene.

Non-alcoholic fatty liver disease is one of the most common hepatic manifestations of metabolic syndrome. Recent evidence connects epigenetic phenomena to the development of non-alcoholic fatty liver disease, which is from simple steatosis through to steatohepatitis, and ultimately cirrhosis. Epigenetic modulation using nutrients or bioactive components has been proposed as a promising approach to reduce the progression of non-alcoholic fatty liver disease. Methyl donor supplementation with choline, vitamin B-12, and folic acid ameliorated high-fat diet-induced hepatic triglyceride accumulation in the liver. This supplementation induced a change in the methylation pattern of the promoter of the fatty acid synthase (*Fasn*) gene in rats [75].

Recently, the beneficial effects of several bioactive food components as epigenetic modifiers in the prevention and treatment of obesity and metabolic syndrome have been proposed (Table 1.4). Genistein, a major phytoestrogen in soy, is an endocrine-disrupting substance that can prevent obesity by decreasing adipose deposition. During the gestation period, maternal dietary supplementation with genistein to dams shifted the coat color of the offspring from heterozygous yellow *agouti* (*Avy/a*) to black pseudoagouti, which represents a non-obese and healthier

phenotype of the offspring. This change of phenotype was accompanied by increased methylation of six cytosine-guanine sites in a retrotransposon site, which is the upstream of the *agouti* gene [76].

Resveratrol is known to protect the liver against non-alcoholic fatty liver disease by reducing fatty acid availability and oxidative stress. In male Sprague-Dawley rats, supplementation with low dose resveratrol in high-fat diet indeed decreased liver fat accumulation and increased fatty acid oxidation. Interestingly, the increase in fatty acid oxidation occurred through the increase in carnitine palmitoyl transferase-1A (CPT-1A) and acyl-CoA oxidase (Aco) activities that require the activation of the 5′ AMP-activated protein kinase (*Ampk*)/*Sirt1* axis [77].

Curcumin, which has an anti-inflammatory function, is a well-known natural compound that can prevent obesity. When treated with curcumin, human monocytes exposed to high glucose conditions reduced activity of HAT and p300, acetylated *CBP/p300* gene, and induced the expression of *CBP/p300* and *HDAC2*. This indicates that curcumin has the ability to diminish the hyperglycemia-induced cytokine production and vascular inflammation derived from diabetic complications [78]. EGCG, a green tea polyphenol, is also useful for the treatment of obesity and metabolic syndrome through its antiinflammatory effects. In an in vitro experiment, EGCG treatment restored the number of regulatory T cells and the production of IL-10, an anti-inflammatory mediator, in obese subjects. This was also associated with a decrease in NF-kB activity and an increase in HDAC activity and HDAC2 expression [79].

1.6 Conclusion and Future Perspectives

Epigenetic phenomena such as DNA methylation, histone modifications, and microRNA can regulate gene transcription. These phenomena interact with each other as well as with environmental factors, including nutrition to influence human phenotypes. Nutritional epigenetic research has recently begun to investigate whether nutrients and bioactive food components improve human health via epigenetic mechanisms.

Epigenetic phenomena are dynamically and reversibly changed throughout a lifetime. Interestingly, aberrant epigenetic changes by unfavorable uterine environment have been suggested to influence the health of offspring later in life. It appears that prenatal nutritional status may affect the aging process and the development of age-associated disease through epigenetic mechanism after birth. However, nutritional epigenetics on aging is still in their infancy, and many questions remain unanswered, because during aging, humans are exposed to diverse environmental factors and lifestyle factors that can modify epigenetic marks. It is also the reason why most studies are limited to cultured cell studies and animal studies that can provide the same environment.

Epigenetic phenomena are also cell-specific, species-specific and age-specific. Even though a nutrient is shown to have a beneficial effect on aging, the specific

target cells or organs need to be determined. Specific timing and dosage of nutritional exposure also need to be clarified.

The question still remains on whether age-associated epigenetic changes occur in a preprogrammed manner or in a random fashion according to exposed environments. Nevertheless, the field of nutritional epigenetics is still promising and significant with respect to the retarding of the aging process and preventing age-associated disease because it is believed that nutrients, bioactive food components, and diet may delay the undesirable age-associated epigenetic changes. Future work studying the underlying nutritional epigenetic mechanisms that govern the effects of specific nutrients will enable us to better achieve healthy aging.

References

1. Boehm M, Slack F (2005) A developmental timing microRNA and its target regulate life span in C. elegans. Science 310(5756):1954–1957
2. Keyes MK, Jang H, Mason JB, Liu Z, Crott JW, Smith DE et al (2007) Older age and dietary folate are determinants of genomic and p16-specific DNA methylation in mouse colon. J Nutr 137(7):1713–1717
3. Waterland RA, Jirtle RL (2003) Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 23(15):5293–5300
4. Choi SW, Friso S, Ghandour H, Bagley PJ, Selhub J, Mason JB (2004) Vitamin B-12 deficiency induces anomalies of base substitution and methylation in the DNA of rat colonic epithelium. J Nutr 134(4):750–755
5. Niculescu MD, Craciunescu CN, Zeisel SH (2006) Dietary choline deficiency alters global and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains. FASEB J. 20(1):43–49
6. Pogribny IP, Ross SA, Wise C, Pogribna M, Jones EA, Tryndyak VP et al (2006) Irreversible global DNA hypomethylation as a key step in hepatocarcinogenesis induced by dietary methyl deficiency. Mutat Res 593(1–2):80–87
7. Waterland RA (2006) Assessing the effects of high methionine intake on DNA methylation. J Nutr 136(6):1706S–1710S
8. Rowling MJ, McMullen MH, Schalinske KL (2002) Vitamin A and its derivatives induce hepatic glycine N-methyltransferase and hypomethylation of DNA in rats. J Nutr 132(3):365–369
9. Wallwork JC, Duerre JA (1985) Effect of zinc deficiency on methionine metabolism, methylation reactions and protein synthesis in isolated perfused rat liver. J Nutr 115(2):252–262
10. Lopes N, Carvalho J, Duraes C, Sousa B, Gomes M, Costa JL et al (2012) 1Alpha, 25-dihydroxyvitamin D3 induces de novo E-cadherin expression in triple-negative breast cancer cells by CDH1-promoter demethylation. Anticancer Res 32(1):249–257
11. Fang MZ, Chen D, Sun Y, Jin Z, Christman JK, Yang CS (2005) Reversal of hypermethylation and reactivation of p16INK4a, RARbeta, and MGMT genes by genistein and other isoflavones from soy. Clin Cancer Res 11(19 Pt 1):7033–7041
12. Vardi A, Bosviel R, Rabiau N, Adjakly M, Satih S, Dechelotte P et al (2010) Soy phytoestrogens modify DNA methylation of GSTP1, RASSF1A, EPH2 and BRCA1 promoter in prostate cancer cells. In Vivo 24(4):393–400
13. Li H, Xu W, Huang Y, Huang X, Xu L, Lv Z (2012) Genistein demethylates the promoter of CHD5 and inhibits neuroblastoma growth in vivo. Int J Mol Med 30(5):1081–1086
14. Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H et al (2003) Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 63(22):7563–7570

15. Mittal A, Piyathilake C, Hara Y, Katiyar SK (2003) Exceptionally high protection of photocarcinogenesis by topical application of (-)-epigallocatechin-3-gallate in hydrophilic cream in SKH-1 hairless mouse model: relationship to inhibition of UVB-induced global DNA hypomethylation. Neoplasia 5(6):555–565
16. Wang LS, Kuo CT, Cho SJ, Seguin C, Siddiqui J, Stoner K et al (2013) Black raspberry-derived anthocyanins demethylate tumor suppressor genes through the inhibition of DNMT1 and DNMT3B in colon cancer cells. Nutr Cancer 65(1):118–125
17. Hsu A, Wong CP, Yu Z, Williams DE, Dashwood RH, Ho E (2011) Promoter de-methylation of cyclin D2 by sulforaphane in prostate cancer cells. Clin epigenetics 3:3
18. Wong CP, Hsu A, Buchanan A, Palomera-Sanchez Z, Beaver LM, Houseman EA et al (2014) Effects of sulforaphane and 3,3′-diindolylmethane on genome-wide promoter methylation in normal prostate epithelial cells and prostate cancer cells. PLoS ONE 9(1):e86787
19. Pogribny IP, Tryndyak VP, Muskhelishvili L, Rusyn I, Ross SA (2007) Methyl deficiency, alterations in global histone modifications, and carcinogenesis. J Nutr 137(1 Suppl):216S–222S
20. Mehedint MG, Niculescu MD, Craciunescu CN, Zeisel SH (2010) Choline deficiency alters global histone methylation and epigenetic marking at the Re1 site of the calbindin 1 gene. FASEB J. 24(1):184–195
21. Majid S, Dar AA, Shahryari V, Hirata H, Ahmad A, Saini S et al (2010) Genistein reverses hypermethylation and induces active histone modifications in tumor suppressor gene B-Cell translocation gene 3 in prostate cancer. Cancer 116(1):66–76
22. Dagdemir A, Durif J, Ngollo M, Bignon YJ, Bernard-Gallon D (2013) Histone lysine trimethylation or acetylation can be modulated by phytoestrogen, estrogen or anti-HDAC in breast cancer cell lines. Epigenomics 5(1):51–63
23. Groh IA, Chen C, Luske C, Cartus AT, Esselen M (2013) Plant polyphenols and oxidative metabolites of the herbal alkenylbenzene methyleugenol suppress histone deacetylase activity in human colon carcinoma cells. J Nutr Metab 2013:821082
24. Kang SK, Cha SH, Jeon HG (2006) Curcumin-induced histone hypoacetylation enhances caspase-3-dependent glioma cell death and neurogenesis of neural progenitor cells. Stem Cells Dev 15(2):165–174
25. Puglia C, Frasca G, Musumeci T, Rizza L, Puglisi G, Bonina F et al (2012) Curcumin loaded NLC induces histone hypoacetylation in the CNS after intraperitoneal administration in mice. Eur Journal Pharm Biopharm: Official Journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV 81(2):288–293
26. Moseley VR, Morris J, Knackstedt RW, Wargovich MJ (2013) Green tea polyphenol epigallocatechin 3-gallate, contributes to the degradation of DNMT3A and HDAC3 in HCT 116 human colon cancer cells. Anticancer Res 33(12):5325–5333
27. Kim SO, Kim MR (2013) (-)-Epigallocatechin 3-gallate inhibits invasion by inducing the expression of Raf kinase inhibitor protein in AsPC1 human pancreatic adenocarcinoma cells through the modulation of histone deacetylase activity. Int J Oncol 42(1):349–358
28. Venturelli S, Berger A, Bocker A, Busch C, Weiland T, Noor S, et al (2013) Correction: resveratrol as a PanHDAC inhibitor alters the acetylation status of jistone proteins in human-derived hepatoblastoma Cells. PloS ONE 8(9)
29. Scuto A, Kirschbaum M, Buettner R, Kujawski M, Cermak JM, Atadja P et al (2013) SIRT1 activation enhances HDAC inhibition-mediated upregulation of GADD45G by repressing the binding of NFkappaB/STAT3 complex to its promoter in malignant lymphoid cells. Cell Death Dis 4:e635
30. Wang RH, Zheng Y, Kim HS, Xu X, Cao L, Luhasen T et al (2008) Interplay among BRCA1, SIRT1, and Survivin during BRCA1-associated tumorigenesis. Mol Cell 32(1):11–20
31. Kai L, Samuel SK, Levenson AS (2010) Resveratrol enhances p53 acetylation and apoptosis in prostate cancer by inhibiting MTA1/NuRD complex. Int J Cancer 126(7):1538–1548
32. Steliou K, Boosalis MS, Perrine SP, Sangerman J, Faller DV (2012) Butyrate histone deacetylase inhibitors. BioResearch Open Access 1(4):192–198

33. Saldanha SN, Kala R, Tollefsbol TO (2014) Molecular mechanisms for inhibition of colon cancer cells by combined epigenetic-modulating epigallocatechin gallate and sodium butyrate. Exp Cell Res 324(1):40–53
34. Tryndyak VP, Ross SA, Beland FA, Pogribny IP (2009) Down-regulation of the microRNAs miR-34a, miR-127, and miR-200b in rat liver during hepatocarcinogenesis induced by a methyl-deficient diet. Mol Carcinog 48(6):479–487
35. Kerek R, Geoffroy A, Bison A, Martin N, Akchiche N, Pourie G et al (2013) Early methyl donor deficiency may induce persistent brain defects by reducing Stat3 signaling targeted by miR-124. Cell Death Dis 4:e755
36. Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, Cimmino A et al (2007) MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene 26(28):4148–4157
37. Giangreco AA, Vaishnav A, Wagner D, Finelli A, Fleshner N, Van der Kwast T et al (2013) Tumor suppressor microRNAs, miR-100 and -125b, are regulated by 1,25-dihydroxyvitamin D in primary prostate cells and in patient tissue. Cancer Prev Res 6(5):483–494
38. Padi SK, Zhang Q, Rustum YM, Morrison C, Guo B (2013) MicroRNA-627 mediates the epigenetic mechanisms of vitamin D to suppress proliferation of human colorectal cancer cells and growth of xenograft tumors in mice. Gastroenterology 145(2):437–446
39. Ma J, Cheng L, Liu H, Zhang J, Shi Y, Zeng F et al (2013) Genistein down-regulates miR-223 expression in pancreatic cancer cells. Curr Drug Targets 14(10):1150–1156
40. Chiyomaru T, Yamamura S, Fukuhara S, Hidaka H, Majid S, Saini S et al (2013) Genistein up-regulates tumor suppressor microRNA-574-3p in prostate cancer. PLoS ONE 8(3):e58929
41. Xu L, Xiang J, Shen J, Zou X, Zhai S, Yin Y et al (2013) Oncogenic microRNA-27a is a target for genistein in ovarian cancer cells. Anti-Cancer Agents Med Chem 13(7):1126–1132
42. Saini S, Arora S, Majid S, Shahryari V, Chen Y, Deng G et al (2011) Curcumin modulates microRNA-203mediated regulation of the Src-Akt axis in bladder cancer. Cancer Prev Res 4 (10):1698–1709
43. Li X, Xie W, Xie C, Huang C, Zhu J, Liang Z et al (2014) Curcumin modulates miR-19/PTEN/AKT/p53 axis to suppress bisphenol A-induced MCF-7 breast cancer cell proliferation. Phytother Res 28(10):1553–1560
44. Dahmke IN, Backes C, Rudzitis-Auth J, Laschke MW, Leidinger P, Menger MD et al (2013) Curcumin intake affects miRNA signature in murine melanoma with mmu-miR-205-5p most significantly altered. PLoS ONE 8(12):e81122
45. Wang H, Bian S, Yang CS (2011) Green tea polyphenol EGCG suppresses lung cancer cell growth through upregulating miR-210 expression caused by stabilizing HIF-1alpha. Carcinogenesis 32(12):1881–1889
46. Chakrabarti M, Khandkar M, Banik NL, Ray SK (2012) Alterations in expression of specific microRNAs by combination of 4-HPR and EGCG inhibited growth of human malignant neuroblastoma cells. Brain Res 1454:1–13
47. Zhou DH, Wang X, Feng Q (2014) EGCG enhances the efficacy of cisplatin by downregulating hsa-miR-98-5p in NSCLC A549 cells. Nutr Cancer 66(4):636–644
48. Altamemi I, Murphy EA, Catroppo JF, Zumbrun EE, Zhang J, McClellan JL et al (2014) Role of microRNAs in resveratrol-mediated mitigation of colitis-associated tumorigenesis in ApcMin/+Mice. J Pharmacol Exp Ther 350(1):99–109
49. Xia J, Duan Q, Ahmad A, Bao B, Banerjee S, Shi Y et al (2012) Genistein inhibits cell growth and induces apoptosis through up-regulation of miR-34a in pancreatic cancer cells. Curr Drug Targets 13(14):1750–1756
50. Bai T, Dong DS, Pei L (2014) Synergistic antitumor activity of resveratrol and miR-200c in human lung cancer. Oncol Rep 31(5):2293–2297
51. Oliveira AM, Hemstedt TJ, Bading H (2012) Rescue of aging-associated decline in Dnmt3a2 expression restores cognitive abilities. Nat Neurosci 15(8):1111–1113
52. Penner MR, Roth TL, Chawla MK, Hoang LT, Roth ED, Lubin FD et al (2011) Age-related changes in Arc transcription and DNA methylation within the hippocampus. Neurobiol Aging 32(12):2198210

53. Ma DK, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N et al (2009) Neuronal activityinduced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. Science 323(5917):1074–1077
54. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J et al (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459(7243):55–60
55. Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, Agis-Balboa RC et al (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328(5979):753–756
56. Ji M, Dong L, Jia M, Liu W, Zhang M, Ju L et al (2014) Epigenetic enhancement of brain-derived neurotrophic factor signaling pathway improves cognitive impairments induced by isoflurane exposure in aged rats. Mol Neurobiol 50(3):937–944
57. Borrelli E, Nestler EJ, Allis CD, Sassone-Corsi P (2008) Decoding the epigenetic language of neuronal plasticity. Neuron 60(6):961–974
58. Chatterjee S, Mizar P, Cassel R, Neidl R, Selvi BR, Mohankrishna DV et al (2013) A novel activator of CBP/p300 acetyltransferases promotes neurogenesis and extends memory duration in adult mice. J Neurosci: Official J Soc Neurosci 33(26):10698–10712
59. Schaefer A, O'Carroll D, Tan CL, Hillman D, Sugimori M, Llinas R et al (2007) Cerebellar neurodegeneration in the absence of microRNAs. J Exp Med 204(7):1553–1558
60. Konopka W, Kiryk A, Novak M, Herwerth M, Parkitna JR, Wawrzyniak M et al (2010) MicroRNA loss enhances learning and memory in mice. J Neurosci: Official J Soc Neurosci 30 (44):14835–14842
61. Malouf R, Grimley Evans J (2008) Folic acid with or without vitamin B12 for the prevention and treatment of healthy elderly and demented people. Cochrane Database Syst Rev 8(4): Cd004514
62. Teather LA, Wurtman RJ (2003) Dietary cytidine (5′)-diphosphocholine supplementation protects against development of memory deficits in aging rats. Prog Neuropsychopharmacol Biol Psychiatry 27(4):711–717
63. Poly C, Massaro JM, Seshadri S, Wolf PA, Cho E, Krall E et al (2011) The relation of dietary choline to cognitive performance and white-matter hyperintensity in the Framingham Offspring Cohort. Am J Clin Nutr 94(6):1584–1591
64. Zhao YN, Li WF, Li F, Zhang Z, Dai YD, Xu AL et al (2013) Resveratrol improves learning and memory in normally aged mice through microRNA-CREB pathway. Biochem Biophys Res Commun 435(4):597–602
65. Kuehnen P, Mischke M, Wiegand S, Sers C, Horsthemke B, Lau S et al (2012) An Alu element-associated hypermethylation variant of the POMC gene is associated with childhood obesity. PLoS Genet 8(3):15
66. Jiang MH, Fei J, Lan MS, Lu ZP, Liu M, Fan WW et al (2008) Hypermethylation of hepatic Gck promoter in ageing rats contributes to diabetogenic potential. Diabetologia 51(8):1525–1533
67. Jiang M, Zhang Y, Liu M, Lan MS, Fei J, Fan W et al (2011) Hypermethylation of hepatic glucokinase and Ltype pyruvate kinase promoters in high-fat diet-induced obese rats. Endocrinology 152(4):1284–1289
68. Yang BT, Dayeh TA, Volkov PA, Kirkpatrick CL, Malmgren S, Jing X et al (2012) Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes. Mol Endocrinol 26(7):1203–1212
69. Dudley KJ, Sloboda DM, Connor KL, Beltrand J, Vickers MH (2011) Offspring of mothers fed a high fat diet display hepatic cell cycle inhibition and associated changes in gene expression and DNA methylation. PLoS ONE 6(7):11
70. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES et al (2008) Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci USA 105(44):17046–17049
71. Iyer A, Fairlie DP, Brown L (2012) Lysine acetylation in obesity, diabetes and metabolic disease. Immunol Cell Biol 90(1):39–46

72. Son YH, Ka S, Kim AY, Kim JB (2014) Regulation of adipocyte differentiation via microRNAs. Endocrinol Metab 29(2):122–135
73. Cooney CA, Dave AA, Wolff GL (2002) Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr 132(8 Suppl):2393S–2400S
74. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J et al (2007) DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci USA 104(49):19351–19356
75. Cordero P, Gomez-Uriz AM, Campion J, Milagro FI, Martinez JA (2013) Dietary supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. Genes Nutr 8(1):105–113
76. Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect 114(4):567–572
77. Alberdi G, Rodriguez VM, Macarulla MT, Miranda J, Churruca I, Portillo MP (2013) Hepatic lipid metabolic pathways modified by resveratrol in rats fed an obesogenic diet. Nutrition 29 (3):562–567
78. Yun JM, Jialal I, Devaraj S (2011) Epigenetic regulation of high glucose-induced proinflammatory cytokine production in monocytes by curcumin. J Nutr Biochem 22 (5):450–458
79. Yun JM, Jialal I, Devaraj S (2010) Effects of epigallocatechin gallate on regulatory T cell number and function in obese v. lean volunteers. Br J Nutr 103(12):1771–1777

Chapter 2
Dietary Restriction, Dietary Design and the Epigenetics of Aging and Longevity

Craig A. Cooney

Abstract As the mechanisms of long-term control of gene expression, it would seem that the various aspects of epigenetics would be important for, even determinants of, aging and longevity. Yet few data connect these directly. Epigenetics changes with age; in particular DNA methylation and histone acetylation have been well studied. For humans, a DNA methylation based "epigenetic clock" has been developed to track the apparent chronological age of people, tissues, stem cells and cancers. Histone acetylation is important for maintaining cognitive memory in animals and restoration of histone acetylation improves memory in older animals. Several aspects of diet and metabolism affect epigenetics. These include the effects of glucose on histone acetylation and methylation, the effects of acetyl-coenzyme A and energy metabolism on histone acetylation, natural histone deacetylase inhibitors found in foods such as broccoli and garlic affecting histone acetylation and DNA methylation, and the effects of methyl metabolism and nutrients such as folate on DNA and histone methylation. Models of greatly extended longevity should be studied for epigenetics to test if epigenetics are preserved when longevity is extended and then studies to manipulate epigenetics in these models should be done to measure their effects on longevity.

Abbreviations

Ac	Acetyl group
AcCoA	Acetyl-coenzyme A
AGE	Advanced glycation end products
AMPK	AMP activated protein kinase
BHB	D-beta-hydroxybutyrate
CR	Calorie restriction
DCCT	Diabetes Control and Complications Trial
DIM	Diindolylmethane
DNMT	DNA methyltransferase

C.A. Cooney (✉)
Research and Development, Central Arkansas Veterans Healthcare System (CAVHS), 4300 West 7th Street, Little Rock, AR 72205-5484, USA
e-mail: cooneycraiga@gmail.com

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DR	Dietary restriction
EDIC	Epidemiology of Diabetes Intervention and Complications
ERV	Endogenous retrovirus
H3K4	H3 histone tail lysine 4
H3K9	H3 histone tail lysine 9
H4K12	H4 histone tail lysine 12
HAT	Histone acetyltransferase
HbA1c	Glycated hemoglobin used as a measure of long-term average blood glucose levels
HDAC	Histone deacetylase
HDACI	Histone deacetylase inhibitor
HERV-K	Human endogenous retrovirus virus K
HMT	Histone methyltransferase
IAP	Intracisternal A particle
iPSC	Induced pluripotent stem cell
L1	LINE1
LINE1	Long interspersed nuclear element 1
L1Md	An L1 sequence of mice
LSD1	Lysine-specific demethylase 1
LTR	Long terminal repeat
MS-275	Entinostat (an HDACI)
MuERV	Murine endogenous retrovirus
NFkB	Nuclear factor kappa-light chain enhancer of activated B cells
p65	Transcription factor p65 encoded by the RELA gene
RAGE	Receptor for advanced glycation end products
RTG	Yeast genes important in communication between the mitochondria and nucleus
SAH	*S*-adenosylhomocysteine
SAHA	Suberoylanilide hydroxamic acid
SAM	*S*-adenosylmethionine
Set7	Enzyme that methylates lysine residues (e.g. on histones)
TCA	Tricarboxylic acid (cycle) or Krebs cycle

2.1 Introduction

The idea that epigenetics needs to be intact to provide a "young" pattern of gene expression is an old one [1, 2]. We know that gene transcription profiles change with age and that dietary restriction (DR) can slow these changes [3]. Numerous studies show epigenetic change with age and it is regularly assumed that this epigenetic "drift" contributes to some or all of the functional decline and disease of aging [2, 4]. However, it is not at all clear what factors are driving epigenetic

change with aging and to what degree epigenetics controls longevity. In some models of extended longevity, developmental factors [5, 6], dietary factors (especially DR) [7–9] and specific genetic factors [5, 6, 10] clearly increase lifespan. Presumably epigenetics is maintained better in these models than in same chronological age controls, yet few data are available on this point. Further, if epigenetics is important for longevity, certain manipulations of epigenetics per se should extend lifespan, although this has not been demonstrated. However it is possible, even likely, that developmental, metabolic and other factors are controlling epigenetics as just one part of a lifespan extending process. Thus, we will look at various dietary and metabolic influences on epigenetics that may influence health and lifespan.

2.2 Epigenetic Mechanisms

Epigenetics is the collection of heritable chromatin modifications, recursive RNA expression and other heritable factors outside of the DNA sequence itself that affect gene expression. Epigenetics also helps guide the health and development of plants and animals from fertilization and cell division through disease and aging. A broad range of factors affect epigenetics. Epigenetics is often reviewed [2, 11–16] and only a broad overview will be given here. Cancer epigenetics, in particular, has been well studied and helps inform aging and lifespan research.

DNA methylation

The dinucleotide CG (called CpG) in polymeric DNA is the main target of DNA methyltransferases (DNMTs) that methylate the 5 position of cytosines to form 5-methylcytosine [17]. The methyl group donor *S*-adenosylmethionine (SAM) is the other substrate in this reaction which ties DNA methylation to methyl metabolism. One product of this reaction is *S*-adenosylhomocysteine (SAH) which is an inhibitor of most methylation reactions but can be recycled back to SAM by methyl metabolism. The CpG sequence is a palindrome and one of the DNMTs, called DNMT1, copies the methylation pattern of a parental DNA strand onto the daughter strand during DNA replication in a process called maintenance methylation. Methylation of DNA also occurs de novo where unmethylated CpGs are methylated by DNMT1 (in a de novo role) and by DNMT3a and DNMT3b (dedicated de novo DNMTs). DNA methylation patterns can be inherited and propagate gene expression patterns in generations of cells and even in generations of animals [11, 18–20].

Generally, DNA methylation near transcription start sites silences gene expression [17, 21] by attracting methylated DNA binding proteins as well as preventing transcription factor access. Protein complexes that modify histones reinforce, or in some cases initiate, transcriptional silence. This can leave a gene silenced or in other cases it can "poise" a silenced region for rapid activation of gene expression [16].

DNA methylation is removed by base excision repair of 5-methylcytosine and/or by multiple steps of methyl group oxidation. Oxidized products, 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxycytosine are found in mammalian DNA and are thought to be intermediates in demethylation [22, 23]. DNA methylation patterns are extensively rewritten post-fertilization and in primordial germ cells, times when demethylation is prominent [22, 23].

Histone methylation

Histones, the major DNA binding proteins of chromatin, are methylated by SAM through the action of histone methyltransferases (HMTs) [16, 24]. Histone methylation can either promote or silence gene activity depending on which lysine and arginine sites in the histone sequence are methylated. HMTs, histone demethylases and methylated histone binding proteins are highly specific for the site (position) and the degree of methylation (number of methyls on an amino acid side chain). There are greater varieties and specificities of these enzymes and binding proteins for histone methylation than for respective enzymes and binding domains involved in histone acetylation and DNA methylation. As discussed elsewhere in this article, without adequate methyl metabolism and SAM both DNA methylation and histone methylation can be expected to drift. Histone methylation can direct both DNA methylation and histone acetylation and thus adequate methyl metabolism and SAM are particularly important for epigenetics.

Histone acetylation

Histones are also modified by acetylation of their lysines, which nearly always promotes gene activity. This depends on histone acetyltransferases (HATs), the acetyl donor acetyl-coenzyme A (AcCoA), and histone deacetylases (HDACs) [12]. There is a broad array of effects of diet and energy metabolism on histone acetylation [12, 25–28]. In addition, many foods contain compounds that are HDAC inhibitors (HDACIs) which tend to preserve histone acetylation and promote gene activation [29–33].

Chromatin non-coding RNAs

Some RNA molecules interact with chromatin to give a range of effects on gene expression. The X-inactive specific transcript and many other RNAs have important roles in epigenetics [34–37]. The expression of some genes encoding protein gene products are affected by small RNA genes that are embedded in the protein coding genes. Transcription of these small RNA coding regions can interfere with and slow the expression of the protein coding genes. Further, RNAs have a range of effects when binding to the chromatin or other nascent RNA transcripts. RNA expression is amenable to direct analysis by next generation sequencing [38].

2.3 Epigenetics with Aging

Epigenetics drifts and breaks down with aging and age-related disease [2, 4]. A fundamental issue in addressing epigenetics in aging is the paradoxical situation where the average DNA methylation over the genome declines with age (global

hypomethylation) concomitant with gradual, age-related DNA hypermethylation of some specific genes.

Early studies mainly showed global hypomethylation with age in animal tissues [39–42] and drift or hypomethylation with mammalian cells in vitro [43, 44]. Later studies of specific genes most often showed gradual DNA hypermethylation with age in normal tissues [45–47].

In a recent statistical and analytical *tour de force*, Steve Horvath [48] used publicly available datasets (from Illumina 27 and 450 K array platforms) covering large numbers of human genes, to show that age-related changes in DNA methylation are roughly half hypo- and half hypermethylation. Each data set contained DNA methylation values for over 21,000 CpGs from which Horvath identified an "epigenetic clock" based on 353 CpGs that closely correlate with a person's chronological age.

Horvath also showed that induced pluripotent stem cells (iPSCs, made from adult donor cells) have an age of zero and thus could be considered an example of age reversal at the epigenetic and cellular level. On the other end of the aging spectrum, some cancers showed an advanced age well beyond that of their host. This advanced age averaged 36 years (past that of the patient) demonstrating age acceleration at the epigenetic level. Age reversal in iPSCs and accelerated aging in cancer are not new ideas yet they are given greater strength and are quantified in Horvath's findings. Expressing the "age" of stem cells and cancer in terms of the "epigenetic clock" designed to track the age of normal human tissues at many ages could be a very useful tool. Among other applications, it seems that such an epigenetic tool could be a marker for the success of interventions into aging and lifespan. Testing this will require considerable research including development of epigenetic clocks in other species (such as mice) that can be used as models of extended lifespan.

The study of DNA methylation has presented many paradoxes over the years [49, 50]. One of these that occurs with aging, as mentioned above, is global hypomethylation concomitant with age-related gene specific hypermethylation [4, 25]. Interestingly, this same phenomenon occurs to an even larger degree in most cancers [4, 25]. However, this particular "DNA methylation paradox" is not a universal feature of human disease as has been recently shown with autoimmunity where mainly DNA hypomethylation occurs [51, 52]. In cancer and aging this concomitant DNA hypo- and hypermethylation may be driven, in part, by other aspects of epigenetics, especially histone acetylation.

While there are fewer studies of histone modification than DNA methylation with age, an increasing number of studies show changes in histone modifications with age [53–55]. Histone acetylation is particularly important here because some interventions can reverse these age-related changes [54, 56, 57]. Epigenetics is important not just for cell memory but for cognitive memory formation and learning [58].

To study memory in aged animals, Peleg et al. [54] measured hippocampus-dependent associative learning in mice at ages of 3, 8 and 16 months. Although all ages of mice performed similarly on many tests, old mice (16 months of age) did poorly in associative learning and a few other tests compared to younger mice.

In young mice, learning increased histone H4 lysine 12 acetylation (H4K12Ac) and changed the expression of 2,229 genes. In old mice H4K12Ac did not change significantly and only 6 genes were differentially expressed. Importantly, treatment with HDACIs increased H4K12Ac and improved learning in 16 month old mice.

Alzheimer's disease has been studied in mouse models using HDACIs [56]. Normal mice were compared with Alzheimer's mice after intraperitoneal HDACI injections. Normal mice were unaffected whereas the memory of Alzheimer's mice was significantly improved. More recent studies show that HDACIs can increase the expression of proteins that degrade, bind or transport amyloid beta-peptide thus reducing its contribution to cell death and Alzheimer's [57]. These studies show that HDACIs can reverse memory loss due to age-related dementia and Alzheimer's in mice.

In order to address the reduced effectiveness of some medications in the elderly, Montalvo-Ortiz et al. [59] studied the effectiveness of haloperidol in aged mice. They discovered that haloperidol efficacy was increased when mice were pretreated with the HDACIs valproate or entinostat (MS-275) and that this increased efficacy was correlated with increased histone acetylation of the c-fos promoter in the nucleus accumbens shell and prefrontal cortex. These combined HDACI and haloperidol treatments increased c-fos expression to levels comparable to those in young mice.

Some studies using cell culture and adult animals show that DNA methylation, histone acetylation and other epigenetic modifications can change over short periods (minutes or days) [58, 60]. With age there seems to be less epigenetic plasticity and flexibility and this leads to the age-related diminution of memory and other processes that require epigenetic change [2, 54, 55]. Several studies discussed here show improved memory in aged animals by use of HDACIs. This raises the very real possibility of using foods (such as broccoli, [32, 33]), supplements (such as butyrate, DIM or sulforaphane) or drugs (such as valproate or SAHA) to slow or reverse age-related memory loss and dementia. Other aspects of aging might also be improved by such treatments.

2.4 Dietary and Metabolic Factors Affecting Epigenetics

Dietary Restriction

Restricting the amount of food while avoiding malnutrition (dietary restriction or DR) or specifically restricting just the number of calories consumed (calorie restriction or CR) can extend lifespan and delay many symptoms of aging in a wide range of animals including yeast, nematodes, fruit flies, mice and rats [61, 62]. DR can also reduce or delay a number of age-related diseases, most notably cancer [63–66]. Although DR has not been shown to extend lifespan in humans, DR does improve glucose regulation and decrease body temperature and inflammation in humans and other mammals [62, 67–70]. Several signaling pathways are affected by DR, or may

mediate DR's effects. These include insulin, growth hormone, insulin-like growth factor-1 and intracellular pathways connected to mTOR activity [71–73]. DR also lowers the levels of a population of circulating micro RNAs that increase in amount with age in mice [38]. While DR probably affects most or all cells, some have argued that many, if not most, of DR's signaling effects and protective effects are mediated by the ventromedial hypothalamus [74]. This question of how DR exerts its effects is important. Does DR firstly act by broad metabolic change or firstly by altering central control by the ventromedial hypothalamus? The answer to this question affects where we should look for epigenetic change and other effects of DR and where we should look for other mechanisms that affect longevity. An important effect of DR is regulation of glucose levels [74]. Glucose in particular has been shown to affect epigenetics in several cell types and, at the whole body level, glucose causes what has been termed "glycemic memory" [13].

Glucose

Much of what we know about epigenetic changes effected by glucose comes from studies of diabetes. The results of these studies are also relevant to DR, diet (especially glycemic index), aging and longevity.

Well-regulated diabetes through intensive therapy (versus conventional therapy) seems to have lasting effects that extend well beyond particular periods of glucose control or specific treatment regimens [75–78]. The Diabetes Control and Complications Trial (DCCT), Epidemiology of Diabetes Interventions and Complications study (EDIC) and the United Kingdom Prospective Diabetes Study indicate that periods of good glycemic control result in large health differences in diabetic subjects (types 1 and 2) many years later [76–80]. These include differences in vascular pathology as well as nephropathy and retinopathy. Several terms are used for this phenomenon including "glycemic memory", "hyperglycemic memory", "legacy effect" and "metabolic memory".

Basic research aimed at understanding hyperglycemic memory often includes cell culture studies and mouse studies comparing high and normal levels of glucose exposure. In particular, in vivo vascular endothelial cells (that line blood vessels) are exposed to high glucose levels from the blood. In vitro, high glucose causes upregulation of extracellular matrix protein expression and upregulation of proinflammatory pathways (e.g. NFkB expression) in vascular endothelial cells [13, 81].

El-Osta, Brownlee and coworkers studied how glucose levels affected gene expression and epigenetics in cultured bovine aortic endothelial cells [82, 83]. They found that when cells were switched from low to high glucose, the level of H3K4 monomethylation increased, and levels of H3K9 di- and trimethylation decreased, on the NFkB-p65 gene. They further showed that high glucose caused the preferential association of the Set7 HMT and the LSD1 histone demethylase on the NFkB-p65 gene and that these changes corresponded with NFkB-p65 transcription. Importantly, this pattern of histone modification, presence of Set7 and LSD1 and NFkB-p65 transcription persisted once glucose levels were returned to normal. This shows that the gene activating histone modifications and the enzymes likely

responsible for them remain with the active NFkB gene well past the period of hyperglycemia. In more recent work, El-Osta and colleagues used primary human aortic cells to show patterns of higher histone acetylation and gene transcription in several locations in the human genome due to transient high glucose [13].

Brasacchio et al. [83] used diabetic, formerly diabetic and control mice to show that several aspects of vascular damage persisted in the diabetic and formerly diabetic mice compared with controls. In similar comparisons, NFkB-p65, vascular adhesion molecule 1, and macrophage chemoattractant protein 1 were all upregulated in the aortas of diabetic and formerly diabetic mice compared with control mice. This indicates that the persistent effects observed in cell culture were also occurring in mice in vivo.

Blood lymphocytes and monocytes are also directly exposed to blood glucose and have been studied for epigenetics and gene expression with normo- and hyperglycemia. Miao et al. [84] did epigenetic studies of inflammation with high glucose in the human monocyte cell line THP-1. They also showed higher H3K9Ac of TNF-alpha and COX-2 promoters in peripheral monocytes of diabetic patients versus those of control subjects. They pointed out that this in vivo evidence provides at least one molecular mechanism by which high glucose and diabetes can promote the expression of inflammatory genes in peripheral monocytes.

Recently, Miao et al. [85] extended their earlier cell culture findings by studying patients treated with intensive versus conventional therapy from the DCCT and EDIC trials. Thirty subjects who received intensive treatment and maintained low glycated hemoglobin (HbA1c) levels in the DCCT and did not progress to retinopathy or nephropathy in the 10-year followup of the EDIC were compared with 30 subjects who received conventional treatment and maintained high HbA1c levels in the DCCT and did progress to retinopathy or nephropathy in the 10-year followup of the EDIC.

Using blood monocytes and lymphocytes and measuring H3K9Ac, H3K9 dimethylation and H3K4 trimethylation, Miao et al. found significantly more promoter regions of monocytes enriched in H3K9Ac in the conventional therapy subject group (high HbA1c in DCCT) compared to the intensive therapy group (low HbA1c in DCCT). Many of the top genes enriched in H3K9Ac were related to the NFkB inflammatory pathway or to diabetes complications. Combining data from the two groups, Miao et al. found that the recently measured H3K9Ac was significantly associated with the mean HbA1c levels measured many years earlier (during the DCCT and EDIC trials). This association was strong with a P value of less than 10^{-15}. These results indicate that past high glucose (as inferred by HbA1c) affects current gene expression (inferred by H3K9Ac) of inflammation and diabetes related genes. Further, this study provides in vivo human evidence for one likely epigenetic mechanism to explain metabolic or glycemic memory.

High glucose causes changes in patterns of gene expression that appear to be somewhat specific to cell type (although large data sets in each cell type to help define this have not been generated). In each case, however the gene expression patterns are one or more of prodiabetic, prohypertensive or proinflammatory and, at least in tissue culture experiments, these epigenetic and gene expression changes

happen quickly (within hours). Because these happen quickly we can ask what aspects of diabetes, inflammation and aging are due to these quick changes in gene expression and which are due to longer term effects of high glucose such as glycated proteins, advanced glycation end products (AGEs) and receptors for advanced glycation end products (RAGEs) [86]. Secondly, we can ask how persistent these changes are and would animals avoid these changes entirely if they were given a low glycemic index diet their whole lives with or without DR. Most rodent diets are made with a high proportion of grains and starches and probably have high glycemic indices [87]. Presumably there are conditions where these epigenetic changes can be partially or largely reversed (DR is a candidate). Conditions that lead to such reversal could be very useful in medicine.

Acetyl and energy metabolism and histone acetylation and methylation
A possible solution to the paradox of genome-wide declines of DNA methylation with age, concomitant with age-related DNA hypermethylation of specific genes, was proposed by Cooney [12, 25]. Similar proposals were made by Wallace et al. [27]. In these proposals, age-related (and cancer related) mitochondrial dysfunction (including the Warburg effect, [88] and other factors), limits AcCoA availability for histone acetylation. Limited histone acetylation limits gene activation resulting in the gradual silencing of genes. Genes not maintained in an active state become targets for DNA hypermethylation which promotes and maintains their silent state. This process may be reversible using diet, nutritional supplements or drugs [12] that improve the availability of AcCoA for histone acetylation and/or that inhibit HDACs to better maintain histone acetylation. For example, the essential nutrient, pantothenate, makes up part of AcCoA and thus pantothenate intake limits AcCoA levels.

To test some of these ideas, Friis et al. [28] studied mitochondrial dysfunction and AcCoA availability in relation to histone acetylation in yeast. They compared control yeast (with mitochondrial DNA) with yeast lacking mitochondrial DNA (rho-zero yeast) as a model of mitochondrial dysfunction. In their study histone acetylation in control yeast was not limited by the supply of AcCoA, however in rho-zero yeast both histone acetylation and AcCoA levels were low. As a workaround to rho-zero status, Friis et al. activated both the AMPK (Snf1) and RTG signaling pathways which increased the supply of AcCoA for HATs and increased histone acetylation.

As a workaround in mammals, Friis et al. suggested that nutritional interventions such as fasting or ketogenic diets might provide therapeutic benefit presumably through increasing histone acetylation and activating silenced genes. Mammalian cells lacking mitochondrial DNA also show increased gene silencing compared to the same cell lines with mitochondrial DNA [89].

Cells that are exposed to high glucose and/or develop mitochondrial dysfunction tend to rely on glycolysis for energy production [12, 25, 27]. Reliance on glycolysis can be expected to lower and change the balance of tricarboxylic acid (TCA) cycle intermediates. Increasingly these TCA cycle intermediates are being recognized as regulators of epigenetics [37]. Interestingly the influences are much more than metabolic with specific TCA cycle intermediates affecting the activities of enzymes

for epigenetic modifications [37, 90]. For example, Tsukada et al. [91] purified a JmjC domain-containing histone demethylase which demethylates histone H3 at lysine 36. Using the substrate alpha-ketoglutarate (2-oxoglutarate) and cofactor Fe2 +, this enzyme oxidizes the methyl group to generate formaldehyde and succinate. Subsequent work has identified additional dependencies between enzymes for epigenetics and TCA cycle intermediates. As reviewed by [37] and Salminen et al. [92], a picture is developing of histone and DNA demethylases using alpha-ketoglutarate as a substrate and being inhibited by their product, succinate and succinate's downstream product in the TCA cycle, fumarate.

Histone acetylation can be maintained by either (or both) increasing acetylation (HAT activity using AcCoA) or inhibiting histone deacetylation (inhibiting HDAC activity). Shimazu et al. [93] reported that d- beta-hydroxybutyrate (BHB, a ketone body) is a specific inhibitor of class I HDACs. BHB is an endogenous metabolite and metabolic conditions will affect its concentration. Shimazu et al. showed in mice that fasting, DR or exogenous BHB increased tissue histone acetylation. Inhibition of HDACs by BHB is another way that cellular metabolic status is coupled to transcriptional regulation.

Dietary HDACIs

Many HDACIs occur naturally in foods such as broccoli (sulforaphane and diindolylmethane) and garlic (allyl mercaptan). These are active against human cancer cells in vitro, against cancers in mice and are in clinical trials against cancer in humans (www.clinicaltrials.gov) [29–33]. HDACI activity in broccoli also appears to be active in young healthy people where eating broccoli sprouts decreased HDAC activity in peripheral white blood cells [29, 32, 94]. HDACIs from everyday foods may cause epigenetic change, possibly with a beneficial trend toward keeping some genes active that are otherwise silenced with cancer and aging. Potential benefits could include slowing or reversing dementia or slowing age- related decline [55] as discussed above in Sect. 2.3. A recent study shows that many genes (thousands of CpG DNA methylation sites) show changes in DNA methylation in normal and cancer human prostate cell lines when treated with sulforaphane or diindolylmethane [95]. Although the potential utility of naturally-occurring and pharmaceutical HDACIs is huge, much more research is needed before we will be able to use these effectively and predictably to prevent disease or affect aging.

Methyl metabolism provides SAM, the substrate for DNA and histone methylation

Diet and metabolism provide methyl groups for epigenetics and the availability of methyl groups to some degree influences the levels of DNA and histone methylation [12, 25, 27]. Further, the relative availability of the various modifying groups (methyl, acetyl, etc.) probably act to broadly influence epigenetics and gene activity [12, 25, 27]. In turn, epigenetics and gene expression affect cell differentiation and animal phenotype [12, 19, 20, 54, 96–98]. The methyl donor SAM is produced by methyl metabolism and is used by DNMTs and HMTs [99]. The product of these methylation reactions, SAH, inhibits DNMTs and probably HMTs [100–103].

SAM is produced from methionine and ATP and the methyl groups for methionine come via dietary methionine or from the recycling of SAH (and homocysteine) by methyl metabolism [25, 27].

Methyl metabolism, and the broader one-carbon metabolism, use folic acid and dietary folates, cobalamin (vitamin B12), zinc, methionine, *S*-methylmethionine, betaine and choline [104–106]. Folate, methionine, vitamin B_{12} and zinc are intermediates used for the transfer and transport of methyl groups in their roles as enzymatic cofactors [25, 27, 107–109]. All of these are obtained from the diet and, except for betaine and *S*-methylmethionine, all are essential nutrients.

2.5 Endogenous Retroviruses (ERVs) and Interspersed Repetitive Elements

Our DNA contains thousands of ERVs which are normal parts of our genomes [110, 111]. ERVs are inherited though the germline (Mendelian inheritance) but are thought to be derived from repeated retroviral infections of our germline cells in the evolutionary past [112]. Some well-studied ERVs include human endogenous retrovirus virus K (HERV-K) and, in mice, IAPs and MuERVs. Generally ERVs tend to be silent in healthy tissue and can remain fixed in the genome for many years and multiple generations [96, 113]. ERVs contain long terminal repeats (LTRs) that can drive viral transcription or that activate or interfere with expression of adjacent "host" genes. Nearby "host" genes can be over expressed, deregulated, silenced and otherwise dysregulated by ERVs [96, 114, 115]. Expression of ERV-encoded proteins, including reverse transcriptase, can cause ERV transposition and other processes that disrupt the genome (e.g. reverse transcription of "host" RNAs) [116]. Epigenetic silencing including DNA methylation of LTRs and many nutritional, metabolic and genetic factors can affect ERV expression [110, 117–120].

Interspersed elements have some features of ERVs such as reverse transcriptase but lack LTRs. These include LINE-1 (L1) elements in humans and L1Md elements in mice. Like ERVs these elements are generally suppressed by DNA methylation [121]. Activation of L1 sequences involves H3K4 trimethylation and H3K9Ac and lower DNMT1 activity—all features of transcriptional activation [122].

ERV and LINE-1 activities are clearly increased, and may be causal, in some human cancers [123, 124]. ERV expression is increased in several autoimmune diseases of humans and mice [111, 125, 126]. ERV expression may be causal or an important mechanist step in autoimmunity. Toxic insult with benzo (a) pyrene [122] or trichloroethylene [127] can increase expression of LINEs and ERVs (respectively) in mice. Due to their high copy number (a few thousand IAPs and even more L1 sequences in the mouse genome), small increases in the expression of repeats could have huge effects if tens or hundreds of repeats per genome increase their

expression. Of course, large increases in expression of just a few repeats could have similar effects.

ERV and L1 hypomethylation and/or transcription have sometimes been associated with aging [128] and are part of the broad hypomethylation of DNA that occurs with aging [1, 41]. The roles of ERV and L1 activation in aging are unclear. At a minimum there is activation of ERVs, L1s and other repeats due to toxic insults, broad hypomethylation or other events that may accumulate over a lifetime [129]. Alternatively, activation of these sequences may play a more specific mechanistic role in aging and/or the induction of senescence [130].

2.6 Conclusions and Future Directions

It should be possible to design combinations of foods, dietary supplements and drugs to shift metabolism and epigenetics in a direction that would maintain healthy patterns of gene expression (i.e. to push patterns of gene expression away from aging and cancer). This dietary design might involve a combination of a low calorie, low glycemic index diet (to control glucose), leafy vegetables and quinoa (for folate, betaine, pantothenate), foods or supplements to supply TCA cycle intermediates. Others have proposed "epigenetic diets" for similar purposes [131, 132].

There are several nonexclusive approaches that are probably useful for designing diets for maintaining epigenetics. However all these approaches should be tested for efficacy.

- Diets designed to provide micronutrients for epigenetics such as an emphasis on foods high in micronutrients and low in calories [105] such as spinach and kale.
- Supplements of micronutrients such as pantothenate, folate, betaine etc.
- Diets and/or supplements high in HDACIs such as those from broccoli and garlic.
- Diets and/or supplements to increase BHB.
- Diets substituting other energy sources such as fats or citric acid cycle intermediates in place of readily digested starches and sugars that raise blood glucose.
- DR, fasting, low glycemic index diets (e.g. paleolithic), or ketogenic diets (e.g. 95 % fat, 5 % protein).

Glucose is clearly important in epigenetics. Even if other aspects of epigenetics are in a healthy range, high glucose will probably still lead to a diabetic pattern of gene expression. Just as certain minimum levels of micronutrients such as pantothenate, folate, betaine and/or choline are needed for the metabolism underlying epigenetics, low to normal glucose will be needed to keep epigenetics in a normal, nondiabetic state. Treatments that would prevent high glucose or prevent the adverse effects of high glucose could be especially valuable for medicine and longevity. Treatments that would reverse the epigenetic effects of transient or

repeated episodes of high glucose would be similarly valuable. Identification of such approaches could be very useful in medicine.

Horvath's epigenetic clock could be a very useful tool, along with yet to be developed epigenetic clocks for mice and other model organisms. Strategies for maintaining or changing epigenetics could be tested for their ability to change the epigenetic clock. Among other applications, it seems that such an epigenetic tool could be a preliminary marker for the success of interventions into aging and lifespan. Subsequent studies could determine if the changed epigenetic clock leads to changes in lifespan.

References

1. Cooney CA (1993) Are somatic cells inherently deficient in methylation metabolism? A proposed mechanism for DNA methylation loss, senescence and aging. Growth Dev Aging 57(4):261–273
2. Issa JP (2014) Aging and epigenetic drift: a vicious cycle. J Clin Invest 124(1):24–29
3. Lee CK, Weindruch R, Prolla TA (2000) Gene-expression profile of the ageing brain in mice. Nat Genet 25(3):294–297
4. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML et al. (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci USA 102 (30):10604–10609.
5. Bartke A, Brown-Borg H (2004) Life extension in the dwarf mouse. Curr Top Dev Biol 63:189
6. Ayyadevara S, Alla R, Thaden JJ, Reis RJS (2008) Remarkable longevity and stress resistance of nematode PI3 K-null mutants. Aging Cell 7(1):13–22
7. Weindruch R (1996) The retardation of aging by caloric restriction: studies in rodents and primates. Toxicol Pathol 24(6):742–745
8. Spindler SR (2010) Caloric restriction: from soup to nuts. Ageing Res Rev 9(3):324–353
9. Lombard DB, Miller RA (2014) Aging, disease, and longevity in mice. Ann Rev Gerontol Geriatr 34:93–138
10. Vera E, de Jesus BB, Foronda M, Flores JM, Blasco MA (2013) Telomerase reverse transcriptase synergizes with calorie restriction to increase health span and extend mouse longevity. PLoS One 8(1):e53760
11. Cooney CA (2007) Epigenetics—DNA-based mirror of our environment? Dis Markers 23(1–2):121–137
12. Cooney CA (2010) Drugs and supplements that may slow aging of the epigenome. Drug Discov Today 7:57–64
13. Cooper ME, El-Osta A (2010) Epigenetics mechanisms and implications for diabetic complications. Circ Res 107(12):1403–1413
14. Cooney CA, Gilbert KM (2012) Toxicology, epigenetics, and autoimmunity. In: Sahu SC (ed) Toxicology and Epigenetics, John Wiley & Sons, Ltd., Chichester, UK, pp. 241–260
15. Gerhäuser C (2012) Cancer cell metabolism, epigenetics and the potential influence of dietary components–a perspective. Biomed Res 23:1–21
16. Dawson MA, Kouzarides T (2012) Cancer epigenetics: from mechanism to therapy. Cell 150 (1):12–27
17. Ooi SKT, O'Donnell AH, Bestor TH (2009) Mammalian cytosine methylation at a glance. J Cell Sci 122(16):2787–2791

18. Cropley JE, Suter CM, Beckman KB, Martin DI (2006) Germ-line epigenetic modification of the murine Avy allele by nutritional supplementation. Proc Natl Acad Sci USA 103 (46):17308–17312
19. Champagne FA, Curley JP (2009) Epigenetic mechanisms mediating the long-term effects of maternal care on development. Neurosci Biobehav R 33(4):593–600
20. Li CCY, Cropley JE, Cowley MJ, Preiss T, Martin DIK, Suter CM (2011) A sustained dietary change increases epigenetic variation in isogenic mice. Plos Genet. 7(4):e1001380
21. Weaver ICG, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ et al (2005) Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. J Neurosci 25(47):11045–11054
22. Hackett JA, Sengupta R, Zylicz JJ, Murakami K, Lee C, Down TA et al (2013) Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. Science 339(6118):448–452
23. Seisenberger S, Peat JR, Hore TA, Santos F, Dean W, Reik W (2013) Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers. Philos T R Soc B 368(1609):20110330
24. Kooistra SM, Helin K (2012) Molecular mechanisms and potential functions of histone demethylases. Nat Rev Mol Cell Bio 13(5):297–311
25. Cooney CA (2008) Cancer and aging: the epigenetic connection. In: Tollefsbol T, (ed) Cancer epigenetics, CRC Press, Boca Raton, pp 303–316
26. Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR, Thompson CB (2009) ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324(5930):1076–1080
27. Wallace DC, Fan WW, Procaccio V (2010) Mitochondrial energetics and therapeutics. Annu Rev Pathol Mech 5:297–348
28. Friis RMN, Glaves JP, Huan T, Li L, Sykes BD, Schultz MC (2014) Rewiring AMPK and mitochondrial retrograde signaling for metabolic control of aging and histone acetylation in respiratory-defective cells. Cell Rep 7(2):565–574
29. Nian H, Delage B, Ho E, Dashwood RH (2009) Modulation of histone deacetylase activity by dietary isothiocyanates and allyl sulfides: studies with sulforaphane and garlic organosulfur compounds. Environ Mol Mutagen 50(3):213–221
30. Meeran SM, Patel SN, Tollefsbol TO (2010) Sulforaphane causes epigenetic repression of HTERT expression in human breast cancer cell lines. PLoS One 5(7):e11457
31. Meeran SM, Patel SN, Li YY, Shukla S, Tollefsbol TO (2012) Bioactive dietary supplements reactivate ER expression in ER-negative breast cancer cells by active chromatin modifications. PLoS One 7(5):e37748
32. Clarke JD, Riedl K, Bella D, Schwartz SJ, Stevens JF, Ho E (2011) Comparison of isothiocyanate metabolite levels and histone deacetylase activity in human subjects consuming broccoli sprouts or broccoli supplement. J Agr Food Chem 59(20):10955–10963
33. Ernst IMA, Schuemann C, Wagner AE, Rimbach G (2011) 3,3′-Diindolylmethane but not indole-3-carbinol activates Nrf2 and induces Nrf2 target gene expression in cultured murine fibroblasts. Free Radical Res 45(8):941–944
34. Kurokawa R, Rosenfeld MG, Glass CK (2009) Transcriptional regulation through noncoding RNAs and epigenetic modifications. RNA Biology 6(3):233–236
35. Collins LJ, Schonfeld B, Chen XS (2011) The epigenetics of non-coding RNA. In: Tollefsbol T. (ed) Handbook of epigenetics: the new molecular and medical genetics. London Academic, pp 49–61
36. Lee JT (2012) Epigenetic regulation by long noncoding RNAs. Science 338(6113):1435–1439
37. Gerhauser C (2012) Cancer cell metabolism, epigenetics and the potential influence of dietary components—a perspective. Biomed Res India 23:69–89
38. Dhahbi JM, Spindler SR, Atamna H, Yamakawa A, Guerrero N, Boffelli D et al (2013) Deep sequencing identifies circulating mouse miRNAs that are functionally implicated in manifestations of aging and responsive to calorie restriction. Aging 5(2):130–141

39. Vanyushin BF, Nemirovsky LE, Klimenko VV, Vasiliev VK, Belozersky AN (1973) The 5-methylcytosine in DNA of rats. Tissue and age specificity and the changes induced by hydrocortisone and other agents. Gerontologia 19(3):138–152
40. Vanyushin BF, Romanenko EB (1979) Changes in DNA methylation in rats during ontogenesis and under the influence of hydrocortisone. Biochem Moscow 44(1):65–70
41. Singhal RP, Mays-Hoopes LL, Eichhorn GL (1987) DNA methylation in aging of mice. Mech Ageing Dev 41(3):199–210
42. Wilson VL, Smith RA, Ma S, Cutler RG (1987) Genomic 5-methyldeoxycytidine decreases with age. J Biol Chem 262(21):9948–9951
43. Reis RJS, Goldstein S (1982) Variability of DNA methylation patterns during serial passage of human—diploid fibroblasts. P Natl Acad Sci-Biol 79(13):3949–3953
44. Wilson VL, Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220(4601):1054–1057
45. Post WS, Goldschmidt-Clermont PJ, Wilhide CC, Heldman AW, Sussman MS, Ouyang P et al (1999) Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. Cardiovasc Res 43(4):985–991
46. Kwabi-Addo B, Chung WB, Shen L, Ittmann M, Wheeler T, Jelinek J et al (2007) Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 13 (13):3796–3802
47. Maegawa S, Hinkal G, Kim HS, Shen LL, Zhang L, Zhang JX et al (2010) Widespread and tissue specific age-related DNA methylation changes in mice. Genome Res 20(3):332–340
48. Horvath S (2013) DNA methylation age of human tissues and cell types. Genome Biol 14 (10):R115
49. Jones PA (1999) The DNA methylation paradox. Trends Genet 15(1):34–37
50. Esteller M (2005) Aberrant DNA methylation as a cancer-inducing mechanism. Ann Rev Pharmacol 45:629–656
51. Fernandez AF, Assenov Y, Martin-Subero JI, Balint B, Siebert R, Taniguchi H et al (2012) A DNA methylation fingerprint of 1628 human samples. Genome Res 22(2):407–419
52. Jeffries MA, Dozmorov M, Tang YH, Merrill JT, Wren JD, Sawalha AH (2011) Genome-wide DNA methylation patterns in CD4(+) T cells from patients with systemic lupus erythematosus. Epigenetics 6(5):593–601
53. Russanova VR, Hirai TH, Howard BH (2004) Semirandom sampling to detect differentiation-related and age-related epigenome remodeling. J Gerontol Biol 59 (12):1221–1233
54. Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, Agis-Balboa RC, Fischer A (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328(5979):753–756
55. Bahari-Javan S, Sananbenesi F, Fischer A (2014) Histone-acetylation: a link between Alzheimer's disease and post-traumatic stress disorder? Front Neurosci 8:160
56. Kilgore M, Miller CA, Fass DM, Hennig KM, Haggarty SJ, Sweatt JD et al (2010) Inhibitors of class 1 histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer's disease. Neuropsychopharmacology 35(4):870–880
57. Nalivaeva NN, Belyaev ND, Kerridge C, Turner AJ (2014) Amyloid-clearing proteins and their epigenetic regulation as a therapeutic target in Alzheimer's disease. Front Aging Neurosci 6:235
58. Miller CA, Sweatt JD (2007) Covalent modification of DNA regulates memory formation. Neuron. 53(6):857–869
59. Montalvo-Ortiz JL, Keegan J, Gallardo C, Gerst N, Tetsuka K, Tucker C et al (2014) HDAC inhibitors restore the capacity of aged mice to respond to haloperidol through modulation of histone acetylation. Neuropsychopharmacology 39(6):1469–1478
60. Metivier R, Gallais R, Tiffoche C, Le Peron C, Jurkowska RZ, Carmouche RP et al (2008) Cyclical DNA methylation of a transcriptionally active promoter. Nature 452(7183):45–50
61. Maeda H, Gleiser CA, Masoro EJ, Murata I, Mcmahan CA, Yu BP (1985) Nutritional influences on aging of Fischer 344 rats. II Pathology. J Gerontol 40(6):671–688

62. Fontana L (2009) Neuroendocrine factors in the regulation of inflammation: excessive adiposity and calorie restriction. Exp Gerontol 44(1–2):41–45
63. Weindruch R, Albanes D, Kritchevsky D (1991) The role of calories and caloric restriction in carcinogenesis. Hematol Oncol Clin N 5(1):79–89
64. Fu PP, Dooley KL, Vontungeln LS, Bucci T, Hart RW, Kadlubar FF (1994) Caloric restriction profoundly inhibits liver-tumor formation after initiation by 6-nitrochrysene in male-mice. Carcinogenesis 15(2):159–161
65. Hart RW, Turturro A (1997) Dietary restrictions and cancer. Environ Health Persp 105:989–992
66. Dhahbi JM, Kim HJ, Mote PL, Beaver RJ, Spindler SR (2004) Temporal linkage between the phenotypic and genomic responses to caloric restriction. Proc Natl Acad Sci USA 101 (15):5524–5529
67. Fontana L, Klein S, Holloszy JO (2010) Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action, and adipokine production. Age 32 (1):97–108
68. Soare A, Cangemi R, Omodei D, Holloszy JO, Fontana L (2011) Long-term calorie restriction, but not endurance exercise, lowers core body temperature in humans. Aging (Albany NY) 3(4):374–379
69. Ingram DK, Roth GS (2011) Glycolytic inhibition as a strategy for developing calorie restriction mimetics. Exp Gerontol 46(2–3):148–154
70. Carrillo AE, Flouris AD (2011) Caloric restriction and longevity: Effects of reduced body temperature. Ageing Res Rev 10(1):153–162
71. Masoro EJ, Mccarter RJM, Katz MS, Mcmahan CA (1992) Dietary restriction alters characteristics of glucose fuel use. J Gerontol 47(6):B202–B208
72. Sell C (2003) Caloric restriction and insulin-like growth factors in aging and cancer. Horm Metab Res 35(11–12):705–711
73. Kapahi P, Chen D, Rogers AN, Katewa SD, Li PWL, Thomas EL et al (2010) With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab 11(6):453–465
74. Dacks PA, Moreno CL, Kim ES, Marcellino BK, Mobbs CV (2013) Role of the hypothalamus in mediating protective effects of dietary restriction during aging. Front Neuroendocrinol 34(2):95–106
75. Shamoon H, Duffy H, Fleischer N, Engel S, Saenger P, Strelzyn M et al (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes-mellitus. New Engl J Med 329(14):977–986
76. Shamoon H, Duffy H, Dahms W, Mayer L, Brillion D, Lackaye M et al (2000) Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. New Engl J Med 342(6):381–389
77. Steffes MW, Chavers BM, Molitch ME, Cleary PA, Lachin JM, Genuth S et al (2003) Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy—the epidemiology of diabetes interventions and complications (EDIC) study. J Am Med Assoc 290(16):2159–2167
78. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA (2008) 10-year follow-up of intensive glucose control in type 2 diabetes. New Engl J Med 359(15):1577–1589
79. Genuth S, Lipps J, Lorenzi G, Nathan DM, Davis MD, Lachin JM et al (2002) Effect of intensive therapy on the microvascular complications of type 1 diabetes mellitus. J Am Med Assoc 287(19):2563–2569
80. Nathan DM, Cleary PA, Backlund JYC, Genuth SM, Lachin JM, Orchard TJ et al (2005) Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. New Engl J Med 353(25):2643–2653
81. Roy S, Sala R, Cagliero E, Lorenzi M (1990) Overexpression of fibronectin induced by diabetes or high glucose—phenomenon with a memory. Proc Natl Acad Sci USA 87(1):404–408

82. El-Osta A, Brasacchio D, Yao DC, Pocai A, Jones PL, Roeder RG et al (2008) Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. J Exp Med 205(10):2409–2417
83. Brasacchio D, Okabe J, Tikellis C, Balcerczyk A, George P, Baker EK et al (2009) Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that coexist on the lysine tail. Diabetes 58(5):1229–1236
84. Miao F, Gonzalo IG, Lanting L, Natarajan R (2004) In vivo chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. J Biol Chem 279 (17):18091–18097
85. Miao F, Chen Z, Genuth S, Paterson A, Zhang LX, Wu XW et al (2014) Evaluating the role of epigenetic histone modifications in the metabolic memory of type 1 diabetes. Diabetes 63 (5):1748–1762
86. Chilelli NC, Burlina S, Lapolla A (2013) AGEs, rather than hyperglycemia, are responsible for microvascular complications in diabetes: a "glycoxidation-centric" point of view. Nutr Metab Cardiovas 23(10):913–919
87. Kim TH, Petrou S, Reid CA (2014) Low glycaemic index diet reduces seizure susceptibility in a syndrome—specific mouse model of generalized epilepsy. Epilepsy Res 108(1):139–143
88. Warburg O (1956) On respiratory impairment in cancer cells. Science 124(3215):269–270
89. Xie CH, Naito A, Mizumachi T, Evans TT, Douglas MG, Cooney CA et al (2007) Mitochondrial regulation of cancer associated nuclear DNA methylation. Biochem Bioph Res Co 364(3):656–661
90. Teperino R, Schoonjans K, Auwerx J (2010) Histone methyl transferases and demethylases; can they link metabolism and transcription. Cell Metab 12(4):321–327
91. Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P et al (2006) Histone demethylation by a family of JmjC domain-containing proteins. Nature 439 (7078):811–816
92. Salminen A, Kaarniranta K, Hiltunen M, Kauppinen A (2014) Krebs cycle dysfunction shapes epigenetic landscape of chromatin: Novel insights into mitochondrial regulation of aging process. Cell Signal 26(7):1598–1603
93. Shimazu T, Hirschey MD, Newman J, He WJ, Shirakawa K, Le Moan N et al (2013) Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science 339(6116):211–214
94. Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E (2007) Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med 232(2):227–234
95. Wong CP, Hsu A, Buchanan A, Palomera-Sanchez Z, Beaver LM, Houseman EA, et al (2014) Effects of sulforaphane and 3,3′-diindolylmethane on genome-wide promoter methylation in normal prostate epithelial cells and prostate cancer cells. PLoS One 9(1): e86787
96. Wolff GL, Kodell RL, Moore SR, Cooney CA (1998) Maternal epigenetics and methyl supplements affect agouti gene expression in A(vy)/a mice. FASEB J 12(11):949–957
97. Cooney CA, Dave AA, Wolff GL (2002) Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr 132(8):2393S–2400S
98. Eilertsen KJ, Floyd ZE, Gimble JM (2008) The epigenetics of adult (somatic) stem cells. Crit Rev Eukar Gene 18(3):189–206
99. Yoo J, Medina-Franco JL (2012) Trimethylaurintricarboxylic acid inhibits human DNA methyltransferase 1: insights from enzymatic and molecular modeling studies. J Mole Model 18(4):1583–1589
100. Yi P, Melnyk S, Pogribna M, Pogribny IP, Hines RJ, James SJ (2000) Increase in plasma homocysteine associated with parallel increases in plasma *S*-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 275(38):29318–29323
101. Jacobs SA, Harp JM, Devarakonda S, Kim Y, Rastinejad F, Khorasanizadeh S (2002) The active site of the SET domain is constructed on a knot. Nat Struct Biol 9:833–838

102. Cohen HM, Griffiths AD, Tawfik DS, Loakes D (2005) Determinants of cofactor binding to DNA methyltransferases: insights from a systematic series of structural variants of S-adenosylhomocysteine. Org Biomol Chem 3(1):152–161
103. Chen NC, Yang F, Capecci LM, Gu ZY, Schafer AI, Durante W et al (2010) Regulation of homocysteine metabolism and methylation in human and mouse tissues. Faseb J 24(8):2804–2817
104. Augspurger NR, Scherer CS, Garrow TA, Baker DH (2005) Dietary S-methylmethionine, a component of foods, has choline-sparing activity in chickens. J Nut 135(7):1712–1717
105. Cooney CA (2006) Maternal nutrition: nutrients and control of expression. In: Kaput J, Rodriguez RL (ed) Nutrigenomics: concepts and technologies. Wiley, Hoboken pp 219–254
106. Scherb J, Kreissl J, Haupt S, Schieberle P (2009) Quantitation of *S*-methylmethionine in raw vegetables and green malt by a stable isotope dilution assay using LC-MS/MS: comparison with dimethyl sulfide formation after heat treatment. J Agr Food Chem 57(19):9091–9096
107. Cooney CA (2009) Nutrients, epigenetics, and embryonic development. In: Choi SW, Friso S (ed) Nutrients and Epigenetics, CRC Press, Boca Raton pp 155–174
108. Millian NS, Garrow TA (1998) Human betaine-homocysteine methyltransferase is a zinc metalloenzyme. Arch Biochem Biophys 356(1):93–98
109. Uthus EO, Brown-Borg HM (2006) Methionine flux to transsulfuration is enhanced in the long living Ames dwarf mouse. Mech Ageing Dev 127(5):444–450
110. Walsh CP, Chaillet JR, Bestor TH (1998) Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat Genet 20(2):116–117
111. Perl A, Fernandez D, Telarico T, Phillips PE (2010) Endogenous retroviral pathogenesis in lupus. Curr Opin Rheumatol 22(5):483–492
112. Bannert N, Kurth R (2004) Retroelements and the human genome: new perspectives on an old relation. Proc Natl Acad Sci USA 5(101):14572–14579
113. Morgan HD, Sutherland HGE, Martin DIK, Whitelaw E (1999) Epigenetic inheritance at the agouti locus in the mouse. Nat Genet 23(3):314–318
114. Rakyan VK, Chong S, Champ ME, Cuthbert PC, Morgan HD, Luu KVK et al (2003) Transgenerational inheritance of epigenetic states at the murine Axin(Fu) allele occurs after maternal and paternal transmission. Proc Natl Acad Sci USA 100(5):2538–2543
115. Druker R, Bruxner TJ, Lehrbach NJ, Whitelaw E (2004) Complex patterns of transcription at the insertion site of a retrotransposon in the mouse. Nucleic Acids Res 32(19):5800–5808
116. Romanish MT, Cohen CJ, Mager DL (2010) Potential mechanisms of endogenous retroviral-mediated genomic instability in human cancer. Semin Cancer Biol 20(4):246–253
117. Gaudet F, Rideout WM, Meissner A, Dausman J, Leonhardt H, Jaenisch R (2004) Dnmt1 expression in pre- and postimplantation embryogenesis and the maintenance of IAP silencing. Mol Cell Biol 24(4):1640–1648
118. Schulz WA, Steinhoff C, Florl AR (2006) Methylation of endogenous human retroelements in health and disease. Curr Top Microbiol 310:211–250
119. Reiss D, Zhang Y, Rouhi A, Reuter M, Mager DL (2010) Variable DNA methylation of transposable elements the case study of mouse early transposons. Epigenetics 5(1):68–79
120. Cherkasova E, Malinzak E, Rao S, Takahashi Y, Senchenko VN, Kudryavtseva AV et al (2011) Inactivation of the von Hippel-Lindau tumor suppressor leads to selective expression of a human endogenous retrovirus in kidney cancer. Oncogene 30(47):4697–4706
121. Bulut-Karslioglu A, De La Rosa-Velazquez IA, Ramirez F, Barenboim M, Onishi-Seebacher M, Arand J et al (2014) Suv39h-dependent H3K9me3 marks intact retrotransposons and silences LINE elements in mouse embryonic stem cells. Mol Cell 55(2):277–290
122. Teneng I, Montoya-Durango DE, Quertermous JL, Lacy ME, Ramos KS (2011) Reactivation of L1 retrotransposon by benzo(a)pyrene involves complex genetic and epigenetic regulation. Epigenetics 6(3):355–367
123. Rodic N, Burns KH (2013) Long interspersed element-1 (LINE-1): passenger or driver in human neoplasms? PLoS Genetics 9(3):e1003402

124. Patnala R, Lee SH, Dahlstrom JE, Ohms S, Chen L, Dheen ST et al (2014) Inhibition of LINE-1 retrotransposon-encoded reverse transcriptase modulates the expression of cell differentiation genes in breast cancer cells. Breast Cancer Res Treat 143(2):239–253
125. Balada E, Vilardell-Tarres M, Ordi-Ros J (2010) Implication of human endogenous retroviruses in the development of autoimmune diseases. Int Rev Immunol 29(4):351–370
126. Baudino L, Yoshinobu K, Morito N, Santiago-Raber ML, Izui S (2010) Role of endogenous retroviruses in murine SLE. Autoimmun Rev 10(1):27–34
127. Gilbert KM, Nelson AR, Cooney CA, Reisfeld B, Blossom SJ (2012) Epigenetic alterations may regulate temporary reversal of CD4(+) T Cell activation caused by trichloroethylene exposure. Toxicol Sci 127(1):169–178
128. Mayes-Hoopes L, Chao W, Butcher HC, Huang RCC (1986) Decreased methylation of the major mouse long interspersed repeated DNA during aging and in myeloma cells. Dev Genet 7(2):65–73
129. St Laurent G, Hammell N, McCaffrey TA (2010) A LINE-1 component to human aging: do LINE elements exact a longevity cost for evolutionary advantage? Mech Ageing Dev 131 (5):299–305
130. Belancio VP, Roy-Engel AM, Pochampally RR, Deininger P (2010) Somatic expression of LINE-1 elements in human tissues. Nucleic Acids Res 38(12):3909–3922
131. Hardy TM, Tollefsbol TO (2011) Epigenetic diet: impact on the epigenome and cancer. Epigenomics 3(4):503–518
132. Bacalini MG, Friso S, Olivieri F, Pirazzini C, Giuliani C, Capri M, et al (2014) Present and future of anti- ageing epigenetic diets. Mech Ageing Dev 136:101–115

Chapter 3
Anti-inflammatory Action of Calorie Restriction Underlies the Retardation of Aging and Age-Related Diseases

Dae Hyun Kim, Eun Kyeong Lee, Min Hi Park, Byoung Chul Kim, Ki Wung Chung, Byung Pal Yu and Hae Young Chung

Abstract Calorie restriction (CR) is known to extend lifespan and has anti-oxidative properties that lead to physiological and biological resistance against diseases and stress. This chapter reviews the molecular mechanisms of CR's anti-inflammatory actions during aging. The crux of CR's ability to attenuate age-related chronic inflammation is related to its power to maintain redox status by modulating oxidative stress during aging. Here, for better molecular insights, key transcription factors such as FoxO, Nrf2, and PPARs induced by CR are described for their modulation of the age-related inflammation response. In addition, recent analyses by systems biology on chronic inflammation, age-related pro-inflammatory gene activation, and CR's suppression produced evidence identifing various target genes, target molecules, and their networks. The wide implication of the proinflammatory process under

Dae Hyun Kim, Eun Kyeong Lee and Min Hi Park contributed equally to this manuscript.

D.H. Kim · E.K. Lee · M.H. Park · B.C. Kim · K.W. Chung · H.Y. Chung (✉)
Molecular Inflammation Research Center for Aging Intervention (MRCA),
College of Pharmacy, Pusan National University, Gumjung-gu, Busan 609-735, Korea
e-mail: hyjung@pusan.ac.kr

D.H. Kim
e-mail: bioimmune@hanmail.net

E.K. Lee
e-mail: goeklee@gmail.com

M.H. Park
e-mail: angelpmh@nate.com

B.C. Kim
e-mail: bcghim@gmail.com

K.W. Chung
e-mail: kieungc@pusan.ac.kr

B.P. Yu
Department of Physiology, The University of Texas Health Science Center,
San Antonio, TX 78229, USA
e-mail: yu6936@sbcglobal.net

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pathophysiological conditions are further detailed in this chapter by including autophagic activity and the activation of the recently recognized inflammasome, a component of key innate immunological defenses and ER stress. This chapter summarizes the evidence that CR suppresses molecular inflammation through the proper maintenance of redox status, NF-κB signaling, inflammasome activation, ER stress, and insulin sensitivity, which in turn lead to the intervention of aging processes and age-related diseases.

3.1 Introduction

The aging process can be described as progressive, physio-pathological deteriorations over time through multiple, complex interactions between genes and environments that ultimately lead to the failure of homeostasis and increased risk of diseases or death. Underlying the aging process is a chronic inflammatory process state that is exacerbated by increased oxidative stress due to augmented reactive oxygen species (ROS), lipid peroxidation, and protein oxidation [1]. Recent advances in medical research recognize well that major age-associated diseases such as Alzheimer's disease, arthritis, osteoporosis, cancer, diabetes and cardiovascular diseases are all likely causally linked to an underlying chronic inflammatory process. Uncontrolled oxidative stress activates redox-sensitive transcription factors that lead to an age-related chronic inflammatory process at the molecular level. Termed, "molecular inflammation", this process has revealed molecular insights into pro-inflammatory events; thereby emphasizing the importance of the molecular mechanisms that act as precursors to fully expressed inflammatory phenomena.

Calorie restriction (CR) has been shown to delay age-related biologic changes and suppress a number of age-associated pathologic abnormalities in both genders and across mammalian and non-mammalian species, and is thus regarded as the gold standard in aging intervention research [1]. Molecular evidence strongly supports CR's ability to reduce age-related oxidative stress and suppress chronic inflammation [2]. Many researchers now regard CR as the only established anti-aging experimental paradigm. CR's anti-aging effects are thought to be due mainly to its powerful regulation of oxidative stress and ability to maintain a proper cellular redox status [1].

In this chapter, we review recently published experimental data on age-related alterations in the redox signaling pathway, the aggravated pro-inflammatory state, the emerging roles of inflammasomes, endoplasmic reticulum (ER) stress, insulin resistance and the ability of CR to modulate these age-related conditions.

3.2 Age-Related Redox Signaling Pathway and Its Modulation by CR

3.2.1 Age-Related Redox Imbalance and Redox Signaling Pathway

Although the precise underlying cause of aging is not well established, a current and widely accepted mechanism of aging is the 'oxidative stress hypothesis', studies of which through the years have provided molecular insights into the possible causative roles of aging [3]. According to the oxidative stress hypothesis, oxidative damage is elicited by the combined effects of uncontrolled production of reactive species (RS) such as ROS, reactive nitrogen species (RNS), and reactive lipid species, and weakened anti-oxidative defense systems, which together form a crucial component of an imbalance in an organism's overall redox state that leads to aging and age-related degenerative diseases [3].

Biological sources of RS production vary widely depending on various cellular activities related to them such as lipoxygenase, cyclooxygenase (COX), plasma membrane-associated NADPH oxidase, mitochondrial electron transport system, ubiquinone, NADH dehydrogenase, cytochrome P450, cytochrome b5, microsomal electron transport, flavoproteins and oxidases in peroxisome, and xanthine oxidase (XOD) in cytosol [4]. One important source of superoxide production comes from the intracellular conversion of xanthine dehydrogenase (XDH) to XOD [5]. We found the conversion of XDH to XOD increased in aged liver or kidney, which showed that XOD-derived RS generation correspondingly increased with age, peaking at 24 months in aged rats. Also, COX, a key enzyme in the prostaglandins (PG) cascade, converts arachidonic acid to Prostaglandin H2 (PGH2). During the conversion of PGG2 to PGH2 by COX, RS are generated. The RS produced by the PG cascade can add significantly to the overall RS pool under both normal and pathological conditions, particularly during aging. In addition, Zou et al. [6] showed that the high level of lysophosphatidylcholines (LPC) observed in old animals is likely a stimulus for the generation of RS through activation of the 5-lipoxygenase pathway and led to their finding of enhanced oxidative stress in old rat aorta.

To forestall oxidative environments, organisms have various antioxidant defenses such as the classical antioxidant enzymes, superoxide dismutase (SOD), glutathione (GSH), and catalase, as well as non-enzymatic ROS scavengers, including vitamin E, vitamin C, β-carotene, and uric acid. Among them, GSH is most abundant and an important intracellular thiol redox regulator that plays a major role in both the maintenance of redox status and in the protection of cells from electrophilic and oxidative attacks [7]. GSH depletion on the other hand causes various biochemical and pathological changes, including mitochondrial dysfunction and swelling [8]. GSH depletion also is linked to aging processes and age-related diseases such as cardiovascular and neurodegenerative diseases. In addition, thioredoxin (Trx) has been described as an another important member of the anti oxidative defense system [8]. Trx modulates redox reactions by the reversible oxidation of its active center

dithiol to disulfide and catalyzes dithiol-disulfide exchange reactions [8] involving many thiol-dependent cellular processes including the gene expression and signal transduction of pro-inflammatory transcription factors such as nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and hypoxia-inducible factor 1 (HIF-1). Kim et al. [9] showed that age-related increases in redox-sensitive transcription factors, such as NF-κB, AP-1, HIF-1, are associated with increases in cellular oxidative stress, nuclear Trx, and redox effector factor-1 (Ref-1).

Redox balance is important as the physiological acid-base buffer system for the body's optimal operation of homeostatic cellular activities. Changes in redox balance are known to affect cellular signaling pathways and transcriptional activities as most of their reactions and activation depend on reduction/oxidation processes [10].

Cellular redox signaling involving kinases generally influences protein tyrosine kinase (PTK)/protein tyrosine phosphatase (PTP) located near the plasma membrane and then travels to serine/threonine kinases/phosphatase distributed close to the nuclei [11]. A defective or inappropriate shift in either PTK or PTP leads to aberrant tyrosine phosphorylation that contributes to the pathogenesis of many diseases, including cancer and diabetes.

Recently, our laboratory reported that a PTK/PTP imbalance due to activated PTK and inactivated PTP occurred from increased age-related oxidative stress in aged kidney [12]. This study further showed that SRC-family PTKs (SFKs) participate in the remarkably increased PTK activity observed during aging and that among the SFKs, lymphocyte-specific protein tyrosine kinase (LCK) in particular plays an important role in the regulation of PTK activity in the aged kidney [13]. In addition, an age-related oxidative stress-induced PTK/PTP imbalance led to an inactivation of Protein phosphatase-2A (PP2A). PP2A inactivation caused by oxidative stress increases the activation of NF-κB-inducing kinase (NIK)/IkB kinase (IKK) and Mitogen-activated protein kinases (MAPKs) that subsequently lead to NF-κB activation, amplifying the NF-κB-related inflammatory process of aging [14].

The well-known, diverse nuclear factor, NF-κB is a ubiquitous transcription factor that plays a crucial role in regulating inflammation, immunity, cell survival, and apoptosis [15], and it is characteristically very sensitive to oxidative stress [16]. It is known now that age-related oxidative stress imposes substantial influence on the entire activation process by regulating the dissociation of IκBα to facilitate NF-κB's nuclear translocation [9]. According to our recent study, oxidation lipids, glycated proteins, and hormones are closely associated with oxidative stress and these products lead to NF-κB activation during aging [16, 17].

4-Hydroxynonenal (HNE), a lipid peroxidation product, is a strong representative of oxidative agents, and HNE or HNE-bound proteins are shown to increase in serum of aged rat compared to that of young rats [17]. HNE is well-known to trigger NF-κB activation by IKK phosphorylation and IκBα degradation. Also, we have shown advanced glycation endproducts (AGE) accumulation and RAGE-induced NF-κB activation as the major culprits responsible for the NF-κB activation in aged rats [18]. Experimental data suggest that age-related NF-κB activation is affected by AGE-induced NADPH oxidase activation [18]. Recent work from Kim et al. [19] has shown that angiotensin II, a major effector of the rennin-angiotensin system,

influences the phosphorylation of p65-mediated NF-κB activation during aging. Increased phosphorylation of p65 at Ser 536 was mediated by the enhanced phosphorylation of IKK, while phosphorylation site Ser 276 of p65 was mediated by upregulated MSK-1. Furthermore, Insulin/IGF1 signalling can promote NF-κB signalling and potentiate aging and age-related degeneration by activation of the phosphoinositide 3 kinase PI3K/AKT pathway [20]. These data reveal that the cellular signaling pathways are modulated through an interdependent network that is readily influenced by the redox state [21].

3.2.2 Redox Modulation by CR during Aging

Recent results from CR research showed that CR prevents the PTK/PTP imbalance caused by increased oxidative stress during aging [12]. In addition, studies showed that a properly maintained balance between PTK and PTP from CR prevented the phosphorylation of NF-κB signaling, including NIK/IKK and MAPKs [12]. Also, the effect of CR on PP2A activation, which can be regulated by a PTK/PTP balance, has been recently reported [14].

Furthermore, we have reported gene expression data with the knowledge obtained from protein–protein interaction (PPI) networks, termed the interactome. This approach allows researchers to analyze global gene expression changes in aging, as well as to select valuable key molecules from a large number of genes using the connections between genes. On the basis of an analysis of the PPI network composed of differentially expressed genes (DEGs) in aging and CR, we selected three genes (LCK, PLSCR I, NDRG I) that are upregulated in aging but downregulated by CR and two genes (MBP, SLC2A4) that are downregulated in aging but upregulated by CR as hubs that have high degree and centrality scores in the PPI network. Our integrative analysis revealed that one of the key molecules is activated SRC-family PTKs, LCK, during aging, but is effectively inactivated by CR [13].

In summary, redox imbalance is due to a gradual oxidant overload over time, as amplified inflammation coupled with weakened anti-oxidative defense systems gives rise to the disruption of the host's homeostasis. It is suggested that CR's anti-aging effects result from its ability to enhance an organism's resistance to oxidative stress and maintenance of normal redox status.

3.3 Modulation of Inflammation-Related Molecular Pathways by CR as a Possible Mechanism of Its Ability to Delay Aging Processes

Among the several well-known hypotheses of aging, the oxidative stress hypothesis currently offers the most plausible mechanistic description of aging and age-related chronic disease processes [1]. More recent research reports provide evidence that

oxidative processes are major factors in the activation of redox-sensitive inflammatory processes and that they link normal aging processes to age-related chronic diseases [22].

A growing body of evidence suggests that the dysregulation of the immune system with age and the impaired redox balance during aging are important causes of unresolved chronic inflammation [23].

NF-κB is known to be activated by a wide variety of stimuli including infection, inflammation, and oxidative stress [24]. NF-κB also transactivates a number of proinflammatory, apoptotic, and oncogenic genes that collectively function to foster cellular adaptation to stress [24]. Studies from our laboratory show that the activation of redox-sensitive NF-κB also plays a pivotal role in modulating the cellular signaling mechanism for oxidative stress-induced inflammation during aging [22]. Stimulus-initiated intracellular signaling cascades lead to phosphorylation of the inhibitory protein κB (IκB) by IKKs. Stimulus-mediated phosphorylation of NF-κB and the subsequent proteolytic degradation of IκB [25] allow the release and nuclear translocation of NF-κB, where it transactivates a number of target genes. Previous studies from our laboratory and others show interaction among NIK/IKK, MAPKs, and NF-κB activation during aging [16].

CR increases lifespan in various species of animals such as fly, yeast, worm, and rat. CR also has been shown to delay age-related biologic changes and suppress a number of age-associated pathologic abnormalities in both sexes and across mammalian and non-mammalian species [26]. However, Demetrius [27] reported no effect of CR in species with late sexual maturity, a broad reproductive span, body size, metabolic rate, and, in human, high entropy. Otherwise, CR is shown to inhibit protein synthesis, oxidize protein load in liver and skeletal muscle, enhance immune function, and inhibit the inflammatory responses associated with aging [28].

Of note, the anti-inflammatory effects of CR, rather than being simply a passive mechanism, is closely linked to the regulation of inflammatory gene expressions such as forkhead transcription O family (FoxO), nuclear factor erythroid 2-related factor 2 (Nrf2), silent information regulator 1 (Sirt1), NF-κB, and peroxisome proliferator activated receptors (PPARs).

FoxO transcription factors play important roles in the aging process [29], in particular, by their ability to suppress the generation of ROS. The evolutionally conserved FoxO family consists of FoxO1, FoxO3, FoxO4, and FoxO6 in mammals [30]. FoxO regulates some genes involved in cell cycle arrest (p21), DNA repair (Gadd45a), apoptosis (Bim), and in the stress response (MnSOD) to oxidative stress in liver. CR exhibits antineoplastic and anti-inflammation effects by regulating the FoxO target genes involved in cell cycle arrest for DNA damage/repair and apoptosis [31]. CR is also shown to affect FoxO1 in its ability to beneficially regulate NF-κB in aged kidney tissues isolated from ad libitum fed and 40 % CR rats. Furthermore, control aged group rats (24 months old) showed higher levels of FoxO1 phosphorylation and NF-κB activation than the CR aged group rats [20]. Age-related phosphorylation of FoxO6 represses catalase and MnSOD expressions. FoxO6 is regulated by PI3K/Akt activation induced by age-related oxidative stress, which in-turn can be modulated by the anti-aging effect of CR [32].

The positive effects of CR on the aging process in human were reviewed by Redman and Ravussin [33], and it is evident that the application of CR has preventive and therapeutic potentials in the treatment of age-related disorders such as obesity, insulin resistance, type 2 diabetes, atherosclerosis, and cancer [34].

Sirt1 are longevity factors that can regulate directly or indirectly the inhibitors of inflammatory factor NF-κB [35], Sirt1 also can enhance cellular defenses against oxidative stress during aging [36]. Recent findings on the activation by CR have attracted the attention of many researchers, because of its newly discovered role as a NAD^+-dependent epigenetic deacetylase. CR increases SIRT1 expression in liver, adipose tissue, brain, and kidney [37], and SIRT1-dependent deacetylation promotes the nuclear trapping of FoxO1 and upregulates the expressions of its downstream genes [38].

Nrf2 plays an important role in vasoprotection and in regulating the aging process by orchestrating the transcriptional response of cells to oxidative stress [39]. Recently, studies of Nrf2 activation and the upregulation of its downstream target enzymes provided data showing vascular protection against oxidative stress by conferring important antioxidant and anti-inflammatory effects [40]. Nrf2 is a redox-sensitive transcription factor that induces endogenous antioxidant molecules such as the phase II antioxidant genes, including glutathione peroxidase (GPx) and glutathione-s-transferase (GST), and stress-response genes, such as heme oxygenase-1 (HO-1), that lead to cytoprotection against oxidative stress [41] with aging. Therefore, Nrf2 is a potential target to increase antioxidant activity. In addition, CR protects the brain against aging and diseases by increasing activities of plasma membrane redox system enzymes (NADH-ascorbate free radical reductase) and antioxidant levels (α-tocopherol and coenzyme Q10) in brain plasma membrane during aging [42].

PPAR nuclear receptors are transcription factors expressed in multiple tissue lineage, including kidney, liver, and adipose tissue. Activation of the PPAR-γ pathway can inhibit the activity of the transcription factors AP-1 and NF-κB in response to pro-inflammatory cytokines such as TNF-α in endothelial cells [43]. The upregulation of NF-κB and associated inflammatory genes in the absence of PPARs is an age-related phenomenon. However, several age-related alterations in metabolism and body fat have been reported in which PPARs may play a major role such as in adipose lipogenesis, unrestrained hepatic gluconeogenesis, defective glucose synthesis and glucose uptake in skeletal muscle, and abdominal obesity with an accrual of visceral fat [44]. PPARs, by regulating mitochondrial function and uncoupling proteins, likewise seem to play a major role in the age-retarding effects of CR [45]. CR improves glucose, insulin levels and lipid metabolism, thereby improving quality of life via PPAR-γ expression [46]. CR also induces adiponectin gene expression through enhanced PPAR-a and suppressed PPARγ in the epididymal fat [47].

Studies involving CR suggest that transcription factors, FoxO, Nrf2, PPARs and NF-κB play an important role in the inflammatory response with progression of age leading to improvement of the quality of aging and longevity (Fig. 3.1).

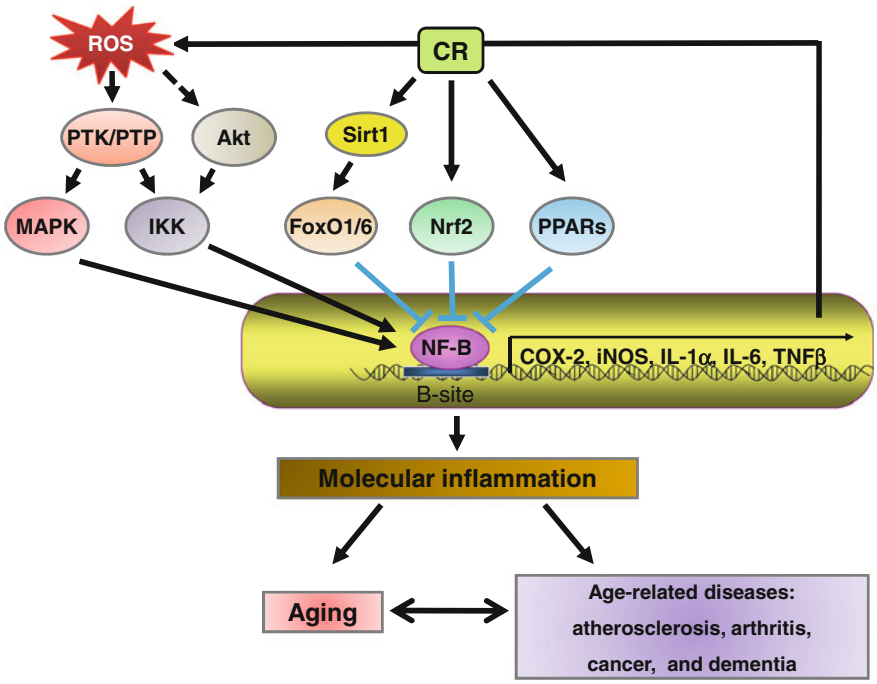

Fig. 3.1 Modulation of proinflammatory NF-κB targeted genes and their regulating transcription factors during aging by CR. CR modulates transcription factors, FoxO, Nrf2, and PPARs negatively regulates NF-κB signaling and lead to molecular inflammation and further aging FoxO, forkhead transcription O family; Nrf2, nuclear factor erythroid 2-related factor 2; Sirt1, silent information regulator 1; NF-κB, nuclear factor kappa B; PPARs, peroxisome proliferator activated receptors; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; CR, calorie restriction; IKK, I κB kinase

3.4 Evidence for Molecular Inflammation in the Aging Process as Revealed by Systems Analysis and NGS Data

Living systems are dynamic networks of interacting components, and their behaviors arise from local interactions that lead to patterns in space and time. Systems biology is a biology-based, cross-disciplinary approach that focuses on complex interactions within biological systems, using quantitative measurements to biological and biomedical research. In these studies, transcriptomics proteomics, metabolomics and high-throughput techniques are used to collect quantitative data. From 2000 onwards, systems biology has been used widely in the biosciences in a variety of contexts. Aging, particularly, is a most complicated physiological process. Because aging is caused by changes in multiple biological systems, the systems biology approach can be an efficient analytical probe for better understanding of altered biological systems that arise during aging.

Microarrays are powerful tools for analyzing gene expression and have increased our understanding of the intricate biological systems involved in normal and diseased organisms. In addition, cDNA microarray technology has been used to identify age-related changes in key pathways, such as inflammatory and mitochondrial dysfunction. A number of cDNA microarray studies have been carried out in mammals, including mice, rats, and humans, to gain an understanding of the transcriptome during the aging process. de Magalhaes et al. [48] performed a meta-analysis of age-related gene expression profiles using 27 datasets from mice, rats and humans, and revealed several common signatures of aging, including 56 genes consistently overexpressed with age, the most significant of which was APOD, and 17 genes that were underexpressed with age. Lu et al. [49] showed through transcriptional profiling of the aged human frontal cortex that DNA damage is markedly increased in the promoters of genes with reduced expression. Zahn et al. [50] constructed an AGEMAP (Atlas of Gene Expression in Mouse Aging Project) gene expression database and found great heterogeneity in the amount of transcriptional changes with age in different tissues.

Age-related dysregulated inflammation plays an essential role as a major risk factor underlying the pathophysiological aging process. Hong et al. [51] applied systems-biology approaches to analyze and elucidate correlations among genes involved in aging and CR, and revealed the systems underlying the crosstalk between aging and the ability of CR to effectively slow-down aging. The authors collected and analyzed 478 aging-related and 586 CR-related mouse genes and found that the transcriptome for both aging and CR were strongly negatively related in the immune response, lipid metabolism, and cell adhesion functions. To identify key molecules controlled by aging and CR, Park et al. [13] integrated data from 84 mouse and rat cDNA microarrays with a protein–protein interaction network, and identified the key molecule as a LCK using integrative analysis.

Despite their usefulness, microarrays have inherent limitations, including a lack of sensitivity to low abundance transcripts and an inability to detect alternative splicing variants and novel transcripts. The ability to detect low abundance transcripts is important, because most gene transcripts are present in low quantities. Particularly important to understanding the aging process is the ability to identify novel RNAs, including non-coding RNAs. RNA-sequencing (RNA-seq) is a advanced technology based on next-generation sequencing used to reveal a snapshot of RNA presence and quantity from a genome at a given moment in time. This technology has significantly accelerated genomic research and has led to a better understanding of changes in total gene expression in many biological systems, including the aging process.

Wood et al. [52] used RNA-seq to sequence the cerebral cortex transcriptome in 6-, 12- and 28-month-old rats, and revealed that protein coding genes related to MHC II presentation and serotonin biosynthesis were differentially expressed in aging. Dillman et al. [53] reported a high-resolution transcriptome dataset of mouse cerebral cortex at embryonic and adult stages using RNA-Seq and found many differences in gene expression, splicing, and RNA editing between embryonic and

adult cerebral cortex, such as cell cycle, DNA damage response and repair in embryonic brain and immune response in adult brain.

Park and his colleagues recently compared 6- and 25-month-old rats and detected age-related differentially expressed genes, novel genes, and alternative splicing events [54]. Notably, they detected the involvement of inflammation-related pathways such as cytokines, which were found upregulated in the aged rats. Furthermore, an up-regulated inflammatory gene analysis identified the involvement of transcription factors, such as STAT4, EGR1, and FOSL1, which regulate cancer as well as inflammation in aging processes (data not shown). Whole transcriptome analysis is very useful for exploring complex pathophysiological phenomena such as aging, and RNA-Seq technology enables us to identify transcriptome changes, including novel genes and alternative splicing forms altered by aging as to identify various biological pathways influencing the aging process. Thus, systems biological approaches might be very powerful tools for identifying target genes and their networks during aging processes as well as the target molecules of anti-aging CR.

3.5 The Emerging Inflammasome as a Crucial Inflammatory Pathway in Aging and Age-Related Disease Processes and Their Modulation by CR

3.5.1 The Emerging Role of Inflammasome as a Crucial Regulator of the Innate Immune System

Inflammasomes are key signaling platforms that detect pathogenic microorganisms as well as sterile stressors [55]. They are a group of multimeric protein complexes that consist of an inflammasome sensor molecule (e.g., NLRP3), the adaptor protein ASC, and caspase1 by outer stressors including infection, damage, or other cellular stresses [55]. Once the protein complexes have formed thoroughly, the inflammasome activates caspase1, which proteolytically activates the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 [56]. More recently, non-canonical inflammasome activation through caspase-11 was also implicated in production of IL-1β and IL-18 [57]. As IL-1β and IL-18 are known to be major cytokines playing an important pathogenic role in many inflammatory diseases, inflammasomes are recognized as a crucial regulator of innate immunity and inflammation [58]. The activity potency of the inflammasome in directing innate immune responses is clearly demonstrated, and a number of recent findings further demonstrated the important role of the inflammasome in various innate immune-related diseases.

3.5.2 The Inflammasome in Aging and Age-Related Diseases

Inflammation is an acute response to infection and tissue damage that limits harm to the host [59]. However, chronic or dysregulated inflammation may result in persistent damage and an improper innate immune response [59]. The inflammasome can recognize a wide range of inflammation-inducing stimuli that include pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patters (DAMPs) [58]. Furthermore, IL-1β and IL-18 produced by the inflammasome are also associated with other important aspects of inflammation and tissue repair such as pyroptosis, a form of cell death [58].

Recent interesting experiments demonstrated the essential role of canonical NLRP3 inflammasome during aging [60]. Rather than using disease models, investigators utilized control and NLRP3$^{-/-}$ mice to observe age-related functional decline. Ablation of NLRP3 inflammasome especially protected mice from age-related increases in innate immune activation, alterations in CNS transciptomes and astrogliosis. Notably, IL-1 mediated only NLRP3 inflammasome dependent improvement in cognitive function and motor performance in aged mice. These studies offered an innovative therapeutic strategy to lower inflammasome activity as to delay multiple age-related chronic diseases [60]. Furthermore, as many age-related diseases such as type 2 diabetes, cardiovascular diseases, arthritis, Alzheimer's disease, and cancer are associated with inflammation, recent findings demonstrated these diseases are also associated with hyper-activation of the inflammasome [61].

3.5.3 The Inflammasome and Metabolic Diseases

Aging is the most universal contributor to the etiologies of metabolic decline and related diseases, including type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD). As metabolic disorders, particularly obesity, T2DM, and CVD constitute the greatest current threat to global human health and welfare, they are recognized as most important age-related diseases [62]. Chronic inflammatory states seem to be a common feature among these metabolic diseases, and many recent findings demonstrate that inflammation plays a pivotal role in mediating obesity and metabolic diseases [61]. As a critical component of chronically activated inflammatory mediators, the inflammasome came into the spotlight in many age-related metabolic diseases [63].

Experimental and clinical evidence strongly links IL-1β and IL-18 to the development of metabolic pathologies and their complications [63]. As metabolic syndromes are thus casually linked with chronic inflammatory alterations, the underlying molecular mechanisms that result in inflammasome activation in the context of obesity and related metabolic diseases are now explained. Inflammasome components and caspase1 activation are increased in the adipose tissue and liver of obese mice and humans, and their levels of expression are directly correlated with the severity of T2DM in obese individuals [64].

Enhanced inflammasome in tissue-infiltrated macrophages might play role in mediating metabolic syndrome [65]. Moreover, inflammasome activation is known to impair β-cell function [66, 67]. IL-1β, preferentially expressed by pancreatic infiltrating macrophages, has been implicated as a critical driver of β-cell death in conditions of chronic exposure to an elevated concentration of glucose [67]. Non-alcoholic fatty liver diseases (NAFLD) and atherosclerosis are also associated with increased inflammasome and consequently hyper-production of IL-1β [68]. These findings suggest that the inflammasome could be a key inflammatory component mediating inflammation and age-related metabolic diseases.

3.5.4 The Inflammasome and Other Age-Related Inflammatory Diseases

The inflammasome is also closely related with other inflammatory diseases. Using a common acute and chronic epithelial injury colitis model, several groups reported decreased disease severity in mice deficient in caspase1 or NLRP3, which correlated with lower IL-1β production during disease [69]. It has been also suggested that inflammasome participates in inflammation-induced tumorigenesis, a common complication of chronic intestinal auto-inflammation. Although it still needs to be more fully elucidated in other age-related diseases, there is emerging evidence showing the importance of the inflammasome in other diseases. Recent studies demonstrated a significant role for the inflammasome in Alzheimer's diseases and other neurological diseases, meaning that inflammasome activation is also associated with decreased brain function during aging [70].

The inflammasome also seems to be increased in arthritis and rheumatism models, which are associated with hyper-activation of autoimmune response in host [71]. A number of endogenous and exogenous stimuli also can provide signals for inflammasome activation in cancer [72]. Although IL-1β can be a good marker for several tumors, the exact role of the inflammasome in tumor developments still needs to be elucidated precisely [72]. However, emerging evidence strongly implicates a role for the inflammasome in various age-related diseases.

3.5.5 Effects of CR on Inflammasome Activation

CR is now accepted as the only established anti-aging experimental paradigm to serve as the gold standard in testing aging interventions [73]. A number of age-associated inflammatory diseases have been shown to be suppressed by CR in animal models. Furthermore, recent CR data on human subjects show promising results in a reduction of the risk factors associated with age-related diseases as well as improvements in several key biomarkers of longevity [73]. Thus, as restricting calories can help in reducing age-related diseases as well as aging, some researchers

have focused on the effects of CR on activation of the inflammasome. Recently, Wen et al. demonstrated that in macrophages, palmitate, a saturated fatty acid, could activate the NLRP3 inflammasome [74]. Interestingly, NLRP3 activation was dependent on oxidative stress, secreted IL-1β, impaired insulin signaling, and promoted insulin resistance in mice. These investigators also observed a reduction in NLRP3 expression in adipose tissue by CR, resulting in a decrease of in the level of inflammation and an increase in insulin sensitivity [74]. It seems that NLRP3 could be a sensor for metabolic stress by recognizing oxidative stress and that CR significantly blunts inflammasome activation. Another interesting aspect of CR is its potential for autophagy activation [75]. Several studies have demonstrated that autophagy is required for CR-mediated lifespan extension. The activation of autophagy was shown to directly regulate clearance of the inflammasome complex, which gives a plausible explanation for the positive effects of CR as an aging intervention [75]. In brief, a decline in autophagy during aging aggravates inflammation through enhanced inflammasome activation, and, alternatively, CR could attenuate these inflammasome activations by inducing autophagy.

Despite great advances in the understanding of the inflammasome, there remain a number of unresolved aspects to the inflammasome regarding aging and age-related diseases. The potent activity of the inflammasome in directing the innate immune response is clearly demonstrated in a number of inflammatory diseases including age-related diseases. Although disease associations in a number of NLRPs suggest important roles in inflammatory disease that provide fertile ground for future research, an association of the inflammasome with aging itself needs further demonstration. However, there is growing evidence indicating that the innate immune regulator inflammasome is implicated in aging and age-associated diseases. A heightened inflammasome with aging provokes inflammation and exposes the host to the risk of age-related inflammatory diseases. Further experimental data demonstrated that efficient autophagic activity by CR can prevent the activation of the inflammasome and induction of inflammatory responses [75]. These recent findings highlight the potential of targeting the molecular pathways regulating inflammasome activation during aging for the management of age-related diseases and chronic inflammation-induced comorbidities.

3.6 Involvement of Inflammation in Lipid Accumulation, ER Stress, Insulin Resistance, and Their Modulation by CR

3.6.1 Inflammation and Lipid Accumulation

Lipids play a wide variety of roles in patho-physiological conditions. A wide-spread abnormal accumulation of lipids in adipose tissue and in ectopic sites such as liver and muscle provides the great opportunity for the activation of proinflammatory genes in

many tissues. Systemic inflammation with lipid accumulation leads to disruptions in protein expressions related to lipid metabolism such as sterol regulatory element binding protein (SREBP) and SREBP cleavage activating protein (SCAP) [76]. SREBP subfamilies are established as transcription factors regulating the transcription of genes involved in cholesterol and fatty acid synthesis [77]. SREBP proteins are initially bound to the rough endoplasmic reticulum (ER) membrane and form a complex with SCAP. These SREBP proteins play a crucial role in the regulation of fatty acid, triglyceride and cholesterol synthesis. Excess lipid accumulation in ectopic sites during aging has a great impact on the aging process and age-related diseases including insulin resistance, pancreatic β-cell apoptosis and heart failure [78]. Therefore, to mediate lipid metabolism through controlling the inflammatory response is one of the most important factors in aging and age-related diseases.

3.6.2 Inflammation and ER Stress

The ER is an organelle comprising a reticular membranous network that extends throughout the cytoplasm and is contiguous with the nuclear envelope. The ER plays a pivotal role in protein modification and lipid biosynthesis and as well as calcium store regulation, which determines its essential role in cell function. The ER stress response can be triggered under conditions that challenge ER function such as hypoxia, over-nutrition, imbalance of redox status, and aberrant calcium regulation, but also can be mediated through three main sensors, namely inositol requiring element-1 (IRE-1), protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6). These pathways, collectively known as the unfolded protein response (UPR), are important for normal cellular homeostasis and organismal development and may play key roles in the pathogenesis of many diseases. In recent years, considerable evidence has demonstrated that pro-inflammatory cytokines can lead to ER stress through enhancing cytosolic Ca^{2+} concentration [79]. Indeed, an excess amount of nitric oxide (NO), which is produced from inducible nitric oxide synthase (iNOS), may directly disturb ER function and activate the ER stress pathway by increasing Ca^{2+} uptake. Aging is associated with increased levels of circulating cytokines [80] and NO production [81]. Thus, inflammatory responses are one of the major triggers of ER stress during aging.

3.6.3 Inflammation and Insulin Resistance

Insulin resistance is a major feature of obesity and is generally accompanied by aging [82]. Therefore, insulin resistance provides a potential explanation for the prevalence of T2DM in elderly subjects. Large studies have shown that insulin resistance precedes the onset of T2DM by many years. Insulin resistance is defined as a reduction in the rate of glucose disposal elicited by a given insulin

concentration compared to the normal range. In the presence of insulin resistance, a normal β-cell will increase its production of insulin, and as long as the compensatory hyperinsulinemia is adequate to overcome insulin resistance, glucose tolerance remains relatively normal. In subjects that develop T2DM, the compensatory β-cell response fails and relative insulin insufficiency develops which then lead to impaired glucose tolerance and eventually marked T2DM. It is well established that obesity-associated lipid accumulation can induce insulin resistance in ectopic sites including the liver and muscle [83].

Pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 that are secreted by immune cells and adipocytes also are thought to play a role in the development of insulin resistance. Pro-inflammatory cytokines activate transcription factors, including NF-κB, and these transcription factors inhibit insulin signaling. Heterozygous $IKK^{+/-}$ mice fed a high fat diet, or crossed with obese ob/ob mice, have an attenuated phenotype in the development of insulin resistance [84]. Moreover, inhibition of NF-κB in macrophages or the liver through tissue-specific expression of a dominant negative form of IκB or tissue-specific deletion of IKKβ, ameliorates inflammatory gene expression and insulin resistance in response to a high fat diet [85].

3.6.4 CR Modulates Lipid Metabolism, ER Stress, and Insulin Resistance by Ameliorating the Inflammatory Response

The inflammatory response impairs lipid metabolism, ER stress, and insulin resistance during aging. So, it is recognized as one of the pivotal risk factors for age-related disease. Only a few means exist to extend lifespan through the attenuation of the inflammatory response. CR is the only known dietary intervention that can impede a great variety of aging processes, extend median and maximum lifespan, and decrease the incidence of age-associated diseases in mammals [86]. CR can improve an imbalance in the redox status by reducing energy flux and metabolism with a consequential lowering of reactive oxygen species and rate of oxidative damage to vital tissues. Normal energy metabolism in aerobic organisms is coupled to the generation of ROS. In fact, 2–5 % of oxygen consumption is not associated with the oxidative metabolism of fuels but is associated with the production of highly reactive oxygen molecules such as the superoxide radical, hydrogen peroxide, and the hydroxyl radical. Therefore, reducing metabolic rate by using CR may in-turn reduce oxygen consumption, which then could decrease ROS formation and potentially increase lifespan. Indeed, several studies suggest that CR delays many age-sensitive parameters in primates. For example, rhesus monkeys show an age-related dysregulation of cytokine levels [87]. Therefore, CR may prove a powerful tool to modulate lipid metabolism, ER stress, and insulin resistance by attenuating inflammatory responses.

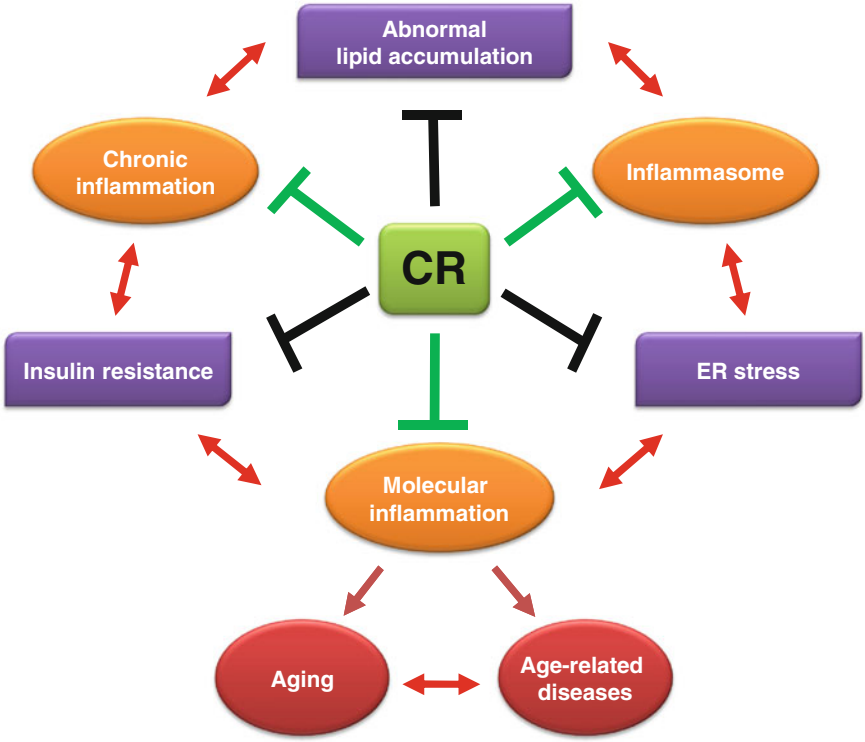

Fig. 3.2 Central role of CR as an anti-inflammatory intervention of aging and major age-related metabolic disorders. Inflammatory responses and metabolic abnormalities are major causes of accelerated aging. The anti-inflammatory effects of CR play a central role in modulating lipid metabolic disorders, leading to the intervention of aging and age-related diseases. CR; calorie restriction

We learned that increased levels of lipid accumulation, ER stress and insulin resistance are closely associated with chronic inflammation during the aging process. However, CR can attenuate these levels by inhibiting the inflammatory response and thereby extend lifespan (Fig. 3.2).

3.7 Concluding Remarks

The evidence described above supports molecular inflammation as an important underlying mechanism of aging processes and age-related diseases. Key molecular events underlying age-related chronic inflammatory state are increased redox status, NF-κB signaling, inflammasome, ER stress, and insulin resistance, all of which can lead to the aging process and age-related diseases, and can be regulated by CR.

We consider molecular inflammation as an important basic concept that outlines a molecular network that underlies and links aging to age-related diseases, such as atherosclerosis, arthritis, cancer, dementia, and vascular diseases. Further exploration of molecular bases for the suppression of chronic inflammation by epigenetic interventions by CR will cast additional support for the anti-aging action of CR as described in this chapter.

References

1. Yu BP (1996) Aging and oxidative stress: modulation by dietary restriction. Free Radic Biol Med 21:651–668
2. Yu BP, Chung HY (2001) Stress resistance by caloric restriction for longevity. Ann NY Acad Sci 928:39–47
3. Yu BP, Yang R (1996) Critical evaluation of the free radical theory of aging. A proposal for the oxidative stress hypothesis. Ann NY Acad Sci 786:1–11
4. Bodamyali T, Stevens CR, Blake DR et al (2000) Reactive oxygen/nitrogen species and acute inflammation: a physiological process. In: Winyard PG, Blake DR, Evans CH (eds) Free radicals and inflammation. Birkha user Verlag, Basel, pp 11–16
5. Chung HY, Cesari M, Anton S et al (2009) Molecular inflammation: underpinnings of aging and age-related diseases. Aging Res Rev 8:18–30
6. Zou Y, Kim DH, Jung KJ et al (2009) Lysophosphatidylcholine enhances oxidative stress via the 5-lipoxygenase pathway in rat aorta during aging. Rejuvenation Res 1:15–24
7. Dickinson DA, Forman HJ (2002) Glutathione in defense and signaling: lessons from a small thiol. Ann NY Acad Sci 973:488–504
8. Holmgren A (1989) Thioredoxin and glutaredoxin systems. J Biol Chem 264:13963–13966
9. Kim HJ, Jung KJ, Yu BP et al (2002) Influence of aging and calorie restriction on MAPKs activity in rat kidney. Exp Gerontol 37:1041–1053
10. Sen CK (2000) Cellular thiols and redox-regulated signal transduction. Curr Top Cell Regul 36:1–30
11. Mustelin T, Vang T, Bottini N (2005) Protein tyrosine phosphatases and the immune response. Nat Rev Immunol 5:43–57
12. Jung KJ, Lee EK, Yu BP et al (2009) Significance of protein tyrosine kinase/protein tyrosine phosphatase balance in the regulation of NF-kappaB signaling in the inflammatory process and aging. Free Radic Biol Med 47:983–991
13. Park D, Lee EK, Jang EJ et al (2013) Identification of the dichotomous role of age-related LCK in calorie restriction revealed by integrative analysis of cDNA microarray and interactome. Age 35:1045–1060
14. Jung KJ, Kim DH, Lee EK et al (2013) Oxidative stress induces inactivation of protein phosphatase 2A, promoting proinflammatory NF-κB in aged rat kidney. Free Radic Biol Med 61C:206–217
15. Kumar A, Takada Y, Boriek AM et al (2004) Nuclear factor-kappaB: its role in health and disease. J Mol Med 82:434–448
16. Bowie A, O'Neill LA (2000) Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 59:13–23
17. Kim CH, Zou Y, Kim DH et al (2006) Proteomic analysis of nitrated and 4-hydroxy-2-nonenal-modified serum proteins during aging. J Gerontol A Biol Sci Med Sci 61:332–338
18. Kim JM, Lee EK, Kim DH et al (2010) Kaempferol modulates pro-inflammatory NF-kappaB activation by suppressing advanced glycation endproducts-induced NADPH oxidase. Age 32:197–208

19. Kim JM, Heo HS, Ha YM et al (2012) Mechanism of Ang II involvement in activation of NF-κB through phosphorylation of p65 during aging. Age 34:11–25
20. Kim DH, Kim JY, Yu BP et al (2008) The activation of NF-kappaB through Akt-induced FOXO1 phosphorylation during aging and its modulation by calorie restriction. Biogerontology 9:33–47
21. Park DU, Kim CH, Hong SE et al (2003) AgingDB: A database for oxidative stress and calorie restriction in the study of aging. J Am Aging Assoc 26:11–17
22. Chung HY, Kim HJ, Kim KW et al (2002) Molecular inflammation hypothesis of aging based on the anti-aging mechanism of calorie restriction. Microsc Res Tech 59:264–272
23. Chung HY, Cesari M, Anton S et al (2009) Molecular inflammation: underpinnings of aging and age-related diseases. Ageing Res Rev 8:18–30
24. Shishodia S, Aggarwal BB (2004) Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates activation of cigarette smoke-induced nuclear factor (NF)-kappaB by suppressing activation of IkappaBalpha kinase in human non-small cell lung carcinoma: correlation with suppression of cyclin D1, COX-2, and matrix metalloproteinase-9. Cancer Res 64:5004–5012
25. Henkel T, Machleidt T, Alkalay I et al (1993) Rapid proteolysis of IkappaB-alpha is necessary for activation of transcription factor NF-kappaB. Nature 365:182–185
26. Yu BP (2005) Calorie restriction as a potent anti-aging intervention: suppression of oxidative stress. In: Rattan Suresh (ed) Aging Intervention and Therapies. World Scientific Publishing, Singapore, pp 193–217
27. Demetrius L (2005) Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. EMBO Rep 6:39–44
28. Longo VD, Finch CE (2003) Evolutionary medicine: from dwarf model systems to healthy centenarians? Science 299:1342–1346
29. Morris BJ (2005) A forkhead in the road to longevity: the molecular basis of lifespan becomes clearer. J Hypertens 23:1285–1309
30. van der Heide LP, Hoekman MFM, Smidt MP (2004) The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J 380:297–309
31. Yamaza H, Komatsu T, Wakita S et al (2010) FoxO1 is involved in the antineoplastic effect of calorie restriction. Aging Cell 9:372–382
32. Kim DH, Park MH, Chung KW et al (2014) The essential role of FoxO6 phosphorylation in aging and calorie restriction. Age 36:9679
33. Redman LM, Ravussin E (2011) Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. Antioxid Redox Signal 14:275–287
34. Yu BP, Chung HY (2006) The inflammatory process in aging. Rev Clin Gerontol 16:179–187
35. Salminen A, Ojala J, Huuskonen J et al (2008) Interaction of aging-associated signaling cascades: inhibition of NF-kB signaling by longevity factors FoxOs and SIRT1. Cell Mol Life Sci 65:1049–1058
36. Van der Horst A, Tertoolen LG, de Vries-Smits LM et al (2004) FoxO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2 (SIRT1). J Biol Chem 279:28873–28879
37. Nisoli E, Tonello C, Cardile A et al (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310:314–317
38. Frescas D, Valenti L, Accili D (2005) Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. J Biol Chem 280:20589–20595
39. Lewis KN, Mele J, Hayes JD et al (2010) Nrf2, a guardian of healthspan and gatekeeper of species longevity. Integr Comp Biol 50:829–843
40. Chen XL, Varner SE, Rao AS et al (2003) Laminar flow induction of antioxidant response element-mediated genes in endothelial cells. A novel anti-inflammatory mechanism. J Biol Chem 278:703–711
41. Mercado N, Thimmulappa R, Thomas CM et al (2011) Decreased histone deacetylase 2 impairs Nrf2 activation by oxidative stress. Biochem Biophys Res Commun 406:292–298

42. Hyun DH, Emerson SS, Jo DG et al (2006) Calorie restriction up-regulates the plasma membrane redox system in brain cells and suppresses oxidative stress during aging. Proc Natl Acad Sci USA 103:19908–19912
43. Wang N, Verna L, Chen NG et al (2002) Constitutive activation of peroxisome proliferator-activated receptor-gamma suppresses pro-inflammatory adhesion molecules in human vascular endothelial cells. J Bio Chem 277:34176–34181
44. Barzilai N, Huffman DM, Muzumdar RH et al (2012) The critical role of metabolic pathways in aging. Diabetes 6:1315–1322
45. de Cavanagh EM, Piotrkowski B, Fraga CG (2007) The interaction between the rennin-angiotensin system and peroxisome proliferator activated receptors: a hypothesis including the participation of mitochondria in aging. Front Biosci 12:1049–1062
46. Rahman M, Halade GV, Bhattacharya A et al (2009) The fat-1 transgene in mice increases antioxidant potential, reduces proinflammatory cytokine levels, and enhances PPAR-gamma and SIRT-1 expression on a calorie restricted diet. Oxid Med Cell Longev 2:307–316
47. Qiao L, Lee B, Kinney B et al (2011) Energy intake and adiponectin gene expression. Am J Physiol Endocrinol Metab 300:E809–E816
48. de Magalhaes JP, Curado J, Church GM (2009) Meta-analysis of age-related gene expression profiles identifies common signatures of aging. Bioinformatics 25:875–881
49. Lu T, Pan Y, Kao SY et al (2004) Gene regulation and DNA damage in the ageing human brain. Nature 429:883–891
50. Zahn JM, Poosala S, Owen AB et al (2007) AGEMAP: a gene expression database for aging in mice. PLoS Genet 3:e201
51. Hong SE, Heo HS, Kim DH et al (2010) Revealing system-level correlations between aging and calorie restriction using a mouse transcriptome. Age 32:15–30
52. Wood SH, Craig T, Li Y et al (2013) Whole transcriptome sequencing of the aging rat brain reveals dynamic RNA changes in the dark matter of the genome. Age 35:763–776
53. Dillman AA, Hauser DN, Gibbs JR et al (2013) mRNA expression, splicing and editing in the embryonic and adult mouse cerebral cortex. Nat Neurosci 16:499–506
54. Park D, Lee EK, Jang EJ et al (2013) Identification of the dichotomous role of age-related LCK in calorie restriction revealed by integrative analysis of cDNA microarray and interactome. Age 35(4):1045–1060
55. Dinarello CA (2009) Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 27:519–550
56. Wilson KP, Black JA, Thomson JA et al (1994) Structure and mechanism of interleukin-1 beta converting enzyme. Nature 370:270–275
57. Kayagaki N, Warming S, Lamkanfi M et al (2011) Non-canonical inflammasome activation targets caspase-11. Nature 479:117–121
58. Davis BK, Wen H, Ting JP (2011) The inflammasome NLRs in immunity, inflammation, and associated diseases. Annu Rev Immunol 29:707–735
59. Medzhitov R (2008) Origin and physiological roles of inflammation. Nature 454:428–435
60. Youm YH, Grant RW, McCabe LR et al (2013) Canonical Nlrp3 inflammasome links systemic low-grade inflammation to functional decline in aging. Cell Metab 18:519–532
61. Vasto S, Candore G, Balistreri CR et al (2007) Inflammatory networks in ageing, age-related diseases and longevity. Mech Ageing Dev 128:83–91
62. Lumeng CN, Saltiel AR (2011) Inflammatory links between obesity and metabolic disease. J Clin Invest 121:2111–2117
63. Strowig T, Henao-Mejia J, Elinav E et al (2012) Inflammasomes in health and disease. Nature 481:278–286
64. Vandanmagsar B, Youm YH, Ravussin A et al (2011) The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nature Med 17:179–188
65. Lumeng CN, Bodzin JL, Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 117:175–184

66. Masters SL, Dunne A, Subramanian SL et al (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. Nature Immunol 11:897–904
67. Dinarello CA, Donath MY, Mandrup-Poulsen T (2010) Role of IL-1β in type 2 diabetes. Curr Opin Endocrinol Diabetes Obes 17:314–321
68. Petrasek J, Bala S, Csak T et al (2012) IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. J Clin Invest 122:3476–3489
69. Bauer C, Duewell P, Mayer C et al (2010) Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. Gut 59:1192–1199
70. Heneka MT, Kummer MP, Stutz A et al (2013) NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature 493:674–678
71. Jin C, Frayssinet P, Pelker R et al (2011) NLRP3 inflammasome plays a critical role in the pathogenesis of hydroxyapatite-associated arthropathy. Proc Natl Acad Sci USA 108:14867–14872
72. Zitvogel L, Kepp O, Galluzzi L et al (2012) Inflammasomes in carcinogenesis and anticancer immune responses. Nat Immunol 13:343–351
73. Chung KW, Kim DH, Park MH et al (2013) Recent advances in calorie restriction research on aging. Exp Gerontol 48:1049–1053
74. Wen H, Gris D, Lei Y et al (2011) Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nat Immunol 12:408–415
75. Shi CS, Shenderov K, Huang NN et al (2012) Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. Nat Immunol 13:255–263
76. Zhang G, Li Q, Wang L et al (2011) The effects of inflammation on lipid accumulation in the kidneys of children with primary nephrotic syndrome. Inflammation 34:645–652
77. Brown MS, Ye J, Rawson RB et al (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell 100:391–398
78. Zhao L, Zou X, Feng Z et al (2014) Evidence for association of mitochondrial metabolism alteration with lipid accumulation in aging rats. Exp Gerontol 56:3–12
79. Philippe J, Ruiz J (2014). The clinical path concept or the difficult revolution of chronic diseases management. Rev Med Suisse 10:1227–1228
80. Michaud M, Balardy L, Moulis G et al (2013) Proinflammatory cytokines, aging, and age-related diseases. J Am Med Dir Assoc 14:877–882
81. Mccann SM, Mastronardi C, De Laurentiis A et al (2005) The nitric oxide theory of aging revisited. Ann NY Acad Sci 1057:64–84
82. Ahima RS (2009) Connecting obesity, aging and diabetes. Nat Med 15:996–997
83. Eckardt K, Taube A, Eckel J (2011) Obesity-associated insulin resistance in skeletal muscle: role of lipid accumulation and physical inactivity. Rev Endocr Metab Disord 12:163–172
84. Yuan M, Konstantopoulos N, Lee J et al (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 293:1673–1677
85. Cai D, Yuan M, Frantz DF et al (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med 11:183–190
86. Wolf G (2006) Calorie restriction increases life span: a molecular mechanism. Nutr Rev 64:89–92
87. Mascarucci P, Taub D, Saccani S et al (2002) Cytokine responses in young and old rhesus monkeys: effect of caloric restriction. J Interferon Cytokine Res 22:565–571

Chapter 4
Hormonal Influence and Modulation in Aging

Isao Shimokawa

Abstract Hormones regulate physiological functions and maintain homeostasis in the body. In aging animals, the levels of many hormones decrease in the blood or target cells become insensitive to certain hormones, thereby causing aging-related disorders. For example, a reduction in the levels of growth hormone (GH) and its peripheral effector, insulin-like growth factor-1 (IGF-1), causes physical frailty due to loss of bone and lean muscle mass in humans. However, inhibition of the GH-IGF-1 axis by genetic manipulation promotes survival in a wide range of animals. Calorie restriction (CR), a nongenetic intervention that extends the lifespan of animals, also inhibits the GH-IGF-1 axis. That is the GH-IGF-1 paradox of aging. This chapter describes our current understanding of the signaling pathways that regulate aging and thus lifespan, particularly focusing on the GH-IGF-1 axis and its downstream signaling. Modulation of neuroendocrine systems by CR is also reviewed in terms of extension of lifespan.

4.1 Introduction

Hormones secreted from endocrine glands into circulation are essential to maintain homeostasis in the body and to adapt to changes in external and internal environments. During the aging process, many water- and lipid-soluble hormones show decreases in synthesis and secretion or target organs become insensitive to hormones, causing functional disorders or diseases in aging animals. Therefore, replacement therapy for deficient hormones has been considered to prevent or improve physical frailty in elderly people [1]. In this chapter, the growth hormone (GH) and insulin-like growth factor (IGF)-1 axis is first described in terms of the modulation of aging processes. GH shows pleiotropic effects in the aging process.

I. Shimokawa (✉)
Department of Pathology, Nagasaki University School of Medicine and Graduate School of Biomedical Sciences, Nagasaki, Japan
e-mail: shimo@nagasaki-u.ac.jp

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In mammals, GH deficiency causes a decrease in the lean muscle mass and an increase in the fat mass, i.e., acceleration of the aging process. In contrast, experimental inhibition of GH signaling results in a longer lifespan, i.e., a delay of the aging process [2]. Thus, investigation of the role of GH-IGF-1 signaling in the aging process might provide insights into the regulation of aging and a healthy lifespan for humans.

Calorie restriction (CR) is a nongenetic intervention to slow aging processes and extend the lifespan of a wide range of organisms [3], indicating the existence of common mechanisms that promote survival in response to limited energy sources in mammals and invertebrates. CR is known to modulate neuroendocrine systems including the GH-IGF-1 axis. Thus, information on the regulation of neuroendocrine systems by CR might broaden our understanding of the regulation of aging processes and a healthy lifespan.

4.2 GH and IGF-1

4.2.1 Aging-Related Reductions of GH Levels and Its Replacement Therapy

Most aging people experience progressive alterations in their body composition such as a decrease in lean muscle mass and an increase in fat mass. Reductions of the skeletal muscle mass and bone density cause physical frailty in elderly people. During the 1980s, it was proposed that reduced bioavailability of GH contributed to these age-dependent changes in body composition [1]. To test this hypothesis, Rudman et al. [1] conducted a one-year clinical study. After the first 6 months of baseline data collection, biosynthetic human GH (hGH) was administered for the next 6 months to 12 healthy men (Group 1) aged between 61 and 81 years. Their plasma IGF-1 concentrations were below 350 U/L. The doses of hGH were adjusted according to the range of plasma IGF-1 between 500 and 1,500 U/L, the range equivalent to that in young adults. The control group did not receive injections of hGH. Within 1 month after the initiation of hGH administration, the mean IGF-1 level rose to 830 U/L, which was maintained a constant level for the next 5 months. At the end of the clinical trial, there were significant increases in lean muscle mass (+3.7 kg), while the adipose tissue mass was decreased in participants by an average of 2.4 kg without significant changes in body weight or bone density. Furthermore, their skin thickness tended to increase (+0.8 mm, $p = 0.07$). There were no signs of hypersomatotropism, such as edema, hypertension, diabetes, or cardiomegaly, although small increases of mean systolic blood pressure and fasting blood glucose concentrations in Group 1 were noted in the published article [1]. This study proved the hypothesis that reduced bioavailability of GH causes an alteration in the body composition of elderly people, which warranted further clinical trials of hGH replacement therapy to improve physical frailty in aging people.

A subsequent study with a protocol involving longer periods of hGH administration and a larger number of subjects identified a high frequency of side effects in subjects whose plasma IGF-1 was over 1,000 U/L, including carpal tunnel syndrome, gynecomastia, and hyperglycemia [4]. The authors suggested that the beneficial effects of hGH may be achieved by maintaining the mean IGF-1 level in the range of 500–1,000 U/L to avoid such side effects. Although anti-aging clinics and websites promise benefits of GH replacement in elderly people, there is no scientific evidence indicating safe doses of hGH. At present, the U.S. Food and Drug Administration has approved GH prescription for a narrow indication, i.e., for children and adults who are truly deficient because of gene mutations or structural defects caused by brain tumors or injuries.

4.2.2 Longevity Models by Inhibition of the GH-IGF-1 Axis

In contrast to GH replacement therapy, scientific evidence confidently indicates that inhibition of the GH-IGF-1 axis increases the lifespans of mice and invertebrates. The findings demonstrating that longevity can by achieved by inhibition of the anabolic pathway were first reported by Friedman and Johnson [5] in *Caenorhabditis elegans*. Genetic analysis indicated that a reduction of functional mutations in a single gene named *age-1* extends lifespan. Subsequent analyses showed that mutations of the *age-1* gene cause a functional reduction of phosphoinositide 3-kinase (PI3K) [6]. PI3K is a key enzyme in IGF-1 and insulin signal transduction. In 1993, *daf-2* mutants were also reported to live longer than wild-type worms [7]. The extension of lifespan in *daf-2* mutants required another gene named *daf-16* [7]. Subsequent studies revealed that mammalian orthologs of *daf-2* and *daf-16* are genes encoding insulin and IGF-1 receptors, and forkhead box O (FoxO) transcription factors, respectively [8, 9]. After a series of studies in *C. elegans*, researchers recognized that insulin or IGF-1 signaling is a key pathway in the regulation of lifespan and thus aging.

In 1996, it was reported that Ames mice, in which pituitary GH, prolactin, and thyroid-stimulating hormone are deficient due to a mutation of the *prop-1* gene, live longer than wild-type mice [10]. This was the first report demonstrating that a single gene mutation is able to extend lifespan even in mammals. At that time, GH was thought to be a key hormone involved in extended lifespans, because overexpression of the GH gene in mice was known to produce some premature aging phenotypes and a shortened lifespan [11].

The significance of GH in regulation of the mammalian lifespan was confirmed by the finding of an extended lifespan in GH receptor/binding protein (GHR/BP)-knockout (KO) mice [12]. Subsequently, there have been reports of similar long-lived mouse models such as Snell mice (*pit-1* gene mutation; [13]), GH-releasing hormone receptor (*Ghrhr*) gene-mutated mice [13], and IGF-1 receptor (*Igf1r*) gene KO heterozygous (+/−) mice [14]. These long-lived models clearly indicate that inhibition of any step in GH-IGF-1 signaling extends the lifespan of mice (Fig. 4.1).

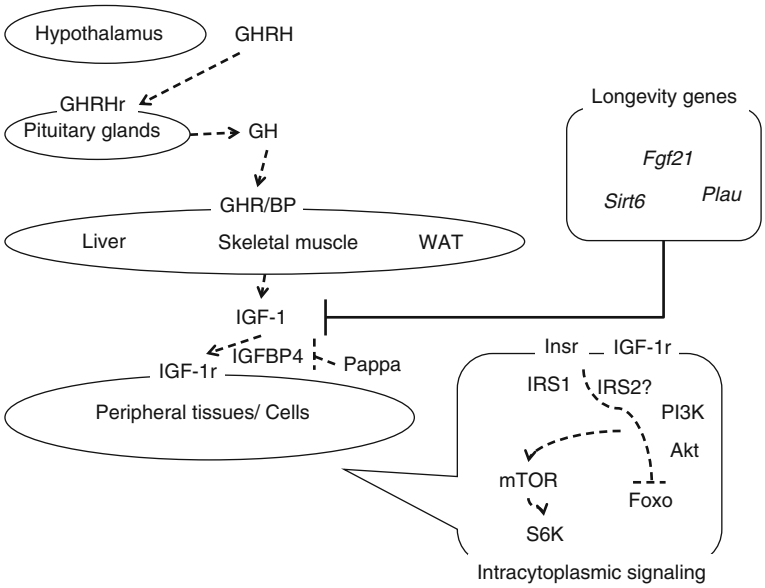

Fig. 4.1 GH-IGF-1 axis as a longevity signal. Inhibition of any step in the GHRH-GH-IGF-1 axis causes an extension in the lifespan of mice. Longevity genes such as *Fgf21*, *Sirt6*, and *Plau* are reported to decrease the plasma concentration of IGF-1, and are thought to affect lifespan through inhibition of the GH-IGF-1 axis. At the intracellular level, attenuation of the Insr/IGF-1r-IRS-PI3K-Akt pathway promotes activation of FoxO transcription factors while inhibiting the mTOR pathway. *Dotted* and *straight lines* represent attenuated and strengthened signals, respectively. *Arrows* and *bars* represent activation and inhibition, respectively

Pregnancy-associated plasma protein A (PAPPA) is a metzincin superfamily metalloproteinase in the IGF system [15]. PAPPA increases IGF bioavailability and mitogenic effectiveness in vitro through regulated cleavage of IGF-binding protein (IGFBP) 4. Pappa-KO mice show dwarfism due to an increase in expression of IGFBP4 that reduces the bioavailability of IGF-1, and outlive wild-type mice (33 % of males, 41 % of females; [16]). In Pappa-KO mice, plasma GH and IGF-1 concentrations tended to be 30 % lower than those in wild-type mice, although the results were statistically insignificant [16]. *Igf1r*$^{+/-}$-mice also live longer than wild-type mice, although the effect was only significant in female mice and not in male mice. In *Igf1r*$^{+/-}$ mice, plasma IGF-1 concentrations were increased probably because of a negative feedback mechanism through the pituitary gland, although the plasma GH levels were not described in the original study. A possible increase in GH levels might have some adverse effects on mice, particularly male mice. Therefore, the life-prolonging effect of reduced intracellular IGF-1 signaling [14] might be minimized in male mice.

In rats, the effect of a reduction in the GH-IGF-1 axis on lifespan and aging remains controversial. A modest reduction of GH and IGF-1 by overexpression of an antisense *GH* gene in Wistar rats also induces dwarfism from neonates [17]. Compared with

wild-type rats, heterozygous rats for the transgene exhibit a 30 % decrease in mean food intake and body weight with a 40 % reduction in plasma IGF-1 [18]. The body size of these rats is comparable to that of wild-type mice subjected to 30 % CR [19]. Furthermore, the transgenic rats live longer than wild-type rats [18], although the life-extending effect is small in comparison with 30 % CR (10 % of transgenic rats versus 18 % of CR rats at the 10th percentile survival point; [19]. In this transgenic model, there are also effects on insulin-glucose homeostasis, such as reduced serum insulin, increased insulin sensitivity, and improved glucose tolerance, which are hallmarks of CR animals [20, 21]. Stress resistance is also enhanced in both transgenic and CR rats [22]. These comparable phenotypes of transgenic and CR rats suggest that the GH-IGF-1 axis plays a role in the anti-aging effect of CR.

In contrast to the long-lived dwarf mouse model, homozygous *dw/dw* rats, originally derived from the Lewis strain, do not show any significant increase in lifespan [23]. However, GH administration between 4 and 15 weeks of age modestly extends the lifespan of *dw/dw* rats. This model is considered to represent adult onset GH deficiency, because the plasma IGF-1 concentration returns to the same level as that in control *dw/dw* mice at 2 weeks after termination of GH replacement [23]. This study suggests the importance of GH during adolescence. However, these findings contradict those in Ames mice in which replacement of GH between 2 and 8 weeks of age abolishes the lifespan extension [24]. Administration of GH for only 7 days also downregulates glutathione S-transferase and mitochondrial thioredoxin systems that affect the protective machineries against oxidative stress in Ames mice [25]. Therefore, the role of GH and IGF-1 in lifespan extension remains somewhat elusive, particularly in rats.

4.2.3 Other Longevity Genes Related to the GH-IGF-1 Axis

αMUPA transgenic mice overexpress the gene encoding urokinase-type plasminogen activator (*Plau*, also known *uPA*) in the brain and outlive wild-type mice [26]. Plau is a serine protease that activates plasminogen by proteolytic cleavage into plasmin. αMUPA mice carry murine Plau cDNA linked downstream from the promoter of the murine α-crystallin gene that is predominantly active in the ocular lens. These transgenic mice unexpectedly express Plau in nerve cells of the central nervous system but not in the peripheral nervous system or non-neuronal tissues [26]. Transgenic Plau is also expressed in the hypothalamic paraventricular nucleus that is involved in regulation of appetite and energy expenditure [27]. αMUPA mice show reduced food intake and body weights. Plasma IGF-1 is also reduced by 30 % of that in wild-type mice at 5 months of age [28]. In addition, the αMUPA mouse exhibits effects similar to those elicited by CR, such as reductions in body temperature and the incidence of spontaneously occurring tumors and carcinogen-induced neoplastic foci [28]. Thus, although the exact mechanisms remain elusive, brain-specific overexpression of Plau may extend lifespan through reductions in food intake and plasma IGF-1 levels, which are similar to the effects of CR.

Extension of lifespans by overexpression of *Fgf21* and *Sirt6* genes in mice can also be achieved by a reduction in plasma IGF-1 or desensitization to IGF-1 bioactivity. Fgf21 is a hormone secreted by the liver during fasting, which is involved in the adaptive response to negative energy balance [29]. Fgf21 increases insulin sensitivity resulting in a decrease in basal insulin concentrations. In addition, Fgf21 blocks somatic growth by induction of GH resistance, a phenomenon associated with physiological adaptation to starvation. Fgf21 transgenic mice are significantly smaller than wild-type mice and show a corresponding decrease in circulating IGF-1 concentrations despite elevated GH levels. Compared with wild-type mice, lifespans are significantly extended in male and female *Fgf21* transgenic mice [30]. *Fgf21* transgenic mice share many phenotypes with CR mice. Gene expression analysis of *Fgf21* transgenic and CR mice has confirmed an overlap of genes significantly regulated in liver, muscle and adipose tissues [30].

Sirt6 is located in the nucleus in association with chromatin [31]. Expression of Sirt6 is found in numerous mouse tissues and at particularly high levels in the thymus, skeletal muscle, and brain [32, 33]. Sirt6 acts in the DNA double-strand break (DSB) repair system. It activates the DNA-DSB repair system via deacetylation of CtBP-interacting protein (CtIp) and ADP ribosylation of poly ADP-ribose polymerase 1 (PARP1). Deletion of the *Sirt6* gene in cells induces genomic instability [33]. *Sirt6*-KO mice exhibit premature aging phenotypes similar to those of XPA/CA or XPA/TTD null mice [33]. Conversely, overexpression of Sirt6 extends the lifespan of male mice but not female mice [34]. Moreover, *Sirt6* transgenic mice show a decrease in plasma IGF-1 levels and an increase in IGFBP1 levels. Activation of IGF-1 signaling is also attenuated as indicated by decreased levels of phosphorylated IGF-1r, Akt, and Foxo1 in epididymal adipose tissue.

Although the mechanism underlying inhibition of IGF-1 signaling by Sirt6 overexpression remains elusive, three longevity mouse models also indicate the significance of GH-IGF-1 axis inhibition for longevity.

4.2.4 Downstream Signaling of the GH-IGF-1 Axis in the Longevity of Mammals

Insulin, IGF-1, and other growth factors bind to their specific tyrosine kinase receptors to trigger autophosphorylation that activates insulin receptor substrate (IRS) proteins by tyrosine phosphorylation, thereby activating PI3K (Fig. 4.1, [35]). PI3K phosphorylates PtdIns(4,5)P3 to produce PtdIns(3,4,5)P3, leading to recruitment of Akt into the cell membrane. Akt is fully activated through conformational changes and additional phosphorylation by PDK1 and mTORC2. Akt phosphorylates multiple substrates to regulate cellular functions, including mechanistic target of rapamycin (mTOR, activated), glycogen synthesis kinase 3 beta (GSK3β, inactivated), and FoxO transcription factors (inactivated).

Some genes encoding intracytoplasmic molecules are also reported to regulate the lifespan of mice. Taguchi et al. [36] demonstrated that whole body and

brain-specific KO mice for the *Irs2* gene outlive wild-type mice. Interestingly, either homozygous or heterozygous brain-specific *Irs2* KO in mice leads to hyperinsulinemia and mild glucose intolerance. However, a reduction in IRS2 signaling prevents aging-related decreases in the protein levels of FoxO1 and its target protein, superoxide dismutase 2 (SOD2). The authors speculated that attenuation of IRS2 signaling in the brain shielded against the negative effects of hyperinsulinemia. In contrast, Selman et al. [37] reported controversial findings in $Irs2^{-/-}$ mice, although they indicated that female $Irs1^{-/-}$ mice show an extended lifespan. In their experiments, $Irs2^{-/-}$ mice displayed diabetic phenotypes and died earlier than wild-type mice. The discrepancy might have been caused by the degree of Irs2 signaling inhibition or different strains or stocks of mice.

$Akt1^{+/-}$ mice are also reported to live longer than wild-type mice (8 % of males and 14 % of females; [38]). In $Akt1^{+/-}$ mice, glucose tolerance and insulin sensitivity are comparable with those in wild-type mice. Nojima et al. [38] showed that the mTOR pathway, which regulates ribosomal biogenesis, protein synthesis, and mitochondrial activity, is downregulated in $Akt1^{+/-}$ mice, although the total protein and phosphorylated form of FoxO3 do not differ between wild-type and $Akt1^{+/-}$-mice.

The mTOR pathway increases protein translation and synthesis via phosphorylation of its main effector proteins, eukaryotic translation initiation factor 4E (eIF-4E)-binding protein 1 (4EBP1) and 70-kDa ribosomal S6 kinase (S6K) [35]. Deletion of the S6K peptide 1 (*S6K1*) gene in mice extends their lifespan, although the effect is only observed in female mice and not in male mice [39]. Compared with wild-type mice, motor functions, bone volume, and glucose tolerance are improved in middle-aged female $S6K1^{-/-}$ mice. Furthermore, $S6K1^{-/-}$ mice show a dwarf phenotype without reductions in plasma IGF-1 and pituitary GH.

FoxO transcription factors, including FoxO1, 3, 4, and 6, are mammalian orthologs of Daf-16 in *C. elegans* [40]. These transcription factors are negatively regulated by signals from growth factor-PI3K-Akt pathways. Target genes of FoxO transcription factors are involved in the cell cycle, DNA repair, stress resistance, apoptosis, autophagy, and metabolism in response to cellular and genotoxic stresses [40]. Overexpression of the *Foxo1* gene induces a loss of muscle proteins, thus leading to sarcopenia [41], but it does not affect the lifespan of mice [42]. Single gene deletion of *Foxo1*, *3*, or *4* does not significantly affect the lifespan or occurrence of tumors in mice under standard ad libitum feeding conditions [43]. Triple knockout of three *Foxo* genes shortens lifespan and increases the incidence of tumors, suggesting functional redundancy of FoxO transcription factors in the effects on lifespan and tumorigenesis. However, our analyses of $Foxo1^{+/-}$-and $Foxo3^{+/-}$-mice with 30 % CR demonstrate differential roles for FoxO1 and FoxO3 in the anti-neoplastic and life-extending effects of CR. $Foxo1^{+/-}$ -mice show a diminished anti-neoplastic effect of CR with maintenance of the life-extending effect [44]. In contrast, there is no life-extending effect of CR, but the prevalence of spontaneously occurring tumors is significantly reduced by CR in $Foxo3^{+/-}$-mice, suggesting a role for Foxo3 in the life-extending effect of CR (Shimokawa, unpublished observation). Numerous human genetic studies indicate a correlation of the minor alleles of the *Foxo3* gene with longevity [45]. However, there is no significant correlation of FoxO1 with longevity.

Therefore, even in humans, inhibition of excess or sustained activation of insulin and IGF-1 signals and promotion of FoxO3 activation may exert some beneficial effects on lifespan. In fact, many studies support this notion.

In summary, longevity models in mice suggest that attenuation of the GH-IGF-1 axis, and therefore inactivation of its downstream intracytoplasmic signaling, favors longevity in mammals, although complete inhibition results in pathological conditions such as diabetes. FoxO transcription factors have functional roles in the regulation of lifespan and tumor incidence under CR conditions, but not ad libitum conditions. Furthermore, the precise mechanisms downstream of FoxO transcription factors remain to be elucidated.

4.3 Physiological Changes Induced by CR

4.3.1 Effect of CR on Neuroendocrine Systems

Information on the modulation of neuroendocrine systems by CR has prompted investigation of the effects of hormones on the aging process. Orexigenic and anorexigenic neurons in the hypothalamic arcuate nuclei competitively regulate appetite and energy expenditure, and second-ordered neurons regulate growth, reproduction, thyroidal thermogenesis, and the adrenal glucocorticoid axis [46]. Orexigenic neurons expressing neuropeptide Y (NPY) and/or agouti gene-related protein (AGRP) are activated by reductions in the plasma concentrations of leptin, insulin, and IGF-1 (Fig. 4.2). These hormonal changes are induced by CR as well as

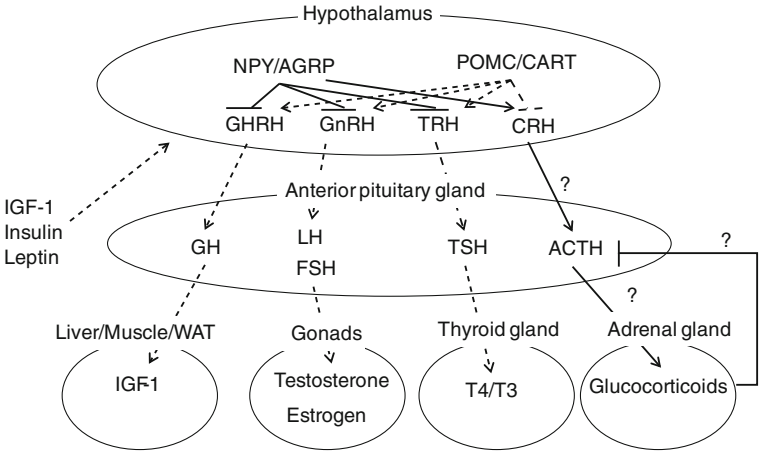

Fig. 4.2 Effects of CR on neuroendocrine systems. Activation of NPY/AGRP neurons and attenuation of POMC/CART neurons inhibit growth, reproduction, and thyroidal functions, but augment adrenal glucocorticoids through modulation of the anterior pituitary as well as peripheral tissues and organs

by fasting [47]. In contrast, the activity of anorexigenic neurons expressing pro-opiomelanocortin (POMC) and cocaine and amphetamine-related transcript (CART) are attenuated under CR conditions [48, 49]. Accordingly, there is inhibition of second-ordered neurons expressing GH-releasing hormone (GHRH), gonadotropin releasing hormone (GnRH), and thyroid-stimulating hormone releasing hormone (TRH), while corticotropin-releasing hormone (CRH)-expressing neurons undergo activation. These alterations in hypothalamic neurons subsequently modulate anterior pituitary hormones. Indeed, these hormones are expectedly regulated by CR, except for ACTH. Plasma ACTH and the ACTH content in the anterior pituitary are reduced in CR rats, probably because of negative feedback due to mild hyperadrenocorticalism [50]. Changes in anterior pituitary hormones modulate some downstream hormones secreted by peripheral organs and endocrine glands. CR also reduces the plasma concentrations of IGF-1, sex steroids, and T4/T3. Accordingly, CR animals display smaller body sizes than those of control animals fed ad libitum. Depending on the severity of CR, reproductive functions are also suppressed in CR animals. For example, there is a reduction in the number of littermates produced by CR females. However, upon termination of CR regimens, female animals show restoration of reproductivity. The fertile lifespan is also extended by CR [51]. Moreover, body temperature is slightly decreased in CR rodents [52]. Plasma corticosterone is increased in aging animals, but CR inhibits this age-related change [53]. However, in CR rodents, there is an increase in the fraction of unbound corticosterone from its binding protein (corticosterone-binding protein), although the total amount of corticosterone does not differ from that in control animals fed ad libitum [53]. CR animals are thought to be in mild hyperadrenocorticalism that is relevant to stress resistance promoted by CR.

Collectively, CR inhibits growth, reproduction, and thyroidal functions while activating the adrenal glucocorticoid axis. These alterations in neuroendocrine systems are considered as physiological responses to manage stresses that are evolutionary conserved for survival in organisms. However, the hormonal changes induced by CR are almost the same as those in aging animals. In this respect, it has been reported that the gene expression profiles of aging mice overlap with those of long-lived mice including CR mice [54]. The aging-related alterations in the neuroendocrine systems of control animals are also considered to be adaptive responses to “aging stress”. The only difference in neuroendocrine systems between the effects of aging and CR is the Npy expression level in the hypothalamus. There is a decrease in the Npy mRNA expression level of aging rats, whereas CR upregulates its expression level [19]. Intriguingly, overexpression of the *Npy* gene slightly but significantly extends lifespan [55]. In contrast, the life-prolonging effect of CR is almost abolished in $Npy^{-/-}$ mice, even though the effects of CR on IGF-1, mTOR, and corticosterone are maintained [56]. In this regard, NPY is considered to be an important neuropeptide in regulation of the aging process and lifespan.

4.3.2 *Effect of CR on Adipose Tissues*

White adipose tissue (WAT) serves as an endocrine organ that responds to both central and peripheral metabolic signals. Adipocytes secrete leptin, adiponectin, resistin, and tumor necrosis factor alpha [57]. Therefore, adipocytes are actively involved in various physiological processes such as regulation of appetite, energy expenditure, growth, reproduction, and inflammation. Leptin is the first identified adipokine that shows increases in circulating blood in parallel with increases in the amount of WAT. Leptin inhibits appetite and augments energy expenditure through regulation of hypothalamic neurons including activation of POMC neurons [46]. Therefore, disruption of such signaling (e.g., mutations of leptin or leptin receptor genes) causes hyperphagia and obesity. Subsequent studies have revealed another physiological role of leptin. Under life-threatening conditions such as fasting, circulating leptin levels are precipitously decreased independently of the amount of body fat, leading to inactivation of gonadal, somatotropic, and thyroidal axes while activating the glucocorticoid system [58]. These physiological responses are considered as one of the stress responses to manage a wide variety of stressors.

Adiponectin also contributes to a decrease in aging-related pathologies and extension of lifespan. The plasma concentration of adiponectin is altered depending on the fat mass [57]. Obese animals show lower concentrations of plasma adiponectin, whereas CR rodents show increased levels of adiponectin in parallel with a reduction in fat mass [59]. FoxO1 is known to upregulate adiponectin gene expression (Fig. 4.3; [60]), and increased plasma adiponectin is associated with insulin-sensitizing, anti-diabetic, and anti-atherosclerotic effects [61]. Overexpression of the human Adiponectin (*Adipoq*) gene extends the lifespan of mice fed a high fat or regular diet [62].

At the early phase of adipogenesis, adipocyte precursor cells develop into preadipocytes. Subsequently, these cells differentiate into immature adipocytes and finally into mature adipocytes. Preadipocytes express the IGF-1 receptor (IGF-1R) predominantly, and adipocytes mainly express GHR [63]. Both cell types express the insulin receptor (IR), although its expression level is higher in adipocytes than that in preadipocytes. These finding suggest that GH, IGF-1, and insulin regulate adipogenesis and lipogenesis. It appears that IR preferentially regulates fuel metabolism, whereas IGF-1R mediates cell proliferation. IGF-1 has been suggested to be a major regulator of preadipocyte proliferation and differentiation. In contrast, GH exerts its effects by increasing the pool of adipocyte precursor cells that can differentiate into mature adipocytes. Insulin promotes lipogenesis through upregulation of glucose and fatty acid uptake. Conversely, GH is also known to reduce the volume of mature adipocytes by inhibiting glucose transport and lipogenesis, and facilitating lipolysis. Therefore, under well-nourished conditions, increases in GH, IGF-1, and insulin levels promote adipogenesis and lipogenesis. If these conditions are sustained, GH counteracts the effects of insulin on lipogenesis in WAT and the whole body, i.e., insulin resistance. Preadipocytes and adipocytes do

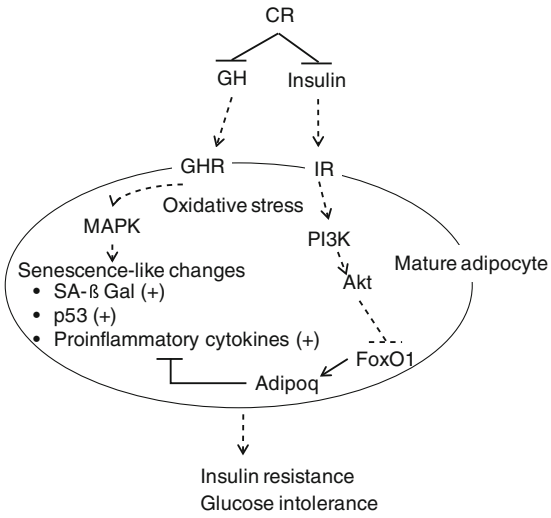

Fig. 4.3 Effects of CR on adipocytes via GH and insulin. GH augments oxidative stress and activates MAPK pathways, leading to senescence-like changes in adipocytes. These senescence-like changes cause whole-body insulin resistance and glucose intolerance. Insulin signaling suppresses activation of the FoxO1 transcription factor. One of the target genes of FoxO1 is *Adipoq* that counteracts senescence-like changes. CR inhibits the senescence-like changes and thus ameliorates insulin resistance and glucose intolerance through attenuation of GH and IR signaling

not express GH, but do express IGF-1. Thus, IGF-1 synthesized in adipocytes acts on these cells in autocrine or paracrine manners.

Excess energy intake leads to the accumulation of oxidative stress in WAT through activation of mitogen-activated protein kinase (MAPK) pathways and promotion of senescence-like changes such as increased expression of SAβ-Gal, p53, and proinflammatory cytokines in WAT (Fig. 4.3; [64]). Expression of p53 induces whole-body insulin resistance and glucose tolerance in experimentally induced obese mice [64]. In the WAT of *Adipoq*-overexpressing mice, p53 and its downstream molecule, p21, are reduced in a manner similar to that in CR mice (Fig. 4.3; Kamohara and Shimokawa, unpublished observation). Concomitantly, there is also an improvement in insulin sensitivity. GH is known to activate p38, probably through augmentation of oxidative stress, leading to upregulation of p53 [65]. Therefore, it appears that CR ameliorates senescence-like changes in WAT via inhibition of GH and insulin signaling (Fig. 4.3).

The net amount of WATs is decreased by CR in rodents. The size of adipocytes is also reduced by CR [66]. The reduction of WAT leads to a decrease in plasma leptin and an increase in adiponectin. These hormonal changes sensitize insulin actions in tissues as described above. Thus, a reduction of WAT is thought to be one of the mechanisms underlying the anti-aging effect of CR. However, the relationship between WAT and the effect of CR is complex. It is thought that CR might inhibit lipogenesis while promoting lipolysis. Indeed, Sirt1 promotes

lipolysis [67] and is considered as a CR mimetic. However, CR facilitates fatty acid synthesis while inhibiting excess lipolysis [68]. These changes are similar to those that occur in obese animals. Under CR conditions, animals store dietary energy in the form of fat and then use the stored fat for ATP production to survive. During this process, energy efficiency appears to be maximized. This obese-like trait may be significant in the anti-aging effect of CR. Recently, Liao et al. [69] reported that over 50 % of inbred strains of mice do not show lifespan extension by 40 % CR. Furthermore, the rate of lifespan extension is negatively correlated with the reduction of WAT by CR, i.e., mouse strains resistant to CR in terms of loss of fat tissues show a significant extension of lifespan in response to CR. These findings suggest that a reduction in WAT does not simply cause an extension of lifespan or the anti-aging effects of CR. In this respect, the findings in $Npy^{-/-}$ mice, i.e., diminution of the life-extending effect of CR [56], is in accordance with the observation reported by Liao et al. [69], because Npy is a neuropeptide that inhibits excess loss of fat by decreasing energy expenditure and lipolysis under conditions of negative energy balance.

4.4 Summary

GH, IGF-1, insulin, and leptin are hormones secreted under well-nourished conditions and communicate those conditions to the hypothalamus. Sustained reductions of these hormones induced by CR induce physiological conditions to maximize the survival of animals, probably by activation of stress response machineries including the adrenal glucocorticoid system concomitantly with inhibiting growth, reproduction, and thermogenesis. At the intracellular level, a reduction in insulin/IGF-1 signaling promotes activation of FoxO transcription factors under stress conditions and concomitantly attenuates the mTOR pathway. Genetic modulation of GH-IGF-1 signaling extends the lifespan of experimental animals, even if the effects are minor compared with those induced by CR. The CR paradigm and longevity gene models provide insights into the regulation of aging and the extension of a healthy lifespan in humans.

References

1. Rudman D et al (1990) Effects of human growth hormone in men over 60 years old. New England J Med 323(1):1–6
2. Shimokawa I et al (2008) Longevity genes: insights from calorie restriction and genetic longevity models. Mol Cells 26(5):427–435
3. Weindruch R, Walford RL (1988) The retardation of aging and disease by dietary restriction. Charles C Thomas Publisher, Springfield, IL
4. Cohn L et al (1993) Carpal tunnel syndrome and gynaecomastia during growth hormone treatment of elderly men with low circulating IGF-I concentrations. Clin Endocrinol 39 (4):417–425

5. Friedman DB, Johnson TE (1988) Three mutants that extend both mean and maximum life span of the Nematode, Caenorhabditis elegans, define the age-1 gene. J Gerontol 43(4):B102–B109
6. Morris JZ, Tissenbaum HA, Ruvkun G (1996) A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. Nature 382 (6591):536–539
7. Kenyon C et al (1993) A C. elegans mutant that lives twice as long as wild type. Nature 366 (6454):461–464
8. Kimura KD et al (1997) daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science 277(5328):942–946
9. Ogg S et al (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature 389(6654):994–999
10. Brown-Borg HM et al (1996) Dwarf mice and the ageing process. Nature 384(6604):33
11. Steger RW, Bartke A, Cecim M (1993) Premature ageing in transgenic mice expressing different growth hormone genes. J Reprod Fertil Suppl 46:61–75
12. Coschigano KT et al (2000) Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. Endocrinology 141(7):2608–2613
13. Flurkey K et al (2001) Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. Proc Nat Acad Sci USA 98(12):6736–6741
14. Holzenberger M et al (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 421(6919):182–187
15. Boldt HB, Conover CA (2007) Pregnancy-associated plasma protein-A (PAPP-A): a local regulator of IGF bioavailability through cleavage of IGFBPs. Growth Horm IGF Res 17 (1):10–18
16. Conover CA, Bale LK (2007) Loss of pregnancy-associated plasma protein A extends lifespan in mice. Aging Cell 6(5):727–729
17. Matsumoto K et al (1993) Growth retardation in rats whose growth hormone gene expression was suppressed by antisense RNA transgene. Mol Reprod Dev 36(1):53–58
18. Shimokawa I et al (2002) Life span extension by reduction in growth hormone-insulin-like growth factor-1 axis in a transgenic rat model. Am J Pathol 160(6):2259–2265
19. Shimokawa I et al (2003) Life span extension by reduction of the growth hormone-insulin-like growth factor-1 axis: relation to caloric restriction. FASEB J 17(9):1108–1109
20. Yamaza H et al (2004) A transgenic dwarf rat model as a tool for the study of calorie restriction and aging. Exp Gerontol 39(2):269–272
21. Yamaza H et al (2007) Involvement of insulin-like growth factor-1 in the effect of caloric restriction: regulation of plasma adiponectin and leptin. J Gerontol Ser A Biol Sci Med Sci 62(1):27–33
22. Komatsu T et al (2011) Acute stress response modified by modest inhibition of growth hormone axis: a potential machinery of the anti-aging effect of calorie restriction. Mech Ageing Dev 132(3):103–109
23. Sonntag WE et al (2005) Adult-onset growth hormone and insulin-like growth factor I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. Endocrinology 146(7):2920–2932
24. Panici JA et al (2010) Early life growth hormone treatment shortens longevity and decreases cellular stress resistance in long-lived mutant mice. FASEB J 24(12):5073–5079
25. Rojanathammanee L, Rakoczy S, Brown-Borg HM (2014) Growth hormone alters the glutathione s-transferase and mitochondrial thioredoxin systems in long-living ames dwarf mice. J Gerontol Ser A Biol Sci Med Sci 69(10):1199–1211
26. Miskin R, Masos T (1997) Transgenic mice overexpressing urokinase-type plasminogen activator in the brain exhibit reduced food consumption, body weight and size, and increased longevity. J Gerontol Ser A Biol Sci Med Sci 52(2):B118–B124
27. Schwartz MW et al (2000) Central nervous system control of food intake. Nature 404 (6778):661–671
28. Miskin R et al (2005) AlphaMUPA mice: a transgenic model for longevity induced by caloric restriction. Mech Ageing Dev 126(2):255–261

29. Potthoff MJ et al (2009) FGF21 induces PGC-1alpha and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. Proc Nat Acad Sci USA 106 (26):10853–10858
30. Zhang Y et al (2012) The starvation hormone, fibroblast growth factor-21, extends lifespan in mice. Elife 1:e00065
31. Gertler AA, Cohen HY (2013) SIRT6, a protein with many faces. Biogerontology 14 (6):629–639
32. Michishita E et al (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell 16(10):4623–4635
33. Mostoslavsky R et al (2006) Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 124(2):315–329
34. Kanfi Y et al (2012) The sirtuin SIRT6 regulates lifespan in male mice. Nature 483:218–221
35. O' Neill C (2013) PI3-kinase/Akt/mTOR signaling: impaired on/off switches in aging, cognitive decline and Alzheimer's disease. Exp Gerontol 48(7):647–653
36. Taguchi A, Wartschow LM, White MF (2007) Brain IRS2 signaling coordinates life span and nutrient homeostasis. Science 317(5836):369–372
37. Selman C et al (2008) Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. FASEB J 22(3):807–818
38. Nojima A et al (2013) Haploinsufficiency of akt1 prolongs the lifespan of mice. PLoS ONE 8 (7):e69178
39. Selman C et al (2009) Ribosomal Protein S6 Kinase 1 signaling regulates mammalian life span. Science 326(5949):140–144
40. Greer EL, Brunet A (2005) FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene 24(50):7410–7425
41. Kamei Y et al (2004) Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. J Biol Chem 279(39):41114–41123
42. Chiba T et al (2009) Overexpression of FOXO1 in skeletal muscle does not alter longevity in mice. Mech Ageing Dev 130(7):420–428
43. Paik J-H et al (2007) FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 128(2):309–323
44. Yamaza H et al (2010) FoxO1 is involved in the antineoplastic effect of calorie restriction. Aging Cell 9(3):372–382
45. Willcox BJ et al (2008) FOXO3A genotype is strongly associated with human longevity. Proc Nat Acad Sci USA 105(37):13987–13992
46. Sainsbury A, Zhang L (2011) Role of the hypothalamus in the neuroendocrine regulation of body weight and composition during energy deficit. Obes Rev 1–24
47. Shimokawa I et al (2008) Longevity genes: insights from calorie restriction and genetic longevity models. Mol Cells 26(5):427–435
48. Shimokawa I et al (2003) Effects of caloric restriction on gene expression in the arcuate nucleus. Neurobiol Aging 24(1):117–123
49. Komatsu T et al (2006) Effect of leptin on hypothalamic gene expression in calorie-restricted rats. J Gerontol Ser A Biol Sci Med Sci 61(9):890–898
50. Han ES et al (1995) Hyperadrenocorticism and food restriction-induced life extension in the rat: evidence for divergent regulation of pituitary proopiomelanocortin RNA and adrenocorticotropic hormone biosynthesis. J Gerontol Ser A Biol Sci Med Sci 50(5): B288–B294
51. Holehan AM, Merry BJ (1985) Lifetime breeding studies in fully fed and dietary restricted female CFY Sprague-Dawley rats 1. Effect of age, housing conditions and diet on fecundity. Mech Ageing Dev 33(1):19–28
52. Himms-Hagen J (1985) Food restriction increases torpor and improves brown adipose tissue thermogenesis in ob/ob mice. Am J Physiol 248(5 Pt 1):E531–E539
53. Sabatino F et al (1991) Assessment of the role of the glucocorticoid system in aging processes and in the action of food restriction. J Gerontol 46(5):B171–B179

54. Schumacher B et al (2008) Delayed and accelerated aging share common longevity assurance mechanisms. PLoS Genet 4(8):e1000161
55. Michalkiewicz M et al (2003) Hypotension and reduced catecholamines in neuropeptide Y transgenic rats. Hypertension 41(5):1056–1062
56. Chiba T et al (2014) A key role for neuropeptide Y in lifespan extension and cancer suppression via dietary restriction. Sci Rep 4:4517
57. Blüher M (2014) Adipokines—removing road blocks to obesity and diabetes therapy. Mol Metab 3(3):230–240
58. Ahima RS et al (1996) Role of leptin in the neuroendocrine response to fasting. Nature 382 (6588):250–252
59. Berg AH et al (2001) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat Med 7(8):947–953
60. Qiao L, Shao J (2006) SIRT1 regulates adiponectin gene expression through Foxo1-C/ enhancer-binding protein alpha transcriptional complex. J Biol Chem 281(52):39915–39924
61. Hui X et al (2012) Adiponectin and cardiovascular health: an update. Br J Pharmacol 165 (3):574–590
62. Otabe S et al (2007) Overexpression of human adiponectin in transgenic mice results in suppression of fat accumulation and prevention of premature death by high-calorie diet. Am J Physiol Endocrinol Metab 293(1):E210–E218
63. Blüher S, Kratzsch J, Kiess W (2005) Insulin-like growth factor I, growth hormone and insulin in white adipose tissue. Best Pract Res Clin Endocrinol Metab 19(4):577–587
64. Minamino T et al (2009) A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nat Med 15(9):1082–1087
65. Bogazzi F et al (2013) Growth hormone is necessary for the p53-mediated, obesity-induced insulin resistance in male C57BL/6 J x CBA mice. Endocrinology 154(11):4226–4236
66. Zhu M et al (2007) Adipogenic signaling in rat white adipose tissue: modulation by aging and calorie restriction. Exp Gerontol 42(8):733–744
67. Picard F et al (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 429(6993):771–776
68. Bruss MD et al (2010) Calorie restriction increases fatty acid synthesis and whole body fat oxidation rates. Am J Physiol Endocrinol Metab 298:E108–E116
69. Liao C-Y et al (2011) Fat maintenance is a predictor of the murine lifespan response to dietary restriction. Aging Cell 10(4):629–639

Chapter 5
Epigenetic Modulation of Gene Expression by Exercise

Sataro Goto, Kyojiro Kawakami, Hisashi Naito, Shizuo Katamoto and Zsolt Radak

Abstract Physical activity has long-lasting beneficial effects by inducing metabolic adaptation, retarding biological ageing and reducing the risk of various age-related disorders and lifestyle-associated diseases such as type 2 diabetes mellitus, cancer, cardiovascular disorders and various types of inflammation, thereby extending healthy lifespan. Recent studies revealed that epigenetic mechanisms such as DNA methylation, histone modifications and microRNA expression are involved in exercise-induced adaptive responses. In this chapter, we first describe the processes of DNA methylation, histone modifications and microRNA, and then overview the effect of exercise on these epigenetic regulatory mechanisms. Finally, we discuss the relevance of epigenetics to ageing.

S. Goto (✉)
Institute of Health, Sports Science and Medicine, Juntendo University Graduate School, Chiba, Japan
e-mail: stgotou@juntendo.ac.jp

K. Kawakami
Research Team for Mechanism of Aging, Molecular Gerontology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan
e-mail: kkawa@tmig.or.jp

H. Naito · S. Katamoto
Department of Exercise Physiology, Juntendo University, Chiba, Japan
e-mail: hnaitou@juntendo.ac.jp

S. Katamoto
e-mail: skatamoto@juntendo.ac.jp

Z. Radak
Institute of Exercise Physiology, Hungarian University of Physical Education, Budapest, Hungary
e-mail: radak@tf.hu

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5.1 Introduction: Genetics and Epigenetics

Gene expression is determined by the nucleotide sequences encoded in the genome and components of the transcriptional machinery. Expression changes during the development and differentiation of cells that have the same genome, starting from a single fertilized egg, and eventually giving rise to an individual consisting of many billions of differentiated and undifferentiated cells in a multi-cellular organism. Each tissue and organ consists of different sets of cells that express both cell-specific and common genes throughout life. Long-term cell-specific gene expression is determined by mechanisms including DNA methylation and histone modifications. Such mechanisms of gene expression are termed epigenetics.

Epigenetics describes a phenomenon in which a fixed pattern of gene expression in a cell or an organism is inherited from one generation to the next in cells or organisms, without changing the nucleotide sequence of the genome. However, this definition has been broadened to include the long-term stable control of cell-specific gene expression without changes in the nucleotide sequence. In addition, the recent development of molecular biology and technologies that allow the detection and quantification of minor RNAs identified the expression of microRNA (miRNA) as an important epigenetic mechanism that modulates gene expression. First, we briefly overview the processes of DNA methylation, histone modifications and miRNA expression, describe the effects of exercise on the epigenetic changes and finally discuss the relevance of epigenetics to ageing.

5.2 DNA Methylation

Genomic DNA methylation is the classical epigenetic modification and has a well-established mechanism of inheritance between generations. It involves the addition of a methyl group to a cytosine base at CpG dinucleotide sequences, although it can also occur in cytosine bases at other sequences. CpG islands are CpG-rich sequences that are often located in the promoter region of genes and are usually hypomethylated compared with CpG sequences downstream of the islands known as CpG shores. The hypermethylation of CpG islands is associated with transcriptional repression. Most CpG sequences in regions other than promoters are also methylated. Methylation is catalysed by a family of DNA methyltransferases, which use the substrate S-adenosylmethionine as the methyl donor. There are two categories of DNA methyltransferases: one that transmits the methylation pattern to the next generation during DNA replication (DNMT1, maintenance DNA methyltransferase) and another that introduces a methyl group to previously un-methylated cytosines to modulate gene expression and other chromatin functions (DNMT3a and DNMT3b, de novo DNA methyltransferases). DNA methylation at promoter regions makes the chromatin conformation more condensed, thereby suppressing transcription, whereas modifications inside the gene enhance transcription. DNA methylation influences histone

modifications, and the two processes exhibit cross-talk. In addition, some miRNAs modulate DNA methyltransferases, thereby affect the status of DNA methylation [1].

The mechanism by which methyl groups are removed from methylated DNA has long been unclear, but it is now thought to be initiated by the oxidation of 5′-methylcytosine into 5-hyroxymethylcytosine. This oxidation is catalysed by the ten-eleven translocation (TET) family enzymes. Oxidised 5′-methylcytosine can be depleted passively during DNA replication or reverted to cytosine actively by base excision repair reactions that are catalysed by thymine DNA glycosylase [2]. Therefore, the methylation and demethylation of DNA can be modulated dynamically by methyltransferases and demethylation reactions.

5.3 Histone Modifications

Histones are highly basic proteins that are associated with nuclear DNA to form nucleosome complexes. These complexes exhibit a compact conformation of beads on a string, which packs DNA in the confined space in the nucleus and thereby represses the expression of most genes. The nucleosome consists of two of each histones, H2A, H2B, H3, and H4 to form an octamer of histones. Another histone, H1, is located between nucleosome particles, and is called the linker histone. A DNA sequence of $\sim$150 nucleotides wraps around the histone octamer. Histones are modified by acetylation (at Lys [K] residues), methylation (K and Arg [R] residues), phosphorylation (Ser [S], Thr [T] and Tyr [Y] residues), ubiquitination (K), deimination (R), sumoylation (K) and carbonylation (unidentified residues). These modifications occur predominantly at the N-terminal regions of histones, which protrude from the globular domains and are therefore named histone tails. The modification of histones alters the conformation of chromatin to influence DNA transcription, replication and repair. The acetylation of K residues is one of the most frequent histone modifications. It neutralizes the positive charge of histones to relax the structure of the chromatin by reducing the electrostatic interaction with negatively charged DNAs, thereby activating transcription.

Histone acetylation is catalysed by histone acetyl transferases (HATs) using acetyl CoA as the substrate. There are many HAT isozymes, including p300 and CBP (cyclic AMP response element binding protein [CREB] binding protein). Acetylated histones are deacetylated by the catalytic action of histone deacetylases (HDACs). HDACs have attracted much attention because deacetylation is likely to play a key role in the dynamics of acetylation. HDACs are categorized as class I to IV. Class II HDACs are characterised by their localisation in both the nucleus and cytoplasm and trafficking between the two compartments, depending on the situation. Class III enzymes, also called silent information regulators (SIRTs), are nicotinamide adenine dinucleotide (NAD)-dependent enzymes whose activity is influenced by the energy status of the cell, as well as metabolic scenarios such as calorie restriction and DNA repair in which the amount of the coenzyme NAD can

change significantly. The HATs and HDACs involved in histone dynamics are also called lysine acetyl transferases (KATs) and lysine deacetylase (KDACs), respectively, because some enzymes catalyse the acetylation and deacetylation of proteins in general, not just histones. For example, many regulatory proteins such as transcription factors and mitochondrial proteins involved in energy metabolism undergo reversible acetylation, which modulates their activity.

Methylation, another frequent histone modification, activates or inactivates the function of chromatin depending on which residues are modified [3]. For example, H3K4 tri-methylation (H3K4me3) at the promoter region activates gene expression [4], whereas H3K27 methylation at the same region suppresses transcription. The methylation is catalysed by histone methyltransferases (HMTs), and can occur as the mono-, di- and tri-methylation of K residues and the mono- and di-methylation of R residues at the ε-amino group. Unlike acetylation, methylation does not change the charge of the histones. The function of chromatin can be induced by the steric effects of methyl group(s) interacting with proteins such as transcription factors. Because of the interaction between histone methyltransferases with SET (Suppressor of variegation 3–9, Enhancer of zeste and Trithorax) domains and DNA methyltransferases, histone methylation influences DNA methylation and vice versa [5].

Other important modifications that can change the architecture of chromatin include the phosphorylation at S, T and Y residues, which can influence the transcription of genes on which the modified chromatin is localised. For example, the phosphorylation of histone H3 S10 enhances acetylation at K14 and suppresses acetylation at K9. The mono-ubiquitination at lysine residues in H2A and H2B also modulates transcription [6].

5.4 MicroRNA

miRNA is a recently identified epigenetic mechanism of gene regulation. miRNAs are non-coding RNAs ~22 nucleotides long, which are derived from the transcription of non-coding DNA. They bind to mRNA at either 3′- or 5′-terminal untranslated regions, decreasing the stability of the mRNA or repressing translation to modulate the amount of protein synthesised. Some miRNAs up-regulate gene expression by increasing the efficiency of translation [7]. miRNA precursors (primary miRNA, pri-miRNA) are transcribed from the miRNA coding regions of genes by RNA polymerase II and are processed to functional mature miRNAs by two endonucleases: Drosha in the nucleus and Dicer in the cytoplasm. The final products then form complexes with proteins that regulate translation of specific mRNAs with complementary sequences to the miRNA [8]. The export of intermediate products from the nucleus to the cytoplasm is dependent on the nuclear export receptor Exportin 5. There are more than 2500 different miRNAs in humans, the number being increasing. A single miRNA can modulate multiple mRNAs, and a single mRNA can be influenced by multiple miRNAs. The expression of more than 60 % of human genes are thought to be modified by miRNAs [9].

In addition to their intracellular roles in the regulation of gene expression, miRNAs are exported into the circulation as microvesicles (exosomes) wrapped in membranes or in complexes with proteins [10]. Circulating miRNAs can play roles in cell-cell communication, and are often used as biomarkers of physiological and pathological conditions. The intercellular transfer of functional miRNAs is mediated by exosomes [11].

5.5 Effects of Exercise on Epigenetics

Regular physical activity exerts long-lasting beneficial effects by inducing metabolic adaptations, thereby reducing the risk of various age-related disorders and extending a healthy life span. These adaptive changes are thought to be induced by altered gene expression. Recent studies revealed that epigenetic mechanisms such as DNA methylation, histone modifications and microRNA expression are involved in this adaptation. Here, we review selected studies as examples of the complex epigenetic regulation that is induced by exercise.

5.5.1 DNA Methylation

Acute and regular exercise influences DNA methylation in skeletal muscle and other tissues by inducing the expression of genes that promote health. Barres et al. performed skeletal muscle biopsies in healthy sedentary individuals, and showed that an acute bout of aerobic exercise decreases the methylation of global DNA and the promoters of peroxisome proliferator-activated receptor gamma coactivator-*1α* (*PGC-1α*) and pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*) genes [12]. In addition, the mRNA expression of these genes was elevated markedly after exercise. Such exercise-induced changes were not seen in other genes such as myocyte enhancer factor 2A (*MEF2a*), myogenic differentiation 1 (*MYOD1*), citrate synthase (*CS*) and glyceraldehyde 3-phosphate dehydrogenase; a house-keeping protein (*GAPDH*). These results were reproduced in a model system where mouse soleus muscle was stimulated by ex vivo contraction, which decreased DNA methylation and increased mRNA expression; these changes were not dependent on factors external to the muscle itself. Therefore, the authors provided evidence that promoter hypomethylation is an early mechanism of the exercise-induced activation of responsive genes in skeletal muscle. In another model system using rat myotube cultures, the authors reported that ionomycin, which activates Ca^{2+} release, or the AMP activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR), similarly induced gene expression without promoter hypomethylation, suggesting that DNA methylation does not exclusively regulate exercise-induced gene activation.

Adipose tissue is important not only as an endocrine organ that produces adipokines and other factors with systemic effects but also as has a central role in preventing obesity and type 2 diabetes [13]. Rönn et al. [14] examined the effects of 6 months of exercise intervention on the genome-wide DNA methylation at CpG sites in subcutaneous adipose tissue biopsies in previously sedentary but healthy males. They found that genes associated with obesity and type 2 diabetes exhibited differential DNA methylation patterns before and after the exercise intervention. They studied three genes (*RALBP1*, *HDAC4* and *NCOR2*) in detail, and showed that DNA methylation was increased in the exercised group. The mRNA expression of these genes was decreased significantly after exercise, as expected from the changes in the methylation levels. Ral-binding protein 1 (RALBP1) is an effector protein of the small GTPases RalA and RalB, which play roles in the pathogenesis of metabolic syndrome. Therefore, the authors assessed whether gene expression was suppressed by exercise. Data revealed that DNA methylation is responsible for the suppression of transcription using a luciferase reporter gene construct linked to a methylated human gene promoter in vitro. HDAC4, a histone deacetylase, suppresses *GLUT4* transcription in adipocytes; therefore, reducing the transcription of *HDAC4* by increasing DNA methylation likely elevates GLUT4 levels and subsequently glucose uptake. NCOR (Nuclear receptor corepressor) is a transcriptional corepressor that binds to and suppresses the enzyme activity of HDAC4. The authors used an in vitro model of 3T3 L1 adipocytes to reveal that silencing *HDAC4* or *NCOR2* increases lipogenesis by reducing the suppression of GLUT4 and increasing adipocyte glucose uptake.

Exercise has beneficial effects on brain functions such as promoting neurogenesis, learning, memory and improving emotions. Gomez-Pinilla et al. [15] studied the effects of 1 week of voluntary exercise on the methylation of brain-derived neurotrophic factor (*BDNF*) gene, as well as histone modifications in the hippocampus of young rats. BDNF is the most abundant neurotrophin in mammalian brains, and it promotes the growth, maintenance, function and protection of neurons. Exercise up-regulates BDNF expression in the hippocampus [16]. The transcription of promoter IV of *BDNF* is suppressed by methyl-CpG-binding protein (MeCP2) when the DNA is methylated in sedentary animals, and is activated by exercise. Therefore, exercise up-regulates BDNF mRNA. The authors found that exercise stimulated DNA demethylation of promoter IV, which is one of multiple promoters and is subjected to epigenetic regulation; therefore, the demethylation led to the dissociation of MeCP2 from the site occupying the promoter. Neuronal depolarisation induces the calcium-calmodulin-dependent protein kinase II (CaMKII) -dependent phosphorylation of MeCP2; phospho-MeCP2 is then dissociated from its binding site on the promoter, which allows transcription to start. As well as decreasing promoter methylation, exercise increased the acetylation of histone H3 in the nucleosomes that were associated with the *BDNF* promoter region (see also "Histone modifications" below). Exercise reduced HDAC5 mRNA and protein levels significantly, possibly by increasing H3 acetylation. Phospho-cyclic AMP-response-element binding protein (CREB) recruits CREB-binding protein, which has HAT-promoting activity. This also contributes to increased H3 acetylation.

Therefore, voluntary exercise changes DNA methylation and histone acetylation at the promoter or *BDNF* gene, which up-regulates the expression of *BDNF* in the hippocampus to promote neural function.

5.5.2 Histone Modifications

McGee et al. [17] studied the effect of a single bout of cycling on global histone modifications in the skeletal muscle of young males. There was no significant change in histone H3 acetylation at K9 and K14 immediately after 1 h of exercise, which was reportedly associated with the initiation of transcription. In contrast, the acetylation of H3K36, which is associated with elongation, was increased. The authors suggested that exercise remodels chromatin to increase the transcription of genes related to energy and other metabolism in skeletal muscle and also increase transcript elongation [18, 19]. The increased acetylation of H3K36 was caused by the transport of class IIa HDACs from the nucleus by exercise. McGee et al. showed that the levels of HDAC4 and HDAC5 in the nucleus were decreased significantly after exercise, even though the total amount of enzymes in the cell was unchanged [17]. The exercise-induced up-regulation of AMP-activated protein kinase (AMPK) and CaMKII was apparently responsible for the increased HDAC phosphorylation that caused their nuclear export, thereby reducing the deacetylation (i.e., increasing the acetylation) of histone H3. Therefore, phosphorylation-dependent nuclear export plays a role in exercise-induced chromatin remodelling. The role of CaMKII activation in H3 hyperacetylation at the MEF2 binding site in the promoter region of *GLUT4* was also suggested to be the mechanism by which *GLUT4* expression is increased in skeletal muscle after intermittent swimming exercise in rats [20].

Much attention has been paid to the beneficial effects of exercise in the ageing brain. However, studies in young brains are limited, particularly regarding the epigenetic effects on gene expression. Able and Rissman studied the effects of 1 week of voluntary wheel running in young (46 days old) mice and found significantly increased H3 global acetylation in the hippocampus and cerebellum compared with sedentary animals [21]. This is consistent with previous findings where exercise increased hippocampal H3 acetylation in the chromatin at the *BDNF* gene promoter in adult rats [15]. Several HDAC mRNAs, including HDAC5, were decreased by exercise in both regions, consistent with the hypothesis that increased H3 acetylation could lead to increased expression of the *BDNF* gene [21]. The expression of *DNMT*s (*DNMT1, DNMT3a* and *3b*) in the hippocampus was down-regulated by exercise. The suppression of *DNMT* gene activity might contribute to increased *BDNF* gene expression by increasing DNA methylation. A strong negative correlation was found between *BDNF* and *HDAC1* expression, supporting the hypothesis that histone acetylation up-regulates the expression of *BDNF*. Therefore, it is possible that exercise promotes brain function by stimulating epigenetic modifications and up-regulating the expression of the genes that are required for neural function in the developing brain in young animals, such as BDNF and

synapsin, which is involved in synaptic vesicle trafficking. These findings highlight the importance of physical activity for stimulating brain function in children. Moreover, it is important to note that running distance correlated positively with *BDNF* expression in both the hippocampus and cerebellum, further emphasizing the importance of physical activity.

The extent of histone acetylation that influences gene expression is dependent on HAT and HDAC activity, as well as the availability of HAT substrate (acetyl CoA). In view of reports demonstrating that histone acetylation in the hippocampus is a significant epigenetic change that is induced by exercise, Elsner et al. [22] studied the effects of a single session of forced treadmill exercise and chronic regular exercise for 2 weeks on the activity of these enzymes in the hippocampus of young rats. A single session of treadmill running suppressed the HDAC activity remarkably compared with sedentary control animals. However, chronic exercise had no significant effect on HDAC activity. HAT activity, which was studied using histone H3 and H4 peptides as substrates, was increased significantly toward H4, but not H3, by a single exercise session, whereas chronic exercise had no effect. These findings are consistent with the observations of other investigators such as Gomez-Pinilla et al. who reported that voluntary exercise reduced HDAC5 mRNA levels in the rat hippocampus, as discussed above. Similarly, McGee and Hargreaves demonstrated that the amount of HDAC4 and HDAC5 proteins was down-regulated in human skeletal muscle after exercise [15, 23].

Histone phosphorylation is generally less well studied than acetylation and methylation. Nevertheless, the phosphorylation of S, T, and Y residues plays an important role in modulating chromatin activity [24]. Chandramohan et al. reported that the acquisition of the behavioural immobility response by forced swimming exercise as a form of psychological stress induced a transient increase in the number of immuno-positive neurons in the dentate gyrus granular cell layer. This was associated with the phosphorylation of histone H3 S10 and the acetylation of H3K14 in the promoter region of the *c-Fos* gene, which led to the induction of *c-Fos* expression [25]. The authors showed that chromatin modifications altered the transcription of genes associated with neural functions by modulating the signal transduction pathways involving N-methyl-D-aspartate (NMDA) receptors and extracellular signal-regulated kinases (ERK), which are associated with learning and memory. The same authors investigated the effects of voluntary exercise on a running wheel for 4 weeks before exposure to stressful conditions on the above parameters [26]. Voluntary exercise increased the number of neurons that were positive for the expression of phospho-acetyl histone H3 and c-Fos in the dentate gyrus of rats exposed to stress compared with control sedentary animals.

Skeletal muscle is composed of mainly fast or slow fibres that express different amounts of myosin heavy chain (MHC). The soleus, a typical slow-type muscle, expresses predominantly type I MHC, while the plantaris, a typical fast-type muscle, expresses primarily type IIb and IIx MHC. *MHC* expression in slow-type fibres undergoes a shift to that typical of fast-type fibres under muscle unloading. Pandorf et al. [27] assessed whether the histone modifications in chromatin at the

MHC gene differ among muscles (soleus and plantaris) or fibre types in rats. They also examined the effect of muscle unloading on possible chromatin remodeling. The association between the modification and the expression of the specific type of MHC was assessed using chromatin immunoprecipitation with antibodies specific for each modification (i.e., diacetylation at H3K9 and 14 and trimethylation at H3K4 [H3K4me3]) followed by PCR to quantify the amount of DNA precipitated. Data revealed high levels of expression of type I MHC in the soleus and type IIb and IIx MHC in the plantaris. Consistent with this differing pattern of gene expression, H3 acetylation was high at the *type I MHC* gene and low at the *type IIb MHC* and *IIx MHC* genes in the soleus. The opposite was true in the plantaris. A similar modification pattern was observed at H3K4me3. Animals were subjected to hind limb suspension to unload the legs for 7 days as an inactivity model, which shifts the muscle fibre type from slow to fast in the soleus. This resulted in a shift in the *MHC* gene expression toward that of the fast type. Therefore, the authors demonstrated that H3 acetylation and H3K4me3 are modulated dynamically in parallel with the shift in fibre type from slow to fast.

5.5.3 miRNA (Micro RNA) Expression

miRNAs play a different role in the epigenetic regulation of gene expression, as compared to DNA methylation and histone modifications, in that it does not involve a direct modification of chromatin. Instead, miRNAs regulate the amount of protein expressed in cells post-transcriptionally by modulating mRNA stability and the efficiency of translation. Nevertheless, they are, of late, becoming increasingly important in the regulation of cell- and tissue-specific gene expression, as well as potential biomarkers for physiological and pathological conditions. In particular, some miRNAs are transported in the blood and, therefore, can be quantified by blood sampling and RT-PCR. As such, there are an increasing number of publications discussing the roles of miRNAs in health and disease, including exercise. Although many are correlative, studies assessing the molecular mechanisms behind the regulation of miRNA expression and consequences of increases or decreases in their expression on the amount of target proteins have emerged.

miRNAs have been studied extensively in skeletal muscle [28]. Many specific miRNAs are enriched in muscle, such as miR-1 and miR-133, which play physiological and pathological roles in myogenesis, muscle growth, differentiation and disease. They are known collectively as myomiRs.

Russell et al. used thigh skeletal muscle biopsies to study the effect of an acute bout of exercise and short-term (10 days) endurance training using cycling on the expression of miRNAs and components involved in miRNA biogenesis; specifically, the two nucleases involved in the processing of precursor miRNAs and exportin 5, which exports the processed products from the nucleus to the cytoplasm [29]. They found a significant increase in miR-1 and miR-133 and a decrease in miR-9, miR-23

and miR-31 after the exercise. The down-regulation of miR-23 would up-regulate the synthesis of PGC-1α, its target protein, which regulates mitochondrial biogenesis. Therefore, miR-23 might be involved in the adaptive response to endurance exercise. miR-1 levels remained high and miR-31 remained low after training, suggesting that exercise had long-lasting effects on adaptation. The predicted target proteins of the miRNAs affected by the exercise were searched using sequence matches with the mRNAs of target proteins using bioinformatics. HDAC4 was a predicted target of miR-1, -133, -9 and -23, whereas nuclear respiratory factor 1 (NRF1) might be a target of miR-9, -23 and -31. Negative correlations were found between miR-9 and HDAC4, miR-31 and HDAC4, and miR-31 and NRF1 protein. These findings prompted the authors to assess the potential relationship between the miRNA levels and protein expression in myotube cultures. Although luciferase reporter assays using the *HDAC4* and *NRF1* genes revealed reduced luciferase activity in cells co-transfected with miR-31, as expected, the expression of *HDAC4* and *NRF1* was not affected. The authors hypothesized that this negative result might be due to the non-physiological conditions in the cell culture system, and suggested that different effects might be observed in human skeletal muscle.

Circulating miRNAs have been used as biomarkers for various physiological and pathological conditions. Physical activity, both acute exercise and endurance training, changes the levels of circulating miRNAs. Nielsen et al. [30] assessed alterations in the miRNA levels in the plasma of exercised humans to assess whether the response to different stimuli could be used as a signature for exercise interventions. They excluded samples that showed signs of haemolysis because red blood cells are an abundant source of miRNAs. The miRNA expression pattern changed immediately after cycling for 60 min (the down-regulation of eight miRNAs, including miR-106a and miR-221, and the upregulation of species including miR-338-3p, miR-330-3p). These findings suggest that circulating miRNAs are adjusted rapidly in response to different exercise stimuli, suggesting that these different patterns could be used to monitor exercise. Interestingly, some miRNAs increased 1 h after exercise, such as miR-143 and miR-145, which were enriched in the liver. Therefore, tissues other than muscle secrete miRNAs in response to exercise, which might affect the function of other organs. Although the liver could be thought of as a tissue that is less responsive to exercise, as compared with skeletal or cardiac muscle, it is important to study tissues such as the brain and liver, which might respond to physical activity to exert systemic beneficial effects. Consistent with this, Radak et al. [31] reported that the oxidative modification of proteins in rat brains was reduced and cognitive function was improved by regular swimming exercise. In addition, Nakamoto et al. [32] showed that regular exercise in rats using treadmill running reduced the oxidative modification of nuclear and mitochondrial DNA and up-regulated the expression of the repair enzyme OGG1 in the liver. Therefore, the systemic effects of exercise have been reported. Nevertheless, it should be noted that the circulating miRNAs might be derived passively, for example from damaged muscle due to exercise; however, not all muscle-enriched miRNAs were detected in the circulation [30]. Therefore, miRNA secretion is likely to be a selective process. As such, although the physiological consequences of

miRNA secretion are not yet fully elucidated, the patterns of miRNAs in the blood might be a good biomarker of different physiological and pathological conditions without the need for tissue isolation.

5.6 Relevance to Ageing

Ageing can be defined as the gradual loss of homeostasis, which leads to decline in physiological function and increased susceptibility to diseases over time. In terms of longevity, the contribution of genetics is estimated to be 25–30 %, and the rest is likely to be due to environmental and lifestyle factors, as well as probability or chance, perhaps with the exception of long-lived cohorts such as centenarians aside. Epigenetic alterations might also contribute to longevity, reduce the risk of ageing-related diseases and also maintain a good quality of life in old age.

A report by Fraga et al. [33] suggests that epigenetic modifications might play a role in human ageing. They demonstrated that there were far more differences in the patterns of DNA methylation and histone acetylation in circulating lymphocytes in older (50 years of age) genetically identical monozygotic twins compared with younger (3 years of age) twins. Importantly, consistent with the epigenetic changes, the differences in gene expression between the older pairs were much greater than were those in young pairs. These findings suggest that an identical genome in early life could undergo different epigenetic modifications throughout life. The relatively small contribution of genetics towards longevity determination might be partly due to variable epigenetic modifications throughout life, which might lead to different disease susceptibility. However, it is important to note that a possible shift in the cell population (e.g. a shift to more memory T cells and fewer naive T cells during ageing) over time might have influenced this result [34].

Ageing is usually associated with reduced levels of global DNA methylation in CpG sequences, as well as the hypermethylation of some areas such as promoter regions. However, the physiological implication of changes in DNA methylation is generally unclear, although the age-related hypermethylation of the promoter regions of tumour suppressor genes increases the risk of carcinogenesis. Maegawa et al. [35] studied the widespread and tissue-specific changes in DNA methylation in mice with age. They found that 21 % of the promoter regions in the intestine exhibited increased methylation, whereas 13 % showed decreased methylation when animals were compared at 3 and 35 months of age. In the human colon, the proportion of autosomal genes which showed age-related hypermethylation was 10 %, while 1 % of genes showed hypomethylation when young (29–41 years) and old (61–72 years) individuals were compared. The authors concluded that the dysregulation of DNA methylation is a common feature of ageing in mammals. Apart of the aberrant methylation of protein-coding genes with age, ribosomal DNA clusters are also hypermethylated in the liver of old rats [36]. This hypermethylation might be associated with reduced gene transcription, which might lead to ageing-related changes in the expression of ribosomal RNA in old animals [37].

Frailty is an important issue for elderly individuals. Although it is not a disease and does not increase the mortality rate directly, it can cause ageing-related diseases. Bellizzi et al. found that worsening frailty status, as measured by loss of bodyweight, sarcopenia, muscle weakness, and reduced physical activity, was associated with decreased global DNA methylation in peripheral blood cells of individuals aged 65–105 over a 7-year-follow-up [38]. It was speculated that environmental factors such as diet and lifestyle might influence the methylation in various tissues, which could affect gene expression and thereby lead to local or systemic frailty.

Changes in the post-translational modification of histones occur with age, which might reduce gene expression. Kawakami et al. [39] reported that the acetylation of H3K9 was decreased and the phosphorylation of H3S10 was increased significantly in rat livers with age. Because these modifications suppress gene activity, these findings suggest that the age-related decline in chromatin functions might be due to such epigenetic changes.

Memory impairment is a common feature of ageing animals. The involvement of epigenetics is correlated with the ageing-related alterations in gene expression in the brain. Peleg et al. [40] studied histone acetylation in the hippocampus of young (3 months) and older (16 months) mice subjected to contextual fear conditioning. The older mice exhibited impaired associative learning, as detected by reduced freezing behaviour upon conditioning. The histone acetylation at H3K9 and K14 or H4K5, K8, K12 or K16 was similar in the two age groups of naïve mice. There was a transient increase in H3K9 and K14 and H4K5, K8 and K12 acetylation in young mice, whereas H4K12 acetylation was not up-regulated in the old mice. The increase in the other sites was similar in both groups of animals, suggesting that memory impairment in older animals correlated with defective learning-induced H4K12 acetylation. Data revealed that the hippocampal transcription of old mice remained almost unchanged in response to fear conditioning, whereas it increased in the young animals. They further used chromatin immunoprecipitation to demonstrate that the high level of gene expression induced by the conditioning correlated with increased H4K12 acetylation along the coding regions of genes, suggesting that transcription elongation was impaired in the old mice. Interestingly, the administration of HDAC inhibitors such as sodium butylate to older mice prior to the conditioning increased H4K12 acetylation significantly in the coding regions of learning-regulated genes. These findings suggest that the dysregulation of H4K12 is causally related to age-associated memory impairment. It is interesting to note that transgenic model mice with induced neuronal loss that are housed continuously in an environmentally enriched cage with wheels for voluntary running and other devices for physical activity exhibited increased histone H3 and H4 acetylation in the hippocampus at multiple sites, including H4K12 [41]. The model mice with neurodegenerative diseases that experienced environmental enrichment re-established access to long-term memories, exhibited dendrite sprouting and had an increased number of synapses, which could be mimicked by treatment with HDAC inhibitors that up-regulate histone acetylation. Therefore, physical exercise in an enriched environment might facilitate recovery from the impaired learning and memory that occurs with ageing by increasing histone acetylation.

Reports on the possible involvement of miRNAs in ageing are limited (see the special issue of Ageing Research Reviews 17: 1–98, 2014). Ageing-related decline in muscle function is a major concern for elderly individuals; therefore, they must maintain physical activity in daily life. Drummond et al. studied the expression of muscle-specific miRNAs and primary transcript pri-miRNAs in muscle biopsies taken from young (29 ± 2 years) and old (70 ± 2 year) males in response to leg extension exercises and the ingestion of leucine-enriched essential amino acid solution as an anabolic stimulus trying to correlate the stimulus with the expression of muscle specific miRNAs, upstream regulators (MyoD and myogenin) and downstream target proteins insulin-like growth factor-1 (IGF-1), HDAC4 and MEF2) that can be related to the promotion of the protein synthesis in the muscle [42]. The levels of pri-miRNA-1-1, -1-2, -133a-1 and 133a-2 were higher in older than younger males at baseline (before exercise). The expression of these miRNAs was reduced 6 h after exercise in young males compared with baseline. Mature miRNA-1 was down-regulated in response to the anabolic stimulus in only young individuals. The authors did not detect ageing-related differences in protein expression of IGF-1, HDAC4 and MEF2 at baseline, all of which are predicted or validated targets of miR-1. Studies in nematodes showed a decrease in miR-1 expression with advancing age [43]. Therefore, studies in worms and humans are contradictory. Drummond et al. [42] speculated that this discrepancy might be because nematodes lack satellite cells, which play an essential role in the muscle growth of mammals and have different types of muscle fibre. The failure of older subjects to down-regulate the expression of miR-1 following anabolic stimuli might be responsible for the reduced muscle protein synthesis observed in elderly subjects. Because they did not detect age-related changes in myogenic regulatory factors such as myogenin and MyoD, which are responsible for miR-1 expression, it is unclear whether transcription factors play a role in miRNA regulation in response to anabolic stimuli.

Age-associated cognitive decline is also a serious problem in older age individuals. The involvement of epigenetics, including miRNAs, in the brains of patients with neurodegenerative disease such as Alzheimer's disease and Parkinson's disease was suggested. Inukai et al. examined the expression of miRNAs in the brains of young (5 month-old) and old (24–25 month-old) mice, and discovered several novel miRNA candidates for predicted target proteins, including components of the insulin signal transduction pathways that are relevant to ageing [44]. Many miRNAs in the brain exhibit dynamic changes in expression by more than two-fold during ageing. The expression of most (80–95 %) of these miRNA was decreased with age, consistent with the findings in the human blood mononuclear cells of young and old individuals [45]. However, this contradicts a report showing the predominant up-regulation of miRNAs in mouse brains during ageing [46]. Therefore, studies on miRNAs in ageing are forming an exciting novel field, but further developments are needed.

One of the issues that attract the interest of researchers in gerontology is predicting the age of individuals and tissues. Recently, Horvath developed a method to predict the age of human cells and tissues using a large number of data sets assessing

the methylation of CpG dinucleotides [47]. He found that the DNA methylation age is close to zero in embryonic and iPS cells and that it correlates with cell passage number. However, this differed from mitotic age because it tracks chronological age in non-proliferative tissues (for example, brain tissue). He stated that this model identified a highly heritable measure of ageing acceleration in studies of twins. This prediction was applicable to chimpanzee tissues as well. Although this prediction is interesting, it remains unclear whether it reflects physiological ageing accurately. These findings did not provide evidence of an association between premature ageing in progeria and accelerated DNA methylation age.

5.7 Concluding Remarks

In this chapter, we described advances in studies assessing the influence of exercise-induced epigenetic changes on gene expression and its relevance to the mechanisms of ageing. It is clear that DNA methylation, histone modifications and microRNA expression alter the phenotypes of many cell types, tissues and organs in response to physical activity or inactivity as well as in ageing. Nevertheless, it will take much more time for the entire picture to emerge regarding the epigenetic mechanisms that regulate gene expression during exercise, nutrition, ageing and different pathologies.

References

1. Fabbri M, Garzon R, Cimmino A et al (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104(40):15805–15810
2. Kohli RM, Zhang Y (2013) TET enzymes, TDG and the dynamics of DNA demethylation. Nature 502(7472):472–479
3. Lee MG, Villa R, Trojer P et al (2007) Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science 318(5849):447–450
4. Mikkelsen TS, Ku M, Jaffe DB et al (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448(7153):553–560
5. Cedar H, Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet 10(5):295–304
6. Zhang Y (2003) Transcriptional regulation by histone ubiquitination and deubiquitination. Genes Dev 17(22):2733–2740
7. Vasudevan S (2012) Functional validation of microRNA-target RNA interactions. Methods 58(2):126–134
8. Winter J, Jung S, Keller S et al (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11(3):228–234
9. Friedman RC, Farh KK, Burge CB et al (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19(1):92–105
10. Mitchell PS, Parkin RK, Kroh EM et al (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 105(30):10513–10518

11. Valadi H, Ekstrom K, Bossios A et al (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9(6):654–659
12. Barres R, Yan J, Egan B et al (2012) Acute exercise remodels promoter methylation in human skeletal muscle. Cell Metab 15(3):405–411
13. Golbidi S, Laher I (2014) Exercise induced adipokine changes and the metabolic syndrome. J Diab Res 2014:726861
14. Rönn T, Volkov P, Davegardh C et al (2013) A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. PLoS Genet 9(6):e1003572
15. Gomez-Pinilla F, Zhuang Y, Feng J et al (2011) Exercise impacts brain-derived neurotrophic factor plasticity by engaging mechanisms of epigenetic regulation. Eur J Neurosci 33 (3):383–390
16. Neeper SA, Gomez-Pinilla F, Choi J et al (1995) Exercise and brain neurotrophins. Nature 373(6510):109
17. McGee SL, Fairlie E, Garnham AP et al (2009) Exercise-induced histone modifications in human skeletal muscle. J Physiol 587(Pt 24):5951–5958
18. Mahoney DJ, Parise G, Melov S et al (2005) Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J 19(11):1498–1500
19. Holloszy JO (2008) Regulation by exercise of skeletal muscle content of mitochondria and GLUT4. J Physiol Pharmacol 59(Suppl 7):5–18
20. Smith JA, Kohn TA, Chetty AK et al (2008) CaMK activation during exercise is required for histone hyperacetylation and MEF2A binding at the MEF2 site on the Glut4 gene. Am J Physiol Endocrinol Metab 295(3):E698–E704
21. Abel JL, Rissman EF (2013) Running-induced epigenetic and gene expression changes in the adolescent brain. Int J Dev Neurosci 31(6):382–390
22. Elsner VR, Lovatel GA, Bertoldi K et al (2011) Effect of different exercise protocols on histone acetyltransferases and histone deacetylases activities in rat hippocampus. Neuroscience 192:580–587
23. McGee SL (1985) Hargreaves M (2011) Histone modifications and exercise adaptations. J Appl Physiol 110(1):258–263
24. Rossetto D, Avvakumov N, Cote J (2012) Histone phosphorylation: a chromatin modification involved in diverse nuclear events. Epigenetics 7(10):1098–1108
25. Chandramohan Y, Droste SK, Arthur JS et al (2008) The forced swimming-induced behavioural immobility response involves histone H3 phospho-acetylation and c-Fos induction in dentate gyrus granule neurons via activation of the N-methyl-D-aspartate/extracellular signal-regulated kinase/mitogen- and stress-activated kinase signalling pathway. Eur J Neurosci 27(10):2701–2713
26. Collins A, Hill LE, Chandramohan Y et al (2009) Exercise improves cognitive responses to psychological stress through enhancement of epigenetic mechanisms and gene expression in the dentate gyrus. PLoS ONE 4(1):e4330
27. Pandorf CE, Haddad F, Wright C et al (2009) Differential epigenetic modifications of histones at the myosin heavy chain genes in fast and slow skeletal muscle fibers and in response to muscle unloading. Am J Physiol Cell Physiol 297(1):C6–C16
28. Sharma M, Juvvuna PK, Kukreti H et al (2014) Mega roles of microRNAs in regulation of skeletal muscle health and disease. Front Physiol 5:239
29. Russell AP, Lamon S, Boon H et al (2013) Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. J Physiol 591(Pt 18):4637–4653
30. Nielsen S, Akerstrom T, Rinnov A et al (2014) The miRNA plasma signature in response to acute aerobic exercise and endurance training. PLoS ONE 9(2):e87308
31. Radak Z, Kaneko T, Tahara S et al (2001) Regular exercise improves cognitive function and decreases oxidative damage in rat brain. Neurochem Int 38(1):17–23
32. Nakamoto H, Kaneko T, Tahara S et al (2007) Regular exercise reduces 8-oxodG in the nuclear and mitochondrial DNA and modulates the DNA repair activity in the liver of old rats. Exp Gerontol 42(4):287–295

33. Fraga MF, Ballestar E, Paz MF et al (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci USA 102(30):10604–10609
34. Martin GM (2005) Epigenetic drift in aging identical twins. Proc Natl Acad Sci USA 102(30):10413–10414
35. Maegawa S, Hinkal G, Kim HS et al (2010) Widespread and tissue specific age-related DNA methylation changes in mice. Genome Res 20(3):332–340
36. Oakes CC, Smiraglia DJ, Plass C et al (2003) Aging results in hypermethylation of ribosomal DNA in sperm and liver of male rats. Proc Natl Acad Sci USA 100(4):1775–1780
37. Mori N, Mizuno D, Goto S (1978) Increase in the ratio of 18S RNA to 28S RNA in the cytoplasm of mouse tissues during aging. Mech Ageing Dev 8(4):285–297
38. Bellizzi D, D'Aquila P, Montesanto A et al (2012) Global DNA methylation in old subjects is correlated with frailty. Age 34(1):169–179
39. Kawakami K, Nakamura A, Ishigami A et al (2009) Age-related difference of site-specific histone modifications in rat liver. Biogerontology 10(4):415–421
40. Peleg S, Sananbenesi F, Zovoilis A et al (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328(5979):753–756
41. Fischer A, Sananbenesi F, Wang X et al (2007) Recovery of learning and memory is associated with chromatin remodelling. Nature 447(7141):178–182
42. Drummond MJ, McCarthy JJ, Fry CS et al (2008) Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. Am J Physiol Endocrinol Metab 295(6):E1333–E1340
43. Ibanez-Ventoso C, Yang M, Guo S et al (2006) Modulated microRNA expression during adult lifespan in Caenorhabditis elegans. Aging Cell 5(3):235–246
44. Inukai S, de Lencastre A, Turner M et al (2012) Novel microRNAs differentially expressed during aging in the mouse brain. PLoS ONE 7(7):e40028
45. Noren Hooten N, Abdelmohsen K, Gorospe M et al (2010) microRNA expression patterns reveal differential expression of target genes with age. PLoS ONE 5(5):e10724
46. Li N, Bates DJ, An J et al (2011) Up-regulation of key microRNAs, and inverse down-regulation of their predicted oxidative phosphorylation target genes, during aging in mouse brain. Neurobiol Aging 32(5):944–955
47. Horvath S (2013) DNA methylation age of human tissues and cell types. Genome Biol 14(10): R115

Chapter 6
Metabolic and Antioxidant Adaptation to Exercise: Role of Redox Signaling

Li Li Ji

Abstract The contraction-induced production of reactive oxygen species (ROS) has been implicated in oxidative stress to skeletal muscle for the past few decades. As research advances more evidence has revealed a more complete role of ROS under both physiological and pathological conditions. The current chapter will review the role of redox sensitive signaling pathways in conferring exercise-induced major physiological and cellular adaptations in skeletal muscle, focusing on mitochondrial function and antioxidant defense systems. Several redox signaling pathways that have direct impact on these adaptations, especially the functional roles, mechanisms of action and crosstalk of the nuclear factor (NF) κB, mitogen-activated protein kinase (MAPK), and peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) will be highlighted.

6.1 Introduction

Physical activity propelled by muscle contraction is one of the most powerful stimuli to physiological adaptations of the body including cardiovascular, pulmonary, neuromuscular, endocrinal and skeletal muscle systems [1]. Among these a healthy and strong muscular system not only ensures that exercise can be performed at proper and sustainable intensity and duration to elicit desirable biological benefits, but also participates in other vital physiological functions such as lowering blood sugar, releasing cytokines (myokines) to regulate other organismic function, and stimulate brain wellness. Furthermore, there is evidence that exercise can modulate cellular degenerative processes normally taking place during aging [2]. Despite decades of investigation, the mechanisms by which exercise induces the above mentioned biological functions are still not entirely clear. However, recent

L.L. Ji (✉)
Laboratory of Physiological Hygiene and Exercise Science, School of Kinesiology, University of Minnesota, 1900 University Avenue, Minneapolis, MN 55455, USA
e-mail: llji@umn.edu

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research reveals that reactive oxygen species (ROS) generated within the cell during muscle contraction play an important role in regulating a wide range of biological functions that directly or indirectly affect the adaptations as mentioned above. Specifically, several relatively stable ROS generated within the various cellular compartments, mainly hydrogen peroxide (H_2O_2) and nitric oxide (NO), are capable of activating or inhibiting enzymes and transcription factors (TF) through a redox-sensitive and reversible manner, which ultimately controls the gene expression of specific gene products (enzymes, TFs, cytokines, etc.) that have functional impact on the organisms. Understanding how these ROS-controlled signal transduction processes (termed redox signaling) operate and interact with each other, and how they confer physiological functions, will not only resolve a major scientific question in biology, but also enable us to design strategies to maximize exercise benefits in health, wellbeing and disease prevention.

Redox signaling is one of the most complicated biological processes that encompassing physiology, biochemistry, immunology, endocrinology, pathology and molecular biology. It is also a constantly developing field with new bioactive substances and pathways being discovered and reported every year. Thus, to make a comprehensive review on exercise-induced adaptations in skeletal muscle is not an easy task. The author has chosen to first provide a summary of the redox signaling mechanisms and introduction of the most relevant pathways related to exercise physiology. Two main aspects of muscle adaptation to exercise, the metabolic system primarily controlled by mitochondria, and the antioxidant system, will be the focus of this review. Readers interested in other aspects of exercise adaptation such as cardiopulmonary, neuromuscular control, angiogenesis and blood flow, bone and brain health are referred to other expert chapters of the current book or elsewhere.

6.2 General Mechanism for Redox Signaling

6.2.1 Cellular Production of ROS

It is now well known that during prolonged muscle contraction, a small portion of the oxygen consumed in the mitochondrion will extract an electron from specific components of the electron transport chain (ETC) to form superoxide radical ($O_2^{-\cdot}$). With the enzyme superoxide dismutase (SOD), $O_2^{-\cdot}$ is reduced to hydrogen peroxide (H_2O_2), which can be further reduced to water by catalase and/or glutathione peroxidase (GPX). However, not all $O_2^{-\cdot}$ and H_2O_2 are completely removed in the cell. The two oxygen species can react and generate hydroxyl radical ($OH^{\cdot}$) via Haber-Weiss reaction. $OH^{\cdot}$ may also be formed between H_2O_2 and a medal ion (such as Fe^{2+}) via Fenton reaction. $O_2^{-\cdot}$, H_2O_2 and $OH^{\cdot}$ are collectively called ROS, whereas some researchers prefer to use reactive oxygen and nitrogen species (RONS) to include NO. The steady state concentration of ROS is determined by their

rate of generation and the activities of the antioxidant enzymes SOD, catalase and GPX [3]. Furthermore, several additional ROS generating pathways may be activated during heavy exercise such as NADPH oxidase, xanthine oxidase (XO) and membrane borne oxidases such as cyclooxygenase (COX)-2 and lipooxygenase, although their contribution to overall ROS production is still unclear [4]. Moreover, rigorous muscle contraction, especially lengthening contraction (LC), can result in myofibrial damage, causing production of various cytokines (and chemokines) that stimulate ROS generation through the inflammatory process [5]. Due to the difficulty to accurately detect and quantitate ROS, the precise concentration of various species of ROS in vivo is uncertain. It is estimated that less than 1–2 % of the oxygen consumed in the cell may turn into ROS mostly in form of H_2O_2 [4].

6.2.2 Molecular Mechanism of Redox Signaling

When cells are exposed to oxidative stress such as UV irradiation, phorbol esters, toxins (such as lipopolysaccharide, LPS), redox-disturbing agents (such as paraquat, menadione), certain growth factors, and anoxia/hypoxia/hyperoxia, intracellular levels of antioxidant defenses are increased. It has been known for some time that increased generation of ROS in the cell associated with the exposure of the above physical and chemical agents may stimulate the gene expression of antioxidant enzymes, immunoreactive proteins such as cytokines and chemokines, and TFs. It is now clear that several redox-sensitive signal transduction pathways are responsible for the observed adaptations, among which nuclear factor (NF)κB, mitogen activated protein kinases (MAPK), the phosphoinositide 3-kinase (PI_3K)/Akt pathway, p53 activation, and the heat shock proteins (HSP) are most recognized and studied in early years [6]. Additional research has identified several other important signaling pathways that show redox sensitivity and have a significant impact on cell function, such as peroxisome proliferator-activated receptor (PPAR) γ receptor coactivor α (PGC-1α), folkhead box protein O (FoxO), and sirtuin (Sirt) family proteins. Cellular functions fulfilled by redox signaling appear to be broad and are difficult to summarize in full, but the most important coverages are (1) to regulate the cell's metabolic rate, which can be accomplished by (a) change of enzyme activity and thus the flux of the pathway the enzyme controls and (b) change of the enzyme protein content by altering the gene expression and/or degradation of the enzyme; (2) to regulate cellular antioxidant defense by (a) changing the gene expression of antioxidant enzymes and altering their catalytic activity and (b) changing the synthesis of nonenzymatic antioxidants such as glutathione (GSH), thiroredoxin (Txn) and uncoupling proteins (UCP) [7]; (3) to regulate cell's catabolic processes such as proteolysis, autophagy (including mitophagy) and apoptosis [8]. Because of the vastly broad functions involved in the subject matter, it is impossible to review all aspect of redox signaling in this short chapter.

Broadly speaking, two physiological and environmental stresses may elicit redox signaling: (1) stresses that directly produce ROS, such as increased oxygen flux in ETC, hypoxia-reoxygenation, and oxidase/oxygnease activation (a common final product of these pathways is H_2O_2); (2) processes that result in activation of intracellular enzymes and pathways sensitive to redox change, such as receptor binding by hormones, certain growth factors, and cytokines, and toxin stimulation, which may but do not always rely on H_2O_2 as the messenger (H_2O_2 contributes to >50 % of redox signaling) [6]. The reason for H_2O_2 to serve as a common signaling molecule is several-fold: (1) H_2O_2 is constantly produced in the mitochondria in living cells; (2) H_2O_2 is a relatively stable molecule; (3) it is a strong oxidant capable of oxidizing a variety of moieties (such as sulfhydryl, hydroxyl, sulfoxide, etc.), yet not highly destructive; and (4) H_2O_2 is small enough to diffuse across most, but not all, biomembrane barriers [3]. Figure 6.1 illustrates the various cellular sources of H_2O_2 production.

6.2.3 Major Redox Signaling Pathways

Redox signaling by nature is a paracrine (also called autocrine) system wherein the signals produced in the cell activate a pathway or multiple pathways in adjacent (target) cells through a chain of local mediators. Target genes contain regulatory sequences (DNA binding sites) in their promoter and/or intron regions that can interact with the TF to modulate transcription rate and/or post-transcriptional processes of gene expression [6]. Dimerization, covalent modification and dissociation of TFs are often required before binding to DNA. Figure 6.1 depicts some of the DNA binding sites by representative redox-sensitive signaling pathways. Details of several important redox signaling pathways are discussed below.

PGC-1α*: *the master regulator of mitochondrial function PGC-1α was first identified as a functional activator of the peroxisome proliferator-activated receptor (PPAR) γ receptor in brown adipose tissue. Subsequent research revealed that it is a master transcriptional cofactor participating in almost all aspects of mitochondrial functions ranging from energy fuel selection, muscle fiber differentiation and transformation, antioxidant gene expression, and fusion/fission dynamics [9, 10]. PGC-1α has been identified ubiquitously in mitochondria-rich tissues, including red skeletal muscle and heart, kidney, liver and brain. The primary function of PGC-1α is to co-activate receptor binding of several nuclear TFs, such as PPAR, nuclear respiratory factor (NRF)-1 and NRF-2, and estrogen-related receptor-α (ERR-α) to enhance the expression of nuclear encoded mitochondrial proteins (NEMP) involved in mitochondrial respiration, biogenesis, fatty acid oxidation, and antioxidant defense. PGC-1α also upregulates mitochondrial transcription factor A (Tfam), which directly stimulates mtDNA replication and transcription [10]. In addition, a PGC-1α isoform resulting from splicing of primary PGC-1α (PGC-1α4) has been found to stimulate muscle hypertrophy due to its ability to activate IGF-1/Akt/mTOR pathway and suppress myostatin [11].

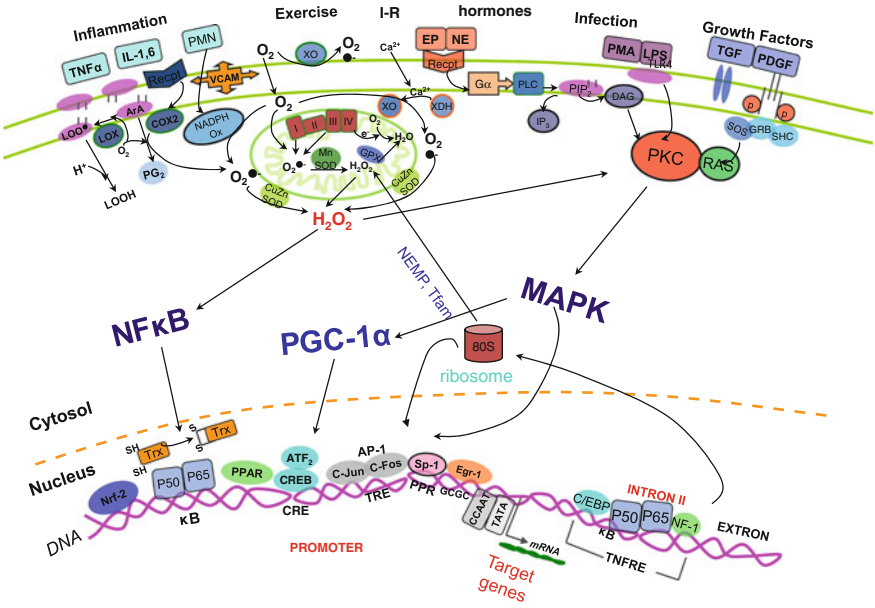

Fig. 6.1 Schematic illustration of major redox signaling pathways in skeletal muscle, including major sources of ROS generation in the cell in response to physiological stimuli, and schematic illustration of the affected nuclear protein binding sites on DNA. Abbreviations used are: *ArA* arachidonic acid. *ARE* Antioxidant response element; *ATF2* activating transcription factor-2; *C/EBP* CCAAT enhancer binding protein; *CRE* cAMP-response element; *DAG* diacylglycerol; *Egr-1* early growth-responsive-1 protein; *Gα* G-protein α subunit; *IP3* inositol triphosphate; *NF-1* nuclear factor1; *Nrf-2* nuclear factor erythroid 2–related factor 2; *PDGF* plasma derived growth factor; *PGC-1α* peroxisome proliferator-activated receptorγ-coactivator-1α; *PKA* protein kinase A; *PKC* protein kinase C; *SOD* superoxidase dismutase; *PMN* polymorphoneutrophil; *PLC* phospholipase C; *TGFβ* transforming growth factor β; *TNFRE* TNFα response element; *XO* xanthine oxidase

NFκB*: *an oxidative stress responder NFκB is a dimeric transcription factor composed of members of the Rel family [12]. It is one of the best known redox sensitive pathways due to the following research evidence. (1) Exposure of certain types of cells (such as T cells, L6 myotubes, 70Z/3 pre-B cells) to H_2O_2 leads to activation of NFκB cascades; (2) the best-known NFκB activators, such as TNFα, IL-1, LPS, and phorbol 1, 2-myristate 13-acetate (PMA) all can lead to increased intracellular levels of H_2O_2; and (3) treatment of cells with antioxidants, such as GSH and N-acetylcysteine (*NAC*) abolishes NFκB activation. NFκB activation by external stimulants as mentioned above result in the phosphorylation and activation of IκB kinase (IKK), which phosphorylates two critical serine residues of IκB and primes IκB for ubiquitination and proteolytic degradation by the 26S proteasome. IκB dissociation unleashes P50/P65 to dimerize and translocate into the nucleus and bind the κB consensus sequence of the target genes. IKK activation involves a Ser/Thr kinase domain at its N-terminal that can be phosphorylated by several

kinases including NFκB-inducing kinase (NIK a member of the MAPK family), transforming growth factor β-activated kinase (TAK-1), and MAPK/ERK kinase (MEKK1), among which NIK activation seems to be the most important step [13]. Other MAPKs may also be selectively involved in NFκB activation. For example, Jiang et al. [14] showed that activation of ERK is required to upregulate the IL-1β induced expression of COX-2 and inducible NO synthetase (iNOS), but not Mn-containing SOD (SOD-2) in cultured rat vascular smooth cells. The best-known proteins and enzymes that require consensus binding of p65 in the promoter are SOD2, glutamylcysteine synthatase (GCS, the rate-limiting enzyme for glutathione synthesis), iNOS, TNF-α, IL-6, COX-2, and vascular cell adhesion molecule-1 (VCAM-1). These genes are involved in a wide variety of biological functions such as antioxidant defense, inflammation, immunity, and anti-apoptosis [15].

MAPK pathways contain JNK, ERK1/2 and $p38^{MAPK}$, each of which is controlled by upstream kinases called MAPK kinase (MEK), which in turn is controlled by MAPK kinase kinase (MKK), forming a hierarchy [6]. The primary extracellular stimulators of the MAPK pathway are growth factors (GF), inflammatory cytokines, and phorbol esters. In the ERK and JNK pathways, receptor binding by GFs initiates conformational changes of member-associated protein Sos, Grb-2 and SHC leading to the activation of Ras, which in turn stimulates the translocation and phosphorylation of Raf-1, the commander of MEK/MKKs. TNFα and IL-1 bypass the Ras pathway by increasing cytosolic H_2O_2 concentration and activating several isoforms of protein kinase C (PKC). PKC appears to serve as a pivot enzyme in activating MAPK pathways by stimulating multiple MEK/MKKs, as well as the NFκB pathway by activating NIK. Thus, there are considerable functional overlaps and crosstalk (see below) between NFκB and MAPK [16]. Furthermore, activating protein (AP)-1 function is largely dependent on MAPK and NFκB signaling pathways. ERK activation induces c-Fos, a subunit of AP-1, whereas phosphorylation of c-Jun by JNK is required before c-fos/c-Jun dimeration. The primary function of the MAPK pathway is to modulate growth, metabolism, differentiation, transcription, translation, and remodeling. This broad MAPK function is outside the scope of this chapter and the readers are referred to other available reviews [6].

SIRT: Sirtuin are a class of proteins that possess either mono-ADP-ribosyltransferase or deacylase activity and have been implicated in influencing a wide range of cellular processes such as mitochondrial biogenesis, transcription, apoptosis, inflammation and energy metabolism. Yeast Sir2 and some, but not all, sirtuins are protein deacetylases. Cellular levels of NAD, NADH and NAD:NADH ratio directly determine Sirt activity, and conversely, Sirt activity may influence cell metabolic status through nicotinamide-linked enzyme activities. As a member of the Sirt family, SIRT1 can be a potential regulator of PGC-1α transcriptional activity and is implicated in a wide range of functions including cellular differentiation, neural- and cardio-protection, skeletal muscle metabolism, and aging [17]. SIRT1 has been reported to directly affect PGC-1α transcriptional activity by physical interaction, deacetylating and activating PGC-1α

both in vitro and in vivo [18]. Thus, SIRT1 acts as a sensor that directly detects metabolic perturbations and regulates transcriptional outputs [19].

FoxO3 belongs to the O subclass of the forkhead family of transcription factors characterized by a distinct fork head DNA-binding domain. When these TFs are phosphorylated by enzymes such as Akt/PKB in the PI3K signaling pathway and translocated out of the nucleus, they are inhibited; whereas post-translational modifications including acetylation and methylation can activate FoxO3 activity. It is well known that muscle IM can downregulate PI3K-Akt pathway, which not only diminishes the signals required to maintain protein synthesis via mTOR transcription factor, but also dephosphorylates FoxO3, a condition that activates atrogin-1 transcription [20]. The PI3K/Akt pathway regulates transcription by phosphorylating FKHRL1 (FoxO3a), leading to FoxO3a sequestration in the cytoplasm, instead of accumulation in the nucleus. On the other hand, if the PI3K/Akt pathway is inactivated such as in immobilized muscle, FoxO3a is dephosphorylated and accumulates in the nucleus where it may activate genes related to apoptosis and protein degradation. Activation of FoxO pathway may stimulate several pathways such as parkin, PINK1, Bnip3 and Bnip3L, eventually leading to mitochondrial degradation (mitophagy) as well [21].

6.2.4 Crosstalk Between Signaling Pathways

In electronics, crosstalk is any phenomenon by which a signal transmitted on one circuit or channel of a transmission system creates an undesired effect in another circuit or channel. In biological systems, crosstalk refers to shared signal transduction components and signals among various pathways. This usually results in synchronized biological effects, such as increased transcription, protein degradation and apoptosis. For example, NFκB and MAPK are distinct signaling pathways in the cell. NFκB is primarily responsive to stress, toxins and cytokines leading to inflammation, apoptosis and adaptation, whereas the primary consequence of MAPK activation is growth, development, transcription, translation, and remodeling. However, there are considerable functional overlaps and crosstalk between the two pathways. For example, ERK and p38 have been shown to play an important role in the temporal regulation of NFκB activation by IL-1β and H_2O_2 [22]. NIK and IKK are members of MEK/MKK family, upstream kinases of MAPK [23]. Furthermore, activating protein (AP)-1 function is largely dependent on MAPK and NFκB signaling pathways, because c-fos, the subunit of AP-1 (c-Fos/c-Jun dimer) is controlled by ERK activation to phosphorylate TCF, and through p90RSK to phosphorylate SRF. Binding of TCF and SRF to SRE is the requirement for c-fos transcription [7]. IL-1 was shown to induce c-Fos and Fra-1 thereby stimulating IL-8 expression, whereas the blockage of MEK1 by PD98059 suppresses the expression of almost all AP-1 subunits, indicating ERK activation was required [24].

Another good example is the crosstalk between various signaling pathways in controlling PGC-1α expression and post-translational modification. Cyclic AMP is the second messenger of adrenergic pathway that activates protein kinase A (PKA) and thus phosphorylating a variety of metabolic enzymes. It also phosphorylates CREB, a required TF for PGC-1α transactivation [25]. CREB phosphorylation is also conferred by CaMK, a calcium activated protein kinase during muscle contraction. Furthermore, p38, a MAPK enzyme that phosphorylates ATF-2, which dimerizes with CREB to bind PGC-1α promoter, also phosphorylates PGC-1α to enhance its co-activating capacity (for details, see Sect. 6.2.3).

Reciprocal activation is often seen in crosstalk activation of a pathway enzyme. For example, H_2O_2 has been shown to oxidize Cys on several PTPs to form disulfide intermediates so as to inhibit the enzymes. The sustained phosphorylation and activation of NIK appears to be accomplished by H_2O_2-induced inhibition of its phosphatase [13]. Inhibition of phosphatases is not only necessary for maintaining its phosphorylation status and activity, but also assures reversal of kinase action without disruption of overall cell function when the stimulus is withdrawn. Reciprocal activation may also affect pathways of opposite metabolic function. For example, Akt (PKB) phosphorylates mTOR and thus promotes protein synthesis. Meanwhile, it also phosphorylates FoxO and the phosphorylated FoxO exits the nucleus avoiding accumulation to activate atrogin-1, a major E3 ligase to cause muscle protein degradation [26].

6.3 Lessons Learned from Muscle Disuse and Misuse

6.3.1 Muscle Immobilization and Atrophy

Aging is associated with decreased activity of muscle contraction. There is evidence that many of the cellular events during the development of sarcopenia are similar to those associated with prolonged muscle disuse, such as bed rest, casting, and microgravity [27]. Thus, study of muscle immobilization (IM) may provide some useful insights into the mechanism of sarcopenia.

Muscle atrophy caused by prolonged IM is characterized by decreased muscle fiber cross-sectional area, reduced force production, increased fatigability and insulin resistance [8]. These alterations also include decreased protein synthesis, increased oxidative stress, increased protein degradation, and suppression of biogenesis. Different signaling pathways may be involved in causing muscle atrophy depending on the upstream perturbations, such as decreased IGF1-AKT-mTOR signaling, inflammatory cytokines and NFκB signaling and reduced nutrition and energy input [26]. An imbalance of ROS production and antioxidant defenses resulting in oxidative stress plays an important role in protein breakdown in skeletal muscle during periods of inactivity with a common transcriptional profile and activation of the ubiquitin-proteasome pathway. Both Atrogin-1 and MuRF-1 genes are highly over-expressed in an animal models of acute muscle atrophy and in the

control the ubiquitination and degradation of both regulatory (e.g., calcineurin and MyoD) and structural (e.g., myosin and troponin I) proteins [28].

There is evidence that mitochondria are a major source of ROS in mechanically ventilated diaphragm muscle atrophy, but XO and NADPH oxidase may also contribute to inactivity-induced ROS production in muscle [29]. Increased ROS can activate the NFκB pathway that controls the gene expression of Atrogin-1 and MuRF-1, as well as TNF-α, IL-1, IL-6, and interferon- α (IFN-α) [20]. By binding to their respective receptors, these cytokines are known to activate the NFκB pathway and further promote their expression through autocrine signaling, thus escalating the vicious cycle within the muscle cell.

A down-regulation of PGC-1α was observed in muscle atrophy of different models and thought to be a major molecular mechanism for FoxO activation, NFαB activation and protein loss [8]. Previous studies suggest that PGC-1α has a protective role against protein catabolism and muscle wasting in a variety of contexts. For instance, denervation-induced muscle atrophy and the effects of Duchenne's muscular dystrophy are greatly ameliorated when the amount of PGC-1α is maintained at normal levels or increased [30]. Inactivity induced deficit of PGC-1α in skeletal muscle results in a chronic systemic inflammatory state, evidenced by increased TNF-α and IL-6 expression, and NFκB activation [31]. Furthermore, PGC-1α KO mice displayed higher basal expression of TNF-α than wild type [32]. Conversely, transgenic mice with PGC-1α over-expression have shown a decreased inflammatory cytokine production and protein degradation caused by denervation and aging [31]. It is noteworthy, however, that although NFκB activation, overproduction of pro-inflammatory cytokines and disturbance of redox status have been found in many previous studies [20, 33, 34], in the absence of chronic disease, inflammation is not consistently shown to be a major pathway leading to disuse muscle atrophy. Alternative pathways may exist explaining muscle protein degradation during IM. For example, it is also known that muscle IM can downregulate the PI3 K-AKT pathway that not only diminishes the signals required to maintain protein synthesis via mTOR transcription factor, but also dephosphorylates FoxO, a condition that activates Atrogin-1 transcription [20]. Furthermore, recent research has revealed that the autophagy-lysosome systems may be activated during muscle IM and contribute to the loss of muscle mass [21]. Activation of the FoxO pathway may stimulate several pathways such as parkin, PINK1, Bnip3 and Bnip3L, and eventually lead to mitochondrial degradation (mitophagy). Figure 6.2 illustrates the redox signaling pathways involved in muscle disuse atrophy.

6.3.2 Muscle Lengthening Contraction and Damage

Eccentric exercise such as downhill running presents a unique challenge to skeletal muscle distinct from those of conventional endurance exercise such as uphill running, and is associated with muscle lengthening contraction (LC) [35]. Due to

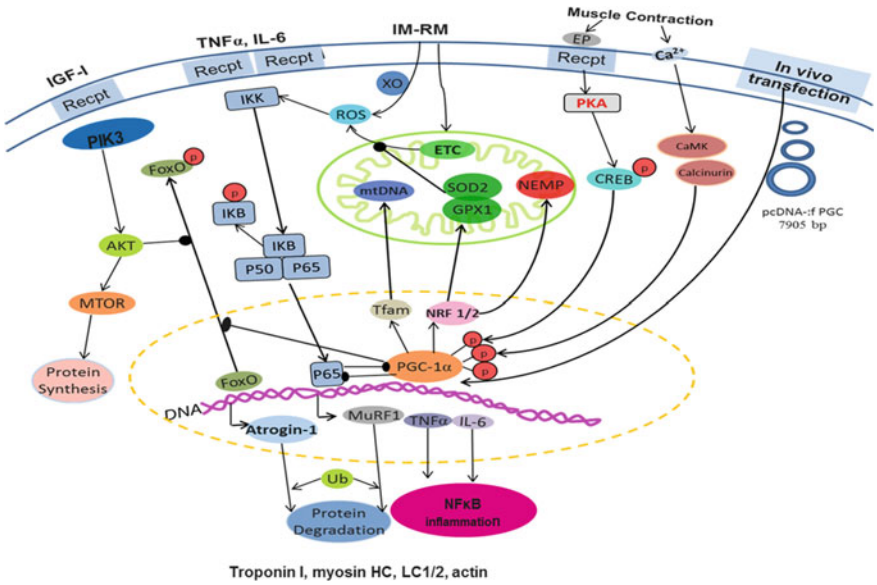

Fig. 6.2 Schematic illustration of various redox signaling pathways in the control of muscle protein degradation caused by immobilization. The illustration did not intend to include all components and pathways, but those mentioned in the text. Abbreviations: *AMPK* AMP activated kinase; *ATF2* activating transcription factor-2; *Atrogin-1* muscle-specific F-box protein; *CaMK* Ca^{2+}/calmedulin dependent protein kinase; *CREB* cAMP response element binding protein; *EP* epinephrine; *ERR* estrogen related receptor; *ETC* electron transport chain; *FoxO* folkhead box O transcription factor; *GPX* glutathione peroxidase; *IKK* IκB activated kinase; *mTOR* mammalian target of rapamycin; *MuRF1* muscle RING-finger protein-1; *NRF2* nuclear respiratory factor 2; *P38* p38 mitogen activated protein kinase; *P50* and *P65* subunit of NFκB; *PGC-1* peroxisome proliferator-activated receptorγ-coactivator-1; *PKA* protein kinase A; *ROS* reactive oxygen species; *SOD* superoxide dismutase; *XO* xanthine oxidase

the relationship between length and tension, stretched sarcomeres will be weakened when myofilaments no longer overlap. LC-caused progressive increase in over-stretched sarcomeres leads to membrane damage [36]. Since the membrane damage involves those of the sarcoplasmic reticulum, transverse tubules, and sarcolemma, Ca^{2+} will enter the sarcoplasm in an uncontrolled manner. With increased sarcoplasmic [Ca^{2+}] muscle contractile function will be affected including a shift in optimum length, a fall in active tension, and a rise in passive tension. Force may remain decreased for a week after an eccentric bout whereas it is recovered within two hours following a concentric bout, indicating damage instead of fatigue [37].

Another consequence of increased sarcoplasmic [Ca^{2+}] is an inflammatory response triggering proteolysis and the breakdown of damaged fibers. The inflammatory process begins with the attraction of leukocytes to the site of injury. The infiltration of neutrophils into the damaged muscle begins within hours and can last 24 h, whereas macrophages may remain in muscles for 2 weeks. The role of

these invading cells is to breakdown damaged tissue similar to the breakdown of xenobiotics during an immune response. The mechanism of degradation is related to the production of ROS by NADPH oxidase and the respiratory burst [38]. Furthermore, they may produce and release pro-inflammatory cytokines that can attract other leukocytes. For instance, IL-6 and IL-1β concentrations were shown to increase following downhill running [39]. IL-1β binding to IL-1R initiates an intracellular signaling cascade [40]. This cascade results in the activation of several transcription factors including NFκB, which further promotes the production of pro-inflammatory cytokines and the progression of inflammation.

6.3.3 Role of Redox Signaling in Inflammation

Inflammation represents a pathophysiological state that substantially alters cellular oxidative-antioxidant homeostasis. The mechanisms and time sequence of injury-induced muscle inflammation have been reviewed extensively. Briefly, muscle fiber damage due to LC can trigger releases of inflammatory cytokines such as TNFα, IL-1 and IL-6 from immune cells such as marcrophages, B cells and T cells and/or damaged muscle tissues. During the early phase of muscle injury, inflammatory cytokines promote the gene expression of adhesion molecules such as VCAM-1, CINC-1 and MCP-1, and NOS expression. In addition, some cytokines can bind with membrane receptors and activate specific ROS-generating enzymes, such as COX-2, NADPH oxidase, and XO. Endothelial cells from injured muscle are known to secret TNF-α, IL-1, IL-6 and IL-8, providing a positive feed-forward cycle. The increased blood flow due to NO production and the chemotactic effects of adhesion molecules facilitate PMN and circulating cytokine infiltration to the affected area. While this process is largely viewed as prooxidative, selective expression of antioxidants is an important part to keep inflammation under control.

It is now clear that redox-sensitive signaling pathway NFκB, AP-1 and MAPK play a critical role in inflammation caused by muscle disuse and misuse. In cultured C2C12 cells, IL-6 production is regulated by IL-1β, and the p38 inhibitor SB-208350 or the ERK inhibitor PD-98059 reduces IL-6 production, suggesting that these two MAPK pathways regulate IL-6 production [41]. ERK has also been shown to play a role in regulating IL-1-induced gene expression of iNOS and COX-2, but not VCAM-1 or MnSOD [14]. Aoi et al. [42] showed that in myotube L6 cells, H_2O_2 stimulated p65 nuclear translocation and expression of CINC-1 and MCP-1, whereas pre-incubation with α-tocopherol limited the increases. An acute bout of exercise increased CINC-1 and MCP-1 levels and nuclear p65 content in rat gastrocnemius muscle, but these changes were less in rats fed a high vitamin E diet. These results indicate that exercise-induced inflammation was caused by phagocyte infiltration and regulated in a redox-sensitive manner.

Recent observations further indicate that PGC-1α may also play a role as an anti-inflammatory agent. Studies in PGC-1α KO animals demonstrate that PGC-1α modulates local or systemic inflammation by regulating the expression of pro-inflammatory cytokines such as TNF-α and IL-6 [43]. PGC-1α KO mice showed higher basal mRNA expression of TNF-α and IL-6 in skeletal muscle, as well as higher serum IL-6 level [44]. In addition, PGC-1α over-expressed mice had lower expression of TNF-α and IL-6 mRNA in skeletal muscle, and reduced serum TNF-α and IL-6 levels [31]. These data suggest that PGC-1α has a protective role in inflammatory response by reducing pro-inflammatory cytokine production. Moreover, a single exercise bout elicited a significant increase in skeletal muscle TNF-α mRNA and serum TNF-α content in PGC-1α KO mice but not wild-type (WT) mice, indicating that PGC-1α normally protects against exercise-induced increases in TNF-α [44]. Previous research has confirmed that PGC-1α is required in the gene expression of mitochondrial ROS-detoxifying enzymes in several types of cells, which can be important in keeping inflammation in check.

6.4 Metabolic Adaptation to Exercise Controlled by Redox Signaling

6.4.1 Mitochondrial Functional Adaptation to Muscle Contraction

When animals are stressed with hypoxia, treated with thyroid hormones, under hypothermia, or engage in long-term physical work with high oxygen consumption, there is a substantial increase in mitochondrial volume, density and oxidative enzyme activity in the skeletal muscle [1]. Increased mitochondrial populations not only increases oxygen consumption to metabolize fuels for ATP production, but also shifts fuels from carbohydrate to fat as a more efficient energy source. In addition, proliferation of mitochondria helps distribute oxygen among increased ETC and reduces the production of ROS. Thus, working muscle is alleviated of both metabolic and oxidative stress resulting from heavy workload.

Mitochondria undergo protein turnover resulting in biochemical, morphological and structural changes. Increased mitochondrial population can be the result of enhanced mitochondrial biogenesis or altered mitochondrial dyanmics (fusion, fission) and mitophagy, or both. Mitochondrial biogenesis is regulated by complex signaling pathways that require the synthesis, import, and incorporation of proteins and lipids to the existing mitochondrial reticulum, as well as replication of the mitochondrial DNA (mtDNA) [45]. In the past decade, PGC-1α emerged as a master transcriptional coactivator, which has provided mechanistic insight into how nuclear regulatory pathways are coupled to the biogenesis of mitochondria, antioxidant

defense, apoptosis and other functions in skeletal muscle. These findings have had profound effects on our understanding of signal transduction pathways related to muscle function.

6.4.2 Mitochondrial Biogenesis: Role of PGC-1α

PGC-1α expression is linked to muscle contraction through Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), and it is known CaMKIV and calcineurin A are activated through calcium ion dynamics within the muscle in response to exercise [46]. The increased calcium signaling during muscle contraction activates several important transcription factors such as cAMP-response element binding protein (CREB), a target of CaMKIV, and myocyte enhancer factor (MEF) 2 [44]. Another factor that regulates PGC-1α expression upon exercise involves p38 MAPK, which activates MEF2 and activating transcription factor 2 (ATF2). ATF-2 and subsequent interactions of ATF2-CREB appears to be an early event in PGC-1α mediated signaling processes [47]. p38 MAPK also stimulates PGC-1α by phosphorylation in response to cytokine stimulation in muscle cells. Finally, as a metabolic energy deprivation sensor, AMPK is activated by exercise due to increased AMP/ATP ratio and Ca^{2+} flux during muscle contraction, enhancing PGC-1α transcription as well as activity. It was demonstrated that activation of p38 MAPK-mediated phosphorylation of CREB and subsequent binding to PGC-1α promoter plays a key role in activating PGC-1α expression in response to increased muscle activity [23].

Endurance exercise is a powerful stimulus to muscle plasticity, and PGC-1α is a major regulator of exercise-induced phenotypic adaptation and fiber transformation. It is well known that chronic exercise training increases PGC-1α levels in the skeletal muscle [11, 23, 48–50]. The training adaptation is usually, but not always accompanied by elevated NRF-1 and Tfam levels. However, endurance training always leads to predictable results in the muscle such as elevated mitochondrial respiratory capacity and ATP production, increased expression of Krebs cycle and ETC enzymes, enhanced fatty acid oxidation and mitochondrial morphological changes [10]. A cross-section study showed that endurance-trained subjects had seven times higher PGC-1α, five times higher Tfam and more than two-fold higher NRF-1 protein contents in VL muscle biopsies than their sedentary counterparts [50]. There is little doubt that the training adaptations were dependent on intact PGC-1α signaling pathways, as PGC-1α KO mice undergoing endurance training showed virtually no change in mitochondrial markers such as NRF-1 and cytochrome c content [49]. It is noteworthy that many of the changes occurring during endurance training can be demonstrated by a single bout of exercise. For example, Kang et al. [51] reported a 6-fold increase in PGC-1α content in rat VL muscle after an acute bout of sprinting on treadmill, along with 200 % increase in NRF-1 and

Tfam levels. Enhanced CREB and p38 phosphorylation were observed, indicating upstream pathways controlling PGC-1α signaling were activated.

6.4.3 Mitochondrial Fusion and Fission Dynamics

Mitochondrial Fusion and Fission Machinery. Morphological data demonstrated that mitochondria are organized into dynamic tubular structures or networks, extending throughout the cytosol, and in close contact with the nucleus, endoplasmic reticulum, Golgi network and cytoskeleton, and undergo constant fusion and fission [52]. Mitochondrial fusion in mammals requires mitofusins (Mfn1, Mfn2), the GTPase family enzymes that are anchored on mitochondrial outer membrane and contain two transmembrane domains connected by a small intermembrane-space loop. Mfn1 seems to act in concert with Optic atrophy protein 1 (OPA1), whereas Mfn2 acts alone [53]. Mitochondrial fission is controlled by the interaction of two proteins: dynamin-related protein 1 (Drp1) and human fission protein 1 (hFis1).

Balanced mitochondrial fusion and fission events are beneficial for oxidative phosphorylation (OXPHOS) and optimal metabolic output in muscle. Fusion provides a chance for mitochondria to mix their contents, thus enabling rapid transmission of Ca^{2+} signals and electrogenic events in large muscle fibers, protein complementation and mtDNA repair [54]. Conversely, fragmented mitochondria resulting from fission are thought to be easily transportable and allow for rapid mitochondrial trafficking to energy-demanding regions of the cell. Recent studies showed that fission of the mitochondrial network into individual units is necessary for efficient mitophagy to eliminate damaged mitochondria [55]. This could occur via proteolytic degradation of the fusion protein OPA1 in energetically compromised mitochondria or by increased activity of the fission proteins, such as Fis1. This in turn stimulates mitochondrial biogenesis to ensure a stable pool of functional mitochondria within each cell.

Role of Redox Signaling in Mitochondrial Remodeling. The exact mechanisms controlling mitochondrial network remodeling are still elusive. However, ROS, PGC-1α and NFκB have been identified as potential regulators in some recent studies. Exercise-induced ROS may contribute to the rapid alteration in mitochondrial fusion/fission protein expression. Koopman et al. [56] reported that vitamin E supplementation restored aberrant mitochondrial morphology in fibroblasts with mitochondrial complex I deficiency, suggesting that ROS are involved in controlling mitochondrial shape in these cells. Mitochondria became fragmented and rounded with the treatment of rotenone or antimycin, known to induce ROS production [57]. Rats subjected to chronic training for 8 weeks had lower Mfn2 protein levels in muscle mitochondria compared to controls, whereas this effect was not seen in trained rats with daily injection of PDTC, an antioxidant, suggesting oxidative process may control Mfn2 expression [49]. PGC-1 has been shown to participate in mitochondrial network remodeling through controlled fusion and fission.

H_2O_2-stimulated Mfn1/2 expression was dramatically attenuated in PGC-1α KO C2C12 muscle cells, whereas PGC-1α over-expression markedly enhances Mfn2 mRNA and protein levels in cultured muscle cells [58]. PGC-1β induces Mfn2 transcription through nuclear receptor ERRα, and increases the length of mitochondrial tubules [45]. This means PGC-1β may predominantly influence the rate of mitochondrial fusion.

Effect of Exercise on Mitochondrial Remodeling. During physical exercise muscle ATP production increases dramatically and so does ROS generation. These changes profoundly change both the morphology and gene expression of mitochondrial fusion and fission proteins. Bo et al. [59] reported that during an acute bout of prolonged exercise with incremental durations, there was an increase in Fis1 expression but decrease in Mfn1/2 expression, and the magnitudes of these alterations depended on exercise duration. These alterations were associated with increased ROS generation and state 4 respiration, but decreased state 3 respiration and attenuated ATP synthase. These findings show that heavy exercise may induce a tendency towards more fragmented mitochondrial network and compromise energy production efficiency. On the other hand, endurance exercise tends to promote expansion of mitochondrial reticulum through a fusion process. Garnier et al. [60] observed increased mRNA levels of Mnf2 and Drp1 in the trained human muscle along with increased mitochondrial respiratory capacity. Increased gene expression appears to occur in the recovery period following an acute exercise bout, with mRNA levels of Mfn1/2 and Fis1 elevated significantly above the resting levels 24 h post-exercise [61]. Similarly, Cartoni et al. [62] showed that Mfn1 and Mfn2 mRNA levels were increased in muscle biopsies obtained from cyclists at 24 h post-exercise. However, Feng et al. [49] recently showed that Mfn2 protein content was decreased in trained rats compared to controls. These results suggest that establishment of a higher level balance of mitochondrial fusion and fission may be an important process behind the functional adaption to endurance training.

6.5 Antioxidant Adaptation to Exercise

6.5.1 Role of NFκB, MAPK and PGC-1α in Antioxidant Enzyme Adaptation

There is an abundance of literature reporting muscle antioxidant adaptation to chronic exercise training. MnSOD (SOD2) shows the most robust increase in both enzyme protein level and activity. Furthermore, an acute bout of exercise has been shown to increase mRNA level of MnDOD, suggesting a transcriptional activation of gene expression [63]. GPX activity also increases after endurance training, but it is not entirely clear whether the increased activity is mainly due to enzyme protein increase or post-translational modification. Training adaptation of antioxidant enzymes is influenced heavily by a number of physiological and environmental

factors, such as sex, age, diet, and drug. For a thorough review on this topic the readers are referred to several previous reviews [4, 7].

There has been evidence that ROS are required molecular signals in exercise-induced redox signaling. An acute bout of exercise has been shown to activate NFκB due to the generation of ROS in rat skeletal muscle [64]. Inhibition of NFκB with the drug, PDTC, severely diminished IKK activation, IκB phosphorylation and p50 nuclear accumulation in response to exercise. Gomez-Cabrera et al. [63] investigated the role of ROS in exercise-activated MAPK and NFκB signaling by injecting rats with allopurinol to inhibit XO prior to a progressive treadmill running. NFκB binding and ERK1/2 and p38 activities were elevated in rat gastrocnemius muscle after exercise, whereas these effects were severely attenuated in the allopurinol-treated rats. Furthermore, allopurinol partially blocked the exercise-induced mRNA of MnSOD and iNOS, both enzymes require NFκB signaling. Kumar et al. [65] reported that passive mechanical stretch in mouse diaphragm muscle activated the NFκB pathway, which could be blocked by NAC. Unloading dramatically decreases NFκB activity, suggesting contractile activity and/or nerve stimulation are required for the basal activity of this signaling pathway.

Recent studies have shown that PGC-1α also has a regulatory mechanism for the expression of endogenous antioxidant proteins. Reduced mRNA levels of SOD1 (CuZn-SOD), SOD2 (Mn-SOD) and/or GPx1, as well as SOD2 protein content were observed in skeletal muscle from PGC-1α KO mice compared to WT, whereas mice with PGC-1α over-expression showed higher SOD2 in skeletal muscle [31]. Also, PGC-1α KO fibroblasts exhibit a decrease in SOD2, catalase and GPx1 mRNA contents compared to WT fibroblasts [58]. Furthermore, PGC-1α promotes SIRT3 gene expression, which deacetylates and activates mitochondrial enzymes including SOD2 through a post-translational mechanism [18]. Taken together, PGC-1α seems to play a mandatory role in upregulating antioxidant enzyme gene expression and activity in response to exercise.

6.5.2 Adaptation of the Glutathione System

GSH:GSSG homeostasis is known to play an important role in muscle antioxidant defense and control of inflammation [7]. Heavy exercise is known to increase GSH oxidation to GSSG while decrease the GSH/GSSG ratio. During the respiratory burst of neutrophils, GSH is consumed due to the increased ROS production by neutrophil NAPDH oxidase. High levels of GSH prevent the inflammatory process partially by inhibiting ICAM-1 expression [66]. Interestingly, both constitutive and TNFα-stimulated ICAM-1 expression was shown to decrease GSH levels in endothelial cells due to suppression of GCL, the rate-limiting enzyme of the γ-glutamyl cycle. However, GSH content was reported to be elevated in rabbit tibialis muscle 24 h after an isokinetic stretch injury, accompanied with elevated GPX and GR activities [67]. Several studies demonstrated that GCS can be induced by endurance training in rat skeletal muscle and liver [68]. Training also increases

muscle GSH content, though the adaptation shows a fiber-specific manner. Surprisingly, little is known about the gene regulation of GCS in skeletal muscle. In mammalian cells, GCS is a heterodimer consisting of the catalytic heavy-chair subunit (GCS-HS) and a regulatory light-chair subunit (GCS-LC). GCS-HS expression is known to be regulated by a redox-sensitive mechanism via a variety of oxidants, phenolic antioxidants and pro-inflammatory cytokines (TNFα and IL-1ß). Both GCS-HC and GCS-LC promoters contain an antioxidant response element (ARE), and NRF-2 binding seems to play a critical role in oxidative stress-induced GCS upregulation. GCS-HC also has NFκB binding sites that are essential for GCS expression in some, but not all cell types [69]. If these signaling pathways are also operational in muscle cells, they could be a potential mechanism for training-induced upregulation of GCS and GSH biosynthesis.

6.5.3 Adaptation of INOS System

NO at low concentration exerts an antioxidant function by neutralizing $O_2^{-\bullet}$. Its vasodilative effect increases blood flow to the working muscle thereby improving the availability of blood-borne energy substrates and antioxidants. Thus, an increase in NO production via the regulation of NOS may be viewed as indirectly enhancing muscle antioxidant defenses during exercise. Unlike the other forms of NOS, inducible iNOS is not regulated by calcium ion and instead is responsive primarily to ROS and inflammatory cytokines through activation of NFκB and MAPK [70]. In rat skeletal muscle myoblasts, the IL-1β-mediated iNOS induction was reduced by blocking ERK1/2 activation and completely abolished by the inhibition of NFκB. iNOS mRNA level has been reported to be elevated after an acute bout of exercise in rat skeletal muscles [63]. While a modest level of iNOS induction may be viewed as improving muscle antioxidant defense, high levels of NO production would lead to the formation of peroxynitrite, a highly reactive ROS contributing to muscle oxidative damage. Thus, iNOS upregualtion may favor either an antioxidant or pro-oxidant state depending on muscle physiological condition.

6.5.4 Adaptation of Uncoupling Proteins

Mitochondrial production of ROS is partly dependent on the cross-membrane proton motive force ($\Delta\psi_m$); thus, increasing mitochondrial membrane permeability with uncouplers (such as dinitrophenol [DNP], trifluorocarbonylcyanide phenylhydrazone [FCCP]) to reduce $\Delta\psi_m$ is viewed as a classic way to reduce ROS production. Located in the mitochondrial inner membrane, uncoupling proteins (UCPs) are a heterogeneous family of proteins that play an important role in partially dissipating the proton electrochemical gradient $\Delta\psi_m$. The best characterized UCP1 is expressed exclusively in the brown adipose tissue of rodents with a key

function of adaptive thermogenesis, whereas UCP2 is expressed ubiquitously [71]. UCP3, expressed primarily in the skeletal muscle, shares 59 % homology to that of UCP1 and is regarded a plausible regulator of trans-membrane proton potential and hence efficiency of oxidative phosphorylation [72]. Several previous studies have shown that UCP3 expression was increased in response to an acute bout of exercise or contractile activity in mammalian skeletal muscle. Goglia and Skulachev [73] postulated that by translocating fatty acid peroxides from inner to the outer membrane leaflet, UCP may fulfill a role in the antioxidant defense of the mitochondria. Jiang et al. [74] reported that in response to a prolonged bout of exercise, UCP3 mRNA and protein expression in rat skeletal muscle was elevated sharply at 45 min and reaching the peak at 150 min, when ROS production and state 4 respiration rate showed a dramatic drop. These data demonstrate that UCP3 expression in rat skeletal can be rapidly upregulated during prolonged exercise, which could reduce ROS production to protect mitochondria from oxidative stress.

The cellular mechanism by which muscle contraction increases UCP expression in the mitochondria is still unclear. Anderson et al. [75] demonstrated that UCP3 gene expression was increased by an acute bout of exercise in mouse gastrocnemius muscle and that this upregulation was dependent on mitochondrial H_2O_2 production, whereas in the UCP3$^{-/-}$ mice, exercise failed to increase mitochondrial uncoupling respiration. St-Pierre et al. [58] showed that while H_2O_2 stimulated UCP3 and UCP2 expression in WT muscle cells, PGC-1α KO, abolished these effects. Thus, UCP gene expression seems to be redox sensitive, and PGC-1α could be an important mediator in the upregulation of UCP3.

6.6 Redox Signaling and Aging

6.6.1 Mitochondrial Adaptation to Exercise in Aging Muscle

Aging has a profound impact on the adaptability of skeletal muscle to exercise intervention. Several relevant questions may be asked: (1) Does aged muscle have decreased levels of protein components in the various redox signaling pathways? (2) Does aged muscle exhibit reduced sensitivity to oxidative challenge and diminished redox signaling potential? (3) What is the functional implication of this impairment and the possible relationship with sarcopenia? A complete review of this important subject is beyond the scope of this chapter. The readers are referred to several recent excellent reviews [27, 76].

The majority of literature seems to agree that training-induced mitochondrial and metabolic adaptations are not entirely abolished by old age and that older animals and humans can still benefit from chronic muscular contraction [76]. This conclusion has been recently confirmed by investigations involving both aged rodents and human subjects [50, 77, 78]. However, it is also widely perceived that aging can attenuate the magnitude of training adaptation seen at a younger age. There is evidence that muscle PGC-1α, NRF-1 and cytochrome c contents from

aged rats did not respond to endurance training as the young rats did, and that the lack of training response was identical to PGC-1α KO mice [48]. Furthermore, it was shown that mitochondrial biogenic markers in aged muscle did not respond to cold exposure or thyroid (T_3) stimulation, the classic PGC-1α stimulators [10]. In contrast, other investigators have recently reported positive training adaptations in muscle mitochondrial biogenesis in aged rats, with increased PGC-1α mRNA and protein levels, and CREB phosphorylation and DNA binding [78]. Several studies using human subjects also provided insightful information. Older subjects showed lower muscle PGC-1α mRNA, NRF-1 and Tfam protein contents than young subjects, whereas a 16-week aerobic exercise training program resulted in a more than two-fold increase in PGC-1α mRNA expression and 1.5-fold increase in NRF-1 content [77]. In a cross-section study, Lanza et al. [50] showed that endurance trained older subjects displayed significantly higher mitochondrial protein levels, mtDNA content and PGC-1α signaling markers (NRF-1, Tfam) than sedentary older subjects, though the magnitude of improvement was consistently lower than that of young subjects. It is unclear whether older subjects did less muscular work and thus were exposed to lower level of stimulus, or older muscles are less capable of responding to a similar exercise challenge. Iversen et al. [79] recently showed that untrained older subjects (71 years) displayed twice as high PGC-1α response ($\sim$12- vs. 6-fold increase) as their trained counterparts after an acute bout of bicycle exercise at 75 % of their matched VO_2max. Both groups showed remarkable increases in the phosphorylation level of AMPK and p38, the two major upstream enzymes that activate PGC-1α expression. These findings clearly indicate that skeletal muscle of elderly subjects maintains the ability of responding to acute exercise and that age should not prevent mitochondrial adaptation to endurance training. Figure 6.3 depicts the potential intervention of exercise on age-related disruption of mitochondria homeostasis in skeletal muscle.

6.6.2 Antioxidant Signaling in Aging Muscle

It is well-established that aging is associated with increased free radical generation and that the resulting oxidative damage accumulated in organisms founds the etiological basis for numerous diseases. It has long been suspected that senescent skeletal muscle may have a compromised antioxidant defense as well as ability to adapt to oxidative stress due to structural and functional impairment. However, after more than a decade of research, simple answers to this question still do not seem to have come forward.

In skeletal muscle antioxidant enzyme activities are increased with old age [80]. However, protein and mRNA levels of CuZnSOD, MnSOD and GPX are found to be either decreased or unaltered in the age muscle [81], indicating post-transcriptional modulation of these enzymes. Furthermore, aged muscle exhibits reduced antioxidant adaptation to training compared to young muscle [7]. Clearly, some biological factors prevent aged skeletal muscle from achieving the higher levels of adaptation normally

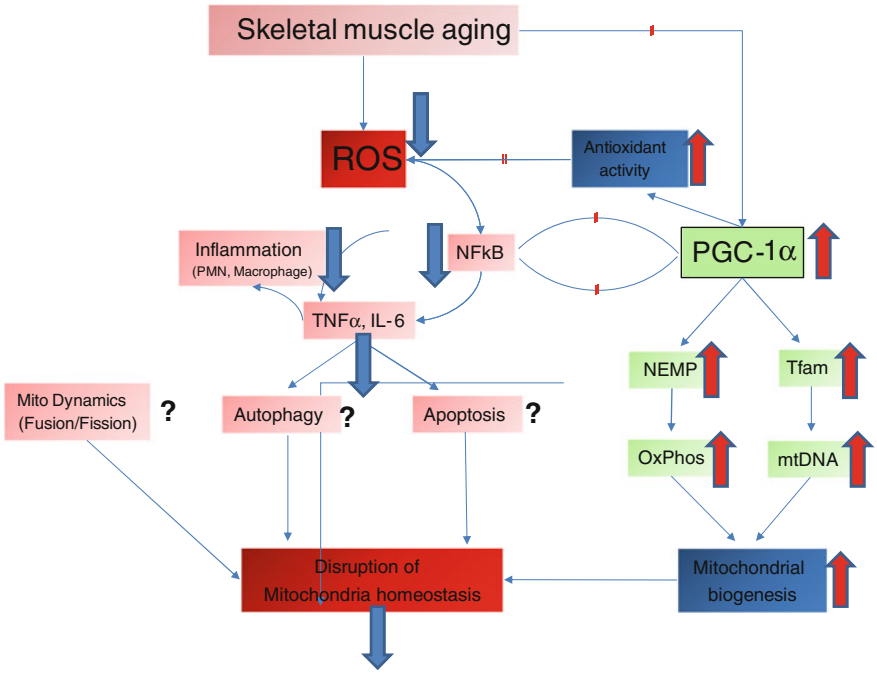

Fig. 6.3 A brief overview of the potential positive intervention of exercise on age-related disruption of muscle mitochondrial homeostasis. The role of several redox signaling pathways are depicted. Arrows indicate exercise effects. Abbreviations: *IL-6* interleukin-6; *NEMP* nuclear encoded mitochondrial protein; *NFκB* nuclear factor κB; *OxPhos* oxidative phosphorylation; *PGC-1α* peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *PMN* polymorphoneutrophil; *ROS* reactive oxygen species; *TNFα* tumor necrosis factor α

seen in the young muscles. These findings led to the hypothesis that aging may impair signal transduction of antioxidant gene expression in response to oxidative stress.

According to the information discussed in previous sections, antioxidant gene expression is controlled by multiple signaling pathways, such as NFκB, MAPK, PGC-1α, FoxO and Sirt, and their crosstalk. Thus, weakening of signals from any of the above-mentioned pathways might compromise the signal transduction and thus the phenol- and genotypic adaptations. Parkington et al. [82] measured ERK1/2 and $p70^{S6K}$ activities in the plantaris and tibialis anterior muscles of young and old rats in response to electric stimulation and concluded that anabolic response to contractile stimulus is attenuated with aging, which may contribute to reduced exercise-induced muscle hypertrophy. In contrast, Williamson et al. [83] reported higher resting activities of ERK1/2, $p90^{RSK}$, p38 and JNK/SAPK in the leg muscle of old men compared to young men. However, aged muscles had decreased MAPK enzyme activities after an acute bout of resistance contraction, whereas young ones had increased levels of these enzyme activities. Total amount of protein in the MAPK pathway were unaltered with age. Hornberger et al. [84] found no difference

in p38, $p70^{S6K}$ and JNK2 activities in the extensor digitorum longu (EDL) muscle between young and old rats. As for NFκB signaling, several authors have reported decreased NFκB binding capacity in aged rodent muscle at rest [81] and in response to stimulated contraction [85]. However, other authors concluded that aged muscles have a chronically high level of inflammation marked by enhanced NFκB expression and signaling. Indeed, aged muscle is known to have high ROS production rate, prone to chronic inflammation and other pathological conditions that may alter oxidant-antioxidant balance, all of which may affect and mask the true aging effect on antioxidant gene expression. Chronic inflammation, especially due to minor injury and/or immobility, is often seen in senescent muscles. NFκB is believed to be constitutively activated at old age, which leads to the higher basal expression of pro-inflammatory cytokines, chemokines, adhesion molecules (ICAM-1, VCAM) and ROS-generating enzymes (COX-2). In fact, chronic activation of NFκB has been identified as a main etiological reason for aged-related muscle wasting and sarcopenia [86]. Since NFκB activation often leads to increased pro-inflammatory cytokine expression, this vicious cycle was hypothesized as the basis for the inflammation theory of aging [87].

6.7 Conclusion

Evolution has turned ROS into an essential component of cellular life through redox signaling. In skeletal muscle, contraction induced ROS production underlies most documented major adaptations that have been observed during the past half century. Muscle contraction can dramatically change the balance between ROS production and antioxidant defense often resulting in a small surplus of ROS, which activates several signal transduction pathways including but not limited to NFκB, MAPK, and PGC-1α. Moderate exercise stimulates PGC-1α signaling that promotes mitochondrial biogenesis and antioxidant adaptation, whereas rigorous exercise containing strenuous eccentric contraction could lead to hyper-activation of NFκB, a negative regulator of PGC-1α and a main stimulative pathway for inflammatory response. However, much is still unknown with regard to the interactions of many ROS controlled processes and the optimal level of ROS that elicit biological functions beneficial to human health. We hope that future research will continue to explore this dose-response relationship and find the best strategy to maximize exercise benefits.

References

1. Holloszy JO, Coyle EF (1984) Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J Appl Physiol 56:831–838
2. Wallace DC (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 39:359

3. Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527–605
4. Powers SK, Jackson MJ (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev 88(4):1243–1276
5. Nieman DC, Davis JM, Henson DA et al (2003) Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run. J Appl Physiol 94:1917–1925
6. Allen RG, Tresini M (2000) Oxidative stress and gene regulation. J Free Rad Biol Med 28:463–499
7. Ji LL (2007) Antioxidant signaling in skeletal muscle: a brief review. Exp Gerontol 42(7):582–593
8. Kandarian SC, Jackman RW (2006) Intracellular signaling during skeletal muscle atrophy. Muscle Nerve 33:155–165
9. Lin J, Wu H, Tarr PT et al (2002) Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. Nature 418:797–801
10. Wu Z, Puigserver P, Andersson U et al (1999) Mechanisms controlling mitochondrial biogenesis and function through the thermogeniccoactivator PGC-1. Cell 98:115–124
11. Ruas JL, White JP, Rao RR et al (2012) A PGC-1α isoform induced by resistance training regulates skeletal muscle hypertrophy. Cell 151:1319–1331
12. Meyer M, Pahl HL, Baeuerle PA (1994) Regulation of the transcription factors NF-kB and AP-1 by redox changes. J Chemico-Biol Interact 91:91–100
13. Li Q, Engelhardt JF (2006) Interlecukin-1β induction of NFκB is partially regulated by H_2O_2-mediated activation of NFκB-inducing kinase. J Biol Chem 281:1495–1505
14. Jiang B, Xu S, Hou X et al (2004) Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-1beta-induced gene expression. J Biol Chem 279:1323–1329
15. Ghosh S, Karin M (2002) Missing pieces in the NF-kappaB puzzle. J Cell 109(Suppl): S81–S96
16. Catani MV, Savini I, Duranti G et al (2004) Nuclear factor kappaB and activating protein 1 are involved in differentiation-related resistance to oxidative stress in skeletal muscle cells. J Free Radic Biol Med 37:1024–1036
17. Dali-Youcef N, Lagouge M, Froelich S et al (2007) Sirtuins: the 'magnificent seven', function, metabolism and longevity. Ann Med 39:335–345
18. Nemoto S, Fergusson M, Finkel T (2005) SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1α. J Biol Chem 280:16456–16460
19. Koltai E, Szabo Z, Atalay M et al (2010) Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats. Mech Ageing Dev 131:21–28
20. Kandarian SC (2002) Activation of an alternative NF-kappaB pathway in skeletal muscle during disuse atrophy. FASEB J 16:529–538
21. Sandri M (2013) Protein breakdown in muscle wasting: Role of autophagy-lysosome and ubiquitin-proteasome. Int J Biochem Cell Biol. doi:10.1016/j.biocel.2013.04.023
22. Durham WJ, Arbogast S, Gerken E et al (2006) Progressive nuclear factor-kappaB activation resistant to inhibition by contraction and curcumin in mdx mice. Muscle Nerve 34:298–303
23. Akimoto T, Pohnert SC, Li P et al (2005) Exercise stimulates Pgc-1α transcription in skeletal muscle through activation of the p38 MAPK pathway. J Biol Chem 280:19587–19593
24. Finkel T, Holbrook N (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247
25. Irrcher I, Ljubicic V, Kirwan AF et al (2008) AMP-activated protein kinase regulated activation of the PGC-1α promoter in skeletal muscle cells. PLoS ONE. doi:10.1371/journal.pone.0003614
26. Sandri M (2008) Signaling in muscle atrophy and hypertrophy. Physiology 23:160–170
27. Marzetti E, Calvani R, Cesari M et al (2013) Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials. Int J Biochem Cell Biol 45:2288–2301
28. Stevenson E, Giresi P, Koncarevic A et al (2003) Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle. J Physiol 551:33–48

29. Whidden MA, McClung JM, Falk DJ et al (2009) Xanthine oxidase contributes to mechanical ventilation-induced diaphragmatic oxidative stress and contractile dysfunction. J Appl Physiol 106:385–394
30. Hanai JI, Cao P, Tanksale P et al (2007) The muscle-specific ubiquitin ligase atrogin-1/MAFbx mediates statin-induced muscle toxicity. J Clin Invest 117:3940–3951
31. Wenz T, Rossi S, Rotundo RL et al (2009) Increased muscle PGC-1α expression protects from sarcopenia and metabolic disease during aging. Proc Nat Acad Sci 106:20405–20410
32. Handschin C, Chin S, Li P et al (2007) Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1α muscle-specific knock-out animals. J Biol Chem 282:30014–30021
33. Peterson JM, Feeback KD, Baas JH et al (2006) Tumor necrosis factor-α promotes the accumulation of neutrophils and macrophages in skeletal muscle. J Appl Physiol 101:1394–1399
34. Kang C, Ji LL (2013) Muscle immobilization and remobilization downregulates PGC-1α signaling and the mitochondrial biogenesis pathway. J Appl Physiol 115:1618–1625
35. Peake J, Nosaka K, Suzuki K (2005) Characterization of inflammatory responses to eccentric exercise in humans. Exerc Immunol Rev 11:64–85
36. Proske U, Allen TJ (2005) Damage to skeletal muscle from eccentric exercise. Exerc Sport Sci Rev 333:98–104
37. Walsh LD, Hesse CW, Morgan DL et al (2004) Human forearm position sense after fatigue of elbow flexor muscles. J Physiol 558:705–715
38. Nguyen HX, Tidball JG (2003) Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro. J Physiol 553:833–841
39. Pedersen BK, Steensber A, Schjerling P (2001) Muscle-derived interleukin-6: possible biological effects. J Physiol 536:329–337
40. Qiang L, Engelhardt JF (2006) Interleukin-1B induction of NFkB is partially regulated by H2O2-mediated activation of NFkB-inducing kinase. J Biol Chem 281:1495–1505
41. Luo G, Hershko DD, Robb BW et al (2003) IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B. Am J Physiol Regul Integr Comp Physiol 284:R1249–R1254
42. Aoi W, Naito Y, Takanami Y et al (2004) Oxidative stress and delayed-onset muscle damage after exercise. Free Radic Biol Med 37:480–487
43. Handschin C (2009) Peroxisome proliferator-activated receptor-γ coactivator-1α in muscle linksmetabolism to inflammation. J Clin Exp Pharmacol Physiol 36:1139–1143
44. Handschin C, Spiegelman BM (2008) The role of exercise and PGC1α in inflammation and chronic disease. J Nature 454:463–469
45. Liesa M, Palacin M, Zorzano A (2009) Mitochondrial dynamics in mammalian health and disease. Physiol Rev 89:799–845
46. Baar K, Wende AR, Jones TE et al (2002) Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J 16:1879–1886
47. Vercauteren K, Pasko RA, Gleyzer N et al (2006) PGC-1-related coactivator: immediate early expression and characterization of a CREB/NRF-1 binding domain associated with cytochrome c promoter occupancy and respiratory growth. Mol Cell Biol 26:7409–7419
48. Derbre F, Gomez-Carbrera C, Nascimento AL et al (2012) Age associated low mitochondrial biogenesis may be explained by lack of response of PGC-1α to exercise training. Age 34:669–679
49. Feng H, Kang C, Dickman J et al (2013) Training-induced Mitochondrial Adaptation: Role of PGC-1α, NFκB and β-Blockade. Exp Physiol 98:784–795
50. Lanza IR, Short DK, Raghavakaimal S et al (2008) Endurance exercise as a countermeasure of aging. Diabetes 57:2933–2942
51. Kang C, O'Moore KM, Dickman JR et al (2009) Exercise activation of muscle peroxisome proliferator-activated receptor-γ coactivator-1α signaling is redox sensitive. Free Rad Biol Med 47:1394–1400
52. Hom J, Sheu SS (2009) Morphological dynamics of mitochondria–a special emphasis on cardiac muscle cells. J Mol Cell Cardiol 46:811–820

53. Song Z, Ghochani M, McCaffery JM et al (2009) Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. J Mol Biol Cell 20:3525–3532
54. Grandemange S, Herzig S, Martinou JC (2009) Mitochondrial dynamics and cancer. Semin Cancer Biol 19(1):50–56
55. Breckenridge DG, Kang BH, Kokel D et al (2008) Caenorhabditis elegans drp-1 and fis-2 regulate distinct cell-death execution pathways downstream of ced-3 and independent of ced-9. J Mol Cell 31:586–597
56. Koopman WJ, Verkaart S, van EV et al (2008) Mitigation of NADH: ubiquinone oxidoreductase deficiency by chronic trolox treatment. Biochim Biophys Acta 1777:853–859
57. Yu T, Robotham JL, Yoon Y (2006) Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci USA 103:2653–2658
58. St-Pierre J, Drori S, Uldry M (2006) Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 127:397–408
59. Bo H, Zhang Y, Ji LL (2010) Redefining the role of mitochondria in exercise: a dynamic remodeling. J Ann NY Acad Sci 1201:121–128
60. Garnier A, Fortin D, Zoll J et al (2005) Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. FASEB J 19:43–52
61. Ding H, Jiang N, Liu H et al (2009) Response of mitochondrial fusion and fission protein gene expression to exercise in rat skeletal muscle. J Biochim Biophys Acta 1800:250–256
62. Cartoni R, Léger B, Hock MB et al (2005) Mitofusins 1/2 and ERR alpha expression are increased in human skeletal muscle after physical exercise. J Physiol 567:349–358
63. Gomez-Cabrera M-C, Borras C, Pallardó FV et al (2005) Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. J Physiol (Lond) 567:113–120
64. Ji LL, Gomez-Cabrera M-C, Steinhafel N et al (2004) Acute exercise activates nuclear factor (NF) κB signaling pathway in rat skeletal muscle. FASEB J 18:1499–1506
65. Kumar A, Boriek AM (2003) Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in duchenne muscular dystrophy. FASEB J 17:386–396
66. Kevil CG, Pruitt H, Kavanagh TJ et al (2004) Regulation of endothelial glutathione by ICAM-1: implications for inflammation. FASEB J 18:1321–1323
67. Best TM, Fiebig R, Corr DT et al (1999) Free radical activity and the response of antioxidant enzymes and glutathione following acute muscle stretch injury in rabbits. J Appl Physiol 87:74–82
68. Ramires P, Ji LL (2001) Glutathione supplementation and training increases myocardial resistance to ischemia-reperfusion in vivo. Am J Physiol 281:H679–H688
69. Chan JY, Kwong M (2000) Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. J Biochim Biophys Acta 1517:19–26
70. Adams V, Nehrhoff B, Spate U et al (2002) Induction of iNOS expression in skeletal muscle by IL-1beta and NFkappaB activation: an in vitro and in vivo study. J Cardiovasc Res 54 (1):95–104
71. Krauss S, Zhang CY, Lowell BB (2005) The mitochondrial uncoupling-protein homologues. Nat Rev Mol Cell Biol 6:248–261
72. Ljubicic V, Adhihetty PJ, Hood DA (2004) Role of UCP3 in state 4 respiration during contractile activity-induced mitochondrial biogenesis. J Appl Physiol 97:976–983
73. Goglia F, Skulachev VP (2003) A function for novel uncoupling proteins: antioxidant defense of mitochondrial matrix by translocating fatty acid peroxides from the inner to the outer membrane leaflet. FASEB J 17:1585–1591
74. Jiang N, Zhang G, Bo H et al (2009) Upregulation of uncoupling protein-3 in skeletal muscle during exercise: a potential antioxidant function. Free Rad Biol Med 46:138–145
75. Anderson EJ, Yamazaki H, Neufer PD (2007) Induction of endogenous uncoupling protein 3 suppresses mitochondrial oxidant emission during fatty acid-supported respiration. J Biol Chem 282:31257–31266

76. Ljubicic V, Joseph A, Saleem A et al (2010) Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging. Biochim Biophys Acta 1800:223–234
77. Ghosh S, Lertwattanarak R, Lefort N et al (2011) Reduction in reactive oxygen species production by mitochondria from elderly subjects with normal and impaired glucose tolerance. Diabetes 60:2051–2060
78. Kang C, Chung E, Diffee G et al (2013) Exercise training attenuates aging-associated mitochondrial dysfunction in rat skeletal muscle: role of PGC-1α. Exp Gerontol 48:1343–1350
79. Iversen N, Krustrup P, Rasmussen H et al (2011) Mitochonddrial biogenesis and agiogenesis in skeletal muscle of the elderly. Exp Gerontol 46:670–678
80. Ji LL, Dillon D, Wu E (1990) Alteration of antioxidant enzymes with aging in rat skeletal muscle and liver. Am J Physiol 258:R918–R923
81. Hollander J, Bejma J, Ookawara T et al (2000) Superoxide dismutase gene expression in skeletal muscle: fiber-specific effect of age. Mech Ageing Dev 116:33–45
82. Parkington JD, LeBrasseur NK, Siebert AP et al (2004) Contraction-mediated mTOR, p70S6 k, and ERK1/2 phosphorylation in aged skeletal muscle. J Appl Physiol 97:243–248
83. Williamson D, Gallagher P, Harber M et al (2003) Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle. J Physiol 547:977–987
84. Hornberger TA, Mateja RD, Chin ER, Andrews JL, Esser KA (2005) Aging does not alter the mechanosensitivity of the p38, p70S6k, and JNK2 signaling pathways in skeletal muscle. J Appl Physiol 98:1562–1566
85. Broome CS, Kayani AC, Palomero J et al (2006) Effect of lifelong overexpression of HSP70 in skeletal muscle on age-related oxidative stress and adaptation after nondamaging contractile activity. FASEB J 20:1549–1551
86. Cai D, Frantz JD, Tawa NE Jr et al (2004) IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. Cell 119:285–298
87. Yu BP, Chung HY (2006) Adaptive mechanisms to oxidative stress during aging. Mech Ageing Dev 127:436–443

Chapter 7
Sarcopenia and Its Intervention

Kunihiro Sakuma and Akihiko Yamaguchi

Abstract The world's elderly population is expanding rapidly, and we are now faced with the significant challenge of maintaining or improving physical activity, independence, and quality of life in the elderly. Sarcopenia, the age-related loss of skeletal muscle mass, is characterized by a deterioration of muscle quantity and quality leading to a gradual slowing of movement, a decline in strength and power, increased risk of fall-related injury, and often, frailty. Muscle loss has been linked with several proteolytic systems, including the ubuiquitin-proteasome and lysosome-autophagy systems. Although many factors are considered to regulate age-dependent muscle loss, this gentle atrophy is not affected by factors known to enhance rapid atrophy (denervation, hindlimb suspension, etc.). In addition, defects in Akt-mammalian target of rapamycin (mTOR) and serum response factor (SRF)-dependent signaling have been found in sarcopenic muscle. Intriguingly, more recent studies indicate an apparent functional defect in autophagy-dependent signaling in sarcopenic muscle. Resistance training combined with amino acid-containing supplements is often utilized to prevent age-related muscle wasting and weakness. Treatment with ursolic acid seems to be effective as therapeutic agents for sarcopenia, because they attenuate the degenerative symptoms of cachexic muscle. Pharmacological, hormonal, and supplemental approaches have been tried to attenuate sarcopenia, but did not obtain outstanding results. In this review, we summarize the current understanding of the adaptation of many regulators in sarcopenia and more recent therapeutic strategies (myostatin inhibition, supplementation with ghrelin or ursolic acid, etc.) for counteracting sarcopenia.

K. Sakuma (✉)
Research Center for Physical Fitness, Sports and Health, Toyohashi University of Technology, 1-1 Hibarigaoka, Tenpaku-Cho, Toyohashi 441-8580, Japan
e-mail: ksakuma@las.tut.ac.jp

A. Yamaguchi
Department of Physical Therapy, Health Sciences University of Hokkaido, Kanazawa, Ishikari-Tobetsu, Hokkaido 061-0293, Japan
e-mail: yama@hoku-iryo-u.ac.jp

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Abbreviations

ACE	angiotensin-converting enzyme
ActRIIB	activin receptor IIB
ALK	activin receptor-like kinase
Atg	autophagy-related genes
atrogin-1	atrophy gene-1
BCAA	branched chain amino acid
CR	caloric restriction
DHEA	dehydroepiandrosterone
DMD	Duchenne muscular dystrophy
eIF	eukaryotic initiation factor
4E-BP	eIF 4E binding protein
FOXO	forkhead box O
GH	growth hormone
GSK	glycogen synthase kinase
IGF-I	insulin-like growth factor-I
IL	interleukin
JAK	Janus kinase
KO	knockout
MRTF	myocardin-related transcription factor
mTOR	mammalian target of rapamycin
mTORC	mTOR signaling complex
MuRF	muscle ring-finger protein
NF-κB	nuclear factor-kappaB
NMJ	neuromuscular junction
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
PI3-K	phosphatidylinositol 3-kinase
p70S6K	p70 ribosomal protein S6 kinase
RDA	recommended dietary allowance
Rheb	Ras homolog enriched in brain
ROS	reactive oxidative species
SRF	serum response factor
STARS	striated muscle activators of Rho signaling
STAT	signal transducer and activator of transcription
TNF-α	tumor necrosis factor-α
TSC	tuberous sclerosis complex
UPS	ubiquitin-proteasome system

7.1 Introduction

Skeletal muscle contractions power human body movements and are essential for maintaining stability. Skeletal muscle tissue accounts for almost half of the human body mass and, in addition to its power-generating role, is a crucial factor in maintaining homeostasis. Given its central role in human mobility and metabolic function, any deterioration in the contractile, material, and metabolic properties of skeletal muscle has an extremely important effect on human health. Aging is associated with a progressive decline of muscle mass, quality, and strength, a condition known as sarcopenia. Although this term is applied clinically to denote loss of muscle mass, it is often used to describe both a set of cellular processes (denervation, mitochondrial dysfunction, inflammatory and hormonal changes) and a set of outcomes, such as decreased muscle strength, mobility, and function, a greater risk of falls, and reduced energy needs. Von Haeling et al. [1] have estimated its prevalence at 5–13 % for elderly people aged 60–70 years and 11–50 % for those aged 80 years or above. Lean muscle mass generally contributes up to ~50 % of total body weight in young adults, but declines with aging to 25 % at 75–80 years old. The loss of muscle mass is most notable in the lower limb muscle groups, with the cross-sectional area of the vastus lateralis being reduced by as much as 40 % between the age of 20 and 80 years old. At the muscle fiber level, sarcopenia is characterized by specific type II muscle fiber atrophy, fiber necrosis, and fiber-type grouping.

Several possible mechanisms of age-related muscle atrophy have been described. Age-related muscle loss is a result of reductions in the size and number of muscle fibers, possibly due to a multi-factoral process that involves physical activity, nutritional intake, metabolic homeostasis, oxidative stress, hormonal changes, and lifespan. The specific contribution of each of these factors is unknown but there is emerging evidence that the disruption of several positive regulators (Akt and SRF) of muscle hypertrophy with age is an important feature in the progression of sarcopenia [2, 3]. Very intriguingly, more recent studies indicated an apparent functional defect in autophagy- and myostatin-dependent signaling in sarcopenic muscle [4–6]. In contrast, many investigators have failed to demonstrate age-related enhancement in the levels of common negative regulators [atrophy gene-1 (atrogin-1), NF-κB (nuclear factor-kappaB), and calpain] in senescent mammalian muscles [2, 3, 7].

Resistance training combined with amino acid-containing supplements is an effective candidate to prevent age-related muscle wasting and weakness [2, 3]. In particular, sarcopenia has been most attenuated by treatment with many essential amino acids plus high leucine. In addition, many researchers have focused on inhibiting myostatin to treat various muscle disorders such as muscular dystrophy, cachexia, and sarcopenia. Furthermore, more recent studies have indicated the possible application of new supplements (e.g. ursolic acid) to prevent muscle atrophy. This chapter aims to outline the molecular mechanism of muscle atrophy in sarcopenia, and to address several recent strategies for inhibiting this phenomenon.

7.2 Molecular Mechanism Regulating Sarcopenia

7.2.1 The Adaptation of Phosphatidylinositol-3-Kinase (PI3-K)/Akt/Mammalian Target of Rapamycin (mTOR) Pathway in Aged Muscle

A central pathway involved in hypertrophy is regulated at the translational level by the serine/threonine kinase Akt. In muscle, Akt is activated by the upstream PI3-K, induced either by receptor binding or by integrin-mediated activation of focal adhesion kinase. The striking effect of Akt1 on muscle size was demonstrated by the transient transfection of a constitutively active inducible Akt1 transgene in skeletal muscle in vivo. Possible downstream regulators of Akt, mTOR and glycogen synthase kinase (GSK)-3β, play a crucial role in the regulation of translation.

mTOR exists in two functionally distinct multi-protein signaling complexes, mTOR signaling complex (mTORC)1 and mTORC2. Akt activates mTOR via phosphorylation and inactivation of tuberous sclerosis complex-2. In general, only signaling by mTORC1 is inhibited by rapamycin, and thus the growth regulatory effects of rapamycin are believed to be primarily exerted through the mTORC1 complex. In skeletal muscle, signaling by mTORC1 has been shown to be regulated by a variety of different stimuli (increased mechanical loading, feeding, or growth factors) that control skeletal muscle mass. mTOR is currently thought to be the major hub for the integration of an array of upstream signaling pathways that, when activated, ultimately result in increased translational efficiency. Two of the most studied mTORC1 targets are the eukaryotic initiation factor 4E binding protein (4E-BP)1 and 70-kDa ribosomal protein S6 kinase (p70S6K), both of which play important roles in the initiation of mRNA translation. mTOR phosphorylates and activates p70S6K, which results in increased translation either directly or indirectly by activating initiation and elongation, elongation initiation factor (eIF)-2, eIF-4E (through 4E-BP), and eEF-2.

Although many researchers consider PI3-K/Akt/mTOR levels to decrease with age, studies using sarcopenic muscles from rats and humans have yielded conflicting results. For example, compared with those in young Fischer 344 × Brown Norway rats, the amounts of phosphorylated mTOR and p70S6K were increased 70–75 % in the tibialis anterior (TA) but not in the plantaris muscle of senescent rats [8]. Kimball et al. [9] showed that, in gastrocnemius muscle, the level of phosphorylated p70S6K, eIF2B activity, and the amount of eIF4E associated with eIF4G increased between 12 and 27 months of age despite an apparent decrease in Akt activity. In addition, other groups [10] also showed the decreased phosphorylation status of Akt in aged mammalian muscle. In contrast, Rahnert et al. [11] showed only significant decrease of phospho-p70S6K (T^{421}/S^{424}) in the aged biceps brachii and no change in phospho-p70S6K (T^{389}), in spite of significant age-related decrease in p70S6K in all head and neck, tongue, and limb muscles (pectoralis, styloglossus, geniohyoid, posterior digastric, and masseter). Therefore, aging did

not commonly modulate the PI3-K/Akt/mTOR-linked molecules in skeletal muscle under sedentary conditions.

Sarcopenic muscle shows a marked defect in the contraction-induced activation of these mediators. Parkingdon et al. [8] reported lower levels of phosphorylated p70S6K and mTOR after high-frequency electrical stimulation [HFES, 3-s trains of pulses (frequency 100 Hz, duration 1 ms at 10–12 V)] in muscle of senescent rats (30 months of age) compared with those in young rats (6 months of age). The same group also demonstrated that 4E-BP1 was markedly phosphorylated in the TA muscle of aged but not young rats at 6 h after HFES. In addition, they suggested no increase in eIF4E-eIF4G association after HFES in aged muscle. Fry et al. [12] demonstrated that acute resistance exercise (eight sets of 10 repetitions of leg-extension at 70 % of 1 repetition maximum with 3 min of rest between each set) increased muscle-protein synthesis rate, and phosphorylation of mTOR, S6K1, and 4E-BP1 only in younger subjects (27 ± 2 years old) but not in elderly ones (70 ± 2 years old). These lines of evidence clearly show that sarcopenic muscle exhibits an impairment of Akt/mTOR/p70S6K signaling after contraction. This defect would explain the limited capacity for hypertrophy after muscle stimulation in aged animals.

7.2.2 The Adaptive Changes in SRF-Linked Molecules with Age

SRF is a ubiquitously expressed member of the MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor family, sharing a highly conserved DNA-binding/dimerization domain, which binds the core sequence of SRF/CArG boxes [CC (A/T)6 GG] as homodimers. SRF-dependent signaling plays a major role in a variety of physiological processes, including cell growth, migration, and cytoskeletal organization. Previous results obtained with specific SRF-knockout models by the Cre-LoxP system emphasize a crucial role for SRF in postnatal skeletal muscle growth and regeneration. More recently, Mokalled et al. [13] demonstrated that members of the myocardin family of transcriptional coactivators, MASTR and myocardin-related transcription factor (MRTF)-A, are up-regulated in satellite cells in response to skeletal muscle injury. In addition, double-knockout satellite cells (MASTR and MRTF-A) impair skeletal muscle regeneration. As proposed by Mokalled et al. [13], the promoting role on muscle regeneration seems to be attributable to both MASTR/myocyte enhancer factor 2 and/or MRTF-A/SRF complexes. In addition, Guerci et al. [14] investigated the functional role of SRF in fiber hypertrophy using $SRF^{flox/flox}$:HAS-Cre-ER^{T2} mice injected with tamoxifen. Guerci et al. [14] showed that the selective lack of SRF in myofibers markedly slows fiber growth after mechanical overloading by modulating the activity of satellite cells. Guerci's hypothesis indicated that IL-4 produced by muscle fibers moves into satellite cells paracrinally to modulate the fusion of satellite cells.

It is proposed that the transcriptional activity of SRF is regulated by muscle ring finger (MuRF) 2 and striated muscle activators of Rho signalng (STARS). At the M-band, the mechanically modulated kinase domain of titin interacts with a complex of the protein products of the atrogenes NBR1, p62/SQSTM-1, and MuRFs. This complex dissociates under mechanical arrest, and MuRF1 and MuRF2 translocate to the cytoplasm and the nucleus. On the other hand, SRF activity is exquisitely sensitive to the state of actin polymerization. G-actin monomers inhibit SRF activity, whereas polymerization of actin occurs in response to serum stimulation and RhoA signaling. In this pathway, signal inputs lower the ratio of globular actin to fibrillar actin, thereby liberating the binding of MRTF-A to globular actin, resulting in the nuclear accumulation of MRTF-A and the subsequent SRF-dependent gene expression.

Mechanical loading for skeletal muscle is widely accepted to determine SRF expression. In humans, Lamon et al. [15] demonstrated that 8 weeks of resistance training (leg presses, squats, and leg extensions) induced increases in SRF mRNA (3-fold) and nuclear protein (1.25-fold) in the vastus lateralis muscle. In the same training period, they also observed a similar increase in the mRNA levels of several SRF-targeted molecules [alpha-actin, myosin heavy chain IIa, and insulin-like growth factor-I (IGF-I)]. Using RT-PCR, crude and fractionated homogenates, and immunofluorescence, our study demonstrated blunted expression of SRF protein in the quadridceps and triceps brachii muscles in aged mice [16]. Immunofluorescence microscopy also indicated the selective down-regulation of SRF immunoreactivity in the cell cytosol but not in Pax7-labeled satellite cells in sarcopenic mice. In addition, our data showed a decrease in MRTF-A mRNA (50–70 %) and protein (76 %) levels in only the nuclear fraction with age. Furthermore, 60 and 40 % decreases in the amount of STARS mRNA were observed in the quadriceps and triceps brachii of 24-month-old mice, respectively [16]. Figure 7.1 shows a schematic diagram of possible SRF-dependent signaling in young and sarcopenic muscle.

7.2.3 The Adaptive Changes of Ubiquitin-Proteasome System (UPS) in Sarcopenic Muscle

The ATP-dependent UPS is essential for regulating protein degradation. The degradation of a protein via the UPS involves two steps: (1) tagging of the substrate by covalent attachment of multiple ubiquitin molecules and (2) degradation of the tagged protein by the 26S proteasome complex with the release of a free and reusable ubiquitin. The ubiquitination of proteins is regulated by at least three enzymes: ubiquitin-activating enzyme (E1); ubiquitin-conjugating enzyme (E2); and ubiquitin ligase (E3). Consistent increases in two important E3 ubiquitin ligases (atrogin-1 and MuRF1) gene expression have been observed in a wide range of in vivo models of skeletal muscle atrophy including diabetes, cancer, renal failure,

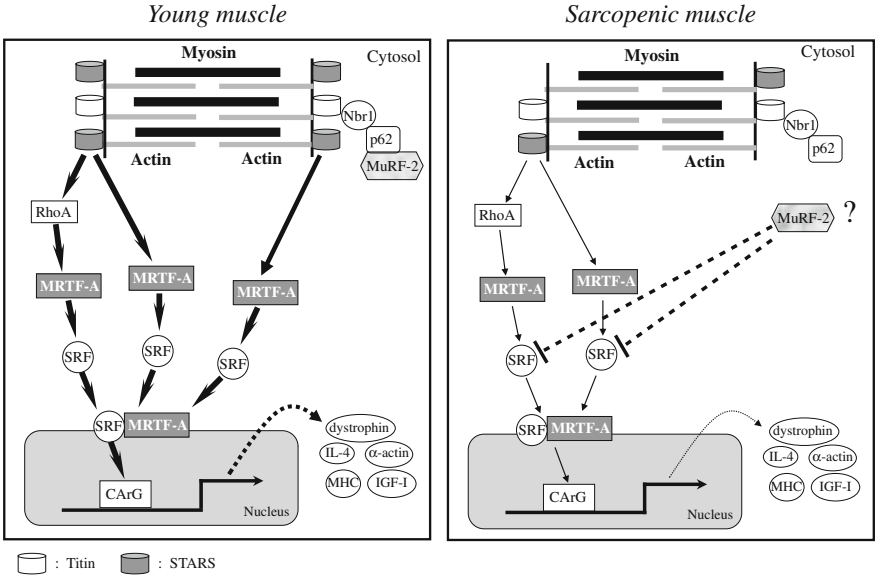

Fig. 7.1 Schematic diagram of SRF-dependent signaling in young and sarcopenic muscle. Mechanical loading produced by muscle contraction causes myosin and actin to interact, which in turn activates STARS and titin. In young muscle, the abundant STARS protein activates MRTF-A indirectly via RhoA or directly. Activated MRTF-A binds to SRF to promote the expression of muscle-specific genes such as those for α-actin, dystrophin, IGF-I, IL-4, and myosin heavy chain. In contrast, the upregulated expression of muscle-specific genes seems to be abrogated by lower levels of STARS, MRTF-A, and SRF in sarcopenic muscle. The zinc-finger protein Nbr1 binds to both titin and p62 at the Nterminal PB1 domain. In young muscle, p62 binds to the ubiquitin ligase MuRF2, via a ubiquitin-associated domain at its C-terminus. With muscle wasting (immobilization, unloading, sarcopenia**?**), nuclear translocation of MuRF2, which is dissociated from p62, leads to a marked reduction in nuclear SRF and transcriptive repression of SRF-dependent genes. Data from Sakuma et al. [7]

denervation, unweighting, and glucocorticoid or cytokine treatment [17]. The importance of these atrophy-regulated genes in muscle wasting was confirmed through knockout studies in mice where an absence of atrogin-1 or MuRF1 attenuated denervation-, fasting-, and dexamethasone-induced muscle atrophy [17].

Only very indirect measurements (small increases in levels of mRNA encoding some components of the UPS or ubiquitin-conjugate accumulation) in old muscles of rodents or humans suggested modest activation of this pathway. Atrogin-1 and/or MuRF1 mRNA levels in aged muscle are reportedly increased or unchanged in humans and rats, or decreased in rats [3, 18]. Even when the mRNA expression of these atrogenes increased in sarcopenic muscles, this was very limited (1.5–2.5-fold) compared with that in other catabolic conditions (10-fold). Although various findings have been made regarding the mRNA levels of both ubiquitin ligases in aged mammalian muscle, the examination of protein levels in sarcopenic muscles did not support age-related increases in the mRNA of several ubiquitin ligases. For instance,

Edström et al. [18] indicated the marked upregulation of phosphorylated Akt and FOXO4 in the gastrocnemus muscle of aged female rats, probably contributing to the downregulation of atrogin-1 and MuRF1 mRNA. This result is further supported by the more recent finding of Léger et al. [10] who, using human subjects aged 70 years old, demonstrated decreases in nuclear FOXO1 and FOXO3a by 73 and 50 %, respectively, although they did not recognize significant age-dependent changes in the expression of atrogin-1 and MuRF1 mRNA. The major peptidase activities of the proteasome (i.e., the chymotrypsin-like, trypsin-like, and caspase-like activities) were always reduced (as reported in other tissues) or unchanged with aging [3]. Interestingly, recent findings indicate that atrogin-1-knockout mice are short-lived and experience higher loss of muscle mass during aging than control mice [19], indicating that the activity of this E3 ubiquitin ligase is required to preserve muscle mass during aging in mice. Moreover, MuRF1-null mice experience higher decay of muscle strength during aging than controls, although muscle mass is at least in part preserved in these mice [20]. As indicated by Sandri et al. [19], chronic inhibition of these atrogenes should not be considered a therapeutic target to counteract sarcopenia because this does not prevent muscle loss but instead exacerbates weakness.

7.2.4 Adaptation of Autophagy-Linked Signaling in Muscle with Age

Macroautophagy (herein autophagy) occurs in all eukaryotic cells and is evolutionarily conserved from yeast to humans. Autophagy is a ubiquitous catabolic process that involves the bulk degradation of cytoplasmic components through a lysosomal pathway [21]. This process is characterized by the engulfment of part of the cytoplasm inside double-membrane vesicles called autophagosomes. Autophagosomes subsequently fuse with lysosomes to form autophagolysosomes in which the cytoplasmic cargo is degraded, and the degradation products are recycled for the synthesis of new molecules. Turnover of most long-lived proteins, macromolecules, biological membranes, and whole organelles, including mitochondria, ribosomes, the endoplasmic reticulum, and peroxisomes, is mediated by autophagy.

At first glance, autophagy was considered a coarse, nonselective, degradative system, but closer investigation revealed a different truth. Autophagy represents an extremely refined collector of altered organelles, abnormal protein aggregates, and pathogens, similar to a selective recycling center rather than a general landfill. The selectivity of the autophagy process is conferred by a growing number of specific cargo receptors, including p62/SQSTM1, Nbr1, Nix (Bnip3L), and optineurin. These adaptor proteins are equipped with both a cargo-binding domain, with the capability to recognize and attach directly to molecular tags on organelles, and at the same time an LC3-interacting region domain, able to recruit and bind essential autophagosome membrane proteins. De novo formation of autophagosomes is regulated by at least three molecular complexes: the LC3 conjugation system and

the regulatory complexes governed by unc51-like kinase-1 and Beclin-1. The conjugation complex is composed of different proteins encoded by autophagy-related genes (Atg). The Atg12-Atg5-Atg16L1 complex, along with Atg7, plays an essential role in the conjugation of LC3 to phosphatidylethanolamine, which is required for the elongation and closure of the isolation membrane.

A decline in autophagy during normal aging has been described for invertebrates and higher organisms. Inefficient autophagy has been attributed a major role in the apparent age-related accumulation of damaged mitochondria. Demontis and Perrimon [22] showed that the function of autophagy/lysosome system of protein degradation declined during aging in the skeletal muscle of *Drosophila*. This results in the progressive accumulation of poly-ubiquitin protein aggregates in senescent *Drosophila* muscle. Intriguingly, overexpression of the FOXO increases the expression of many autophagy genes, preserves the function of the autophagy pathway, and prevents the accumulation of poly-ubiquitin protein aggregates in sarcopenic *Drosophila* muscle [22]. Several investigators reported autophagic changes in aged mammalian skeletal muscle [5, 23, 24]. Compared with those in young male Fischer 344 rats, amounts of Beclin-1 were significantly increased in the plantaris muscles of senescent rats [5]. In contrast, aging did not influence the amounts of Atg7 and Atg9 proteins in rat plantaris muscle [5]. Indeed, Western blot analysis by Wohlgemuth et al. [5] clearly showed a marked increase in the amount of LC3 in muscle during aging. However, they could not demonstrate an aging-related increase of the ratio of LC3-II to LC3-I, a better biochemical marker to assess ongoing autophagy. In contrast, Wenz et al. [24] recognized a significant increase in the ratio of LC3-II to LC3-I during aging (3 versus 22 months) in the biceps femoris muscle of wild-type mice. None of the studies determining the transcript level of autophagy-linked molecules found a significant increase with age [5, 23]. Not all contributors to autophagy signaling seem to change similarly at both mRNA and protein levels in senescent skeletal muscle. Therefore, sarcopenia may include a partial defect of autophagy signaling, although more exhaustive investigation is needed in this field.

Life-long caloric restriction (CR) alone, or combined with voluntary exercise, resulted in mild reduction of LC3 expression and lipidation coupled with increased LAMP-2 (lysosomal marker) expression, suggesting a potential increase in autophagy flux. No significant age-related increase in autophagy-linked molecules was observed in MCK- peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) mice. PGC-1α may also enhance autophagic flux. More recently, GSK-3α was proposed as a critical regulator of aging in various organs (skeletal muscle, heart, liver, bone, etc.) via modulating mTORC1 and autophagy. Intriguingly, mice with null mutation of GSK-3α showed premature death and acceleration of age-related pathologies such as vacuolar degeneration, large tubular aggregates, sarcomere disruption, and striking sarcopenia in cardiac and skeletal muscle [6]. These GSK-3α KO mice exhibited marked activation of mTORC1 and associated suppression of several autophagy molecules. Indeed, unrestrained activation of mTORC1 leads to profound inhibition of autophagy. Therefore, it is expected that pharmacological inhibition (everolimus) of mTORC1 rescued the muscular disorder

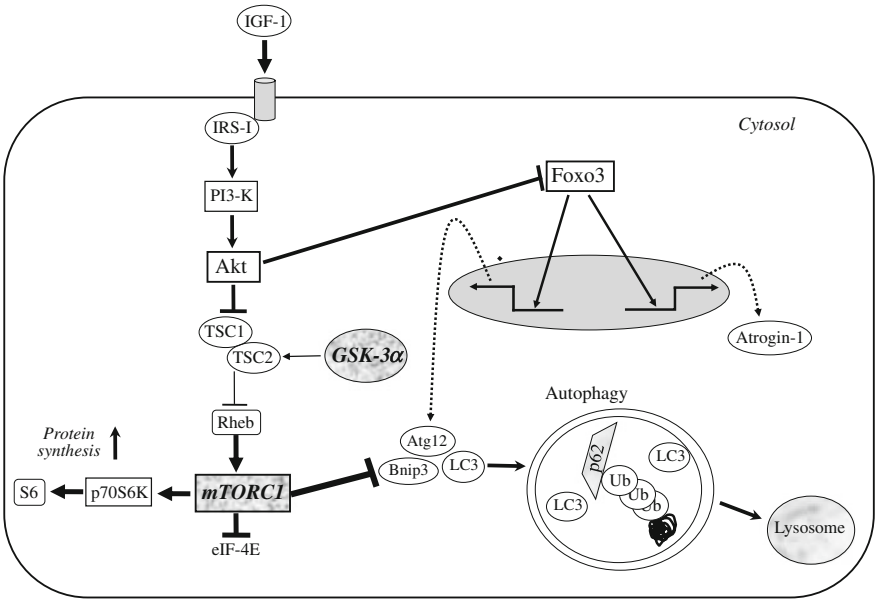

Fig. 7.2 The relationship between PI3-K-Akt-mTOR signaling and autophagy in young and sarcopenic muscle. The major anabolic pathway regulating protein synthesis in skeletal muscle is mTOR/TORC1 signaling. Upstream trigger (IGF-1) activates mTOR signaling through a number of different intermediary proteins such as IRS-I, Akt, and Ras homolog enriched in brain (Rheb). Less anabolic stimulation reduces the amount of activated Akt, which does not block the nuclear translocation of FOXO3 to enhance the expression of autophagy-related genes (Bnip, LC3, Atg12) and atrogin-1, and the consequent protein degradation. In contrast, mTORC1 can inhibit autophagy-related molecules not through Foxo3. In young muscle, abundant GSK-3α protein modulates the activity of mTORC1 by enhancing TSC2. In sarcopenic muscle, lower GSK-3α protein does not activate TSC2 and therefore hyperactivates Rheb and mTORC1. Unnecessary activated mTORC1 would extremely enhance protein synthesis and block autophagy-dependent signaling. Data from Sakuma et al. [7]

resembling sarcopenia in GSK-3α KO mice [6]. Enhancement of autophagy flux (exercise, CR, etc.) would be a potential strategy attenuating sarcopenia as well as various type of muscular dystrophy with autophagy defect [25, 26]. Figure 7.2 shows a schematic diagram of the possible relationship between PI3-K-Akt-mTOR signaling and autophagy including GSK-3α in skeletal muscle. Figure 7.2 shows a schematic diagram of the possible relationship between PI3-K-Akt-mTOR signaling and autophagy including GSK-3α in skeletal muscle.

7.2.5 Myostatin

Myostatin was first discovered during screening for novel members of the transforming growth factor-β superfamily, and shown to be a potent negative regulator

of muscle growth. Mutations in myostatin can lead to massive hypertrophy and/or hyperplasia in developing animals, as evidenced by KO experiments in mice. Moreover, mouse skeletal muscles engineered to overexpress the myostatin propeptide, the naturally occurring myostatin inhibitor follistatin, or a dominant-negative form of activin receptor IIB (ActRIIB: the main myostatin receptor) all display similar, if not greater, increase in size.

Myostatin levels increase with muscle atrophy due to unloading in mice and humans [3], and with severe muscle wasting in HIV patients. The increased levels of myostatin are widely accepted to lead to muscle wasting [27]. Although many researchers consider myostatin levels to increase with age, studies using sarcopenic muscles have yielded conflicting results such as in studies [7, 10, 28]. Intriguingly, Carlson et al. [28] showed enhanced levels of Smad3 (possible myostatin-downstream regulator) but not myostatin in sarcopenic muscles of mice. More recently, McKay et al. [4] observed more abundant myostatin-positive type II-associated stem cells in older than in younger males after muscle loading in spite of no difference in stem cell-specific myostatin levels at baseline. Therefore, it is possible that myostatin-dependent signaling is activated in sarcopenic mammalian muscles.

7.3 Therapeutic Strategy Attenuating Sarcopenia

7.3.1 Exercise (Resistance Training)

One resistance exercise bout can, within 1 h, increase muscle protein synthesis, which can last up to 72 h after exercise. Resistance training has shown the most promise among interventions aimed to decrease the effects of sarcopenia, as it enhances strength, power, and mobility function and induces varying degrees of skeletal muscle hypertrophy [29]. For example, 12 weeks of whole-body resistance training resulted in an increase in type II muscle fiber area in men aged 64–86 years and 65–72 years. A 2-year longitudinal trial of resistance training found increases in leg press (32 %) and military press (90 %) 1 repetition maximum and knee extensor muscle cross-sectional area (9 %) in 60–80-year-old men and women [30]. The functional benefits of resistance training have been evaluated in a large-scale trial of 72–98-year olds and frail nursing home residents, with resistance training increasing muscle strength (113 %), gait velocity (12 %), stair-climbing power (28 %), and spontaneous physical activity [29]. In addition, 6 weeks of training for elderly persons (68.4 + 5.4 years) improved their physical activity profile (6-min walk, 30-s chair stand, chair sit and reach and back scratch) as well as muscle strength.

In the elderly, resistance training induces the muscle expression of IGF-I, myogenic regulatory factors, and IL-6, which contribute to muscular hypertrophy by regulating the activation, proliferation, and differentiation of satellite cells. One bout of resistance exercise for elders can enhance the rate of synthesis of muscle

protein. However, several studies using humans and rodents indicated a lower degree of activation in mitogen-activated protein kinase and Akt-mTOR pathways after muscle contraction or mechanical overload than occurs in young adults [31]. More recently, Mayhew et al. [32] indicated that one bout of resistance exercise elicited a similar extent of activation in translational signaling [Akt, p70S6K, ribosomal protein S6, and 4E-BP1] between young and old subjects. In contrast, physical activity can affect muscle inflammation. Recent evidence shows that chronic resistance physical training contributes to the control of locally-derived inflammation through adaptations to repeated and acute increases in proinflammatory mRNA within muscle. Several studies [33] have shown that excess intensive strength training for the elderly impairs the effective gain of muscle strength and mass particularly in women. Therefore, careful attention should be paid when determining the amount and frequency of resistance training for the elderly.

7.3.2 Supplemental Approach

7.3.2.1 Amino Acid Supplementation

Many Americans consume more than the recommended dietary allowance (RDA) of protein; however, research shows that a significant number of elderly people do not meet the estimated average requirement, let alone the RDA; 32–41 % of women and 22–38 % of men aged 50 and older consume less than the RDA of protein. Epidemiological studies show that protein intake is positively associated with preservation of muscle mass. For example, in a recent study, 38 healthy, normal-weight, sedentary women aged 57–75 were recruited to determine whether a higher muscle mass index was associated with animal or vegetal protein intake.

Many reviews indicate that certain nutritional interventions such as a high protein intake or an increased intake of essential amino acids and the branched chain amino acid (BCAA) leucine with resistance training may help to attenuate fiber atrophy in sarcopenic muscle by the modulation of both anabolic and catabolic pathways [34]. In particular, leucine can be considered a regulatory amino acid with unique characteristics. It plays several roles in muscle metabolism regulation, which includes translational control of protein synthesis and glucose homeostasis. In addition, leucine has been demonstrated to be a nitrogen donor for the synthesis of muscle alanine and glutamine. Considering these findings, the use of leucine as an anti-atrophic agent is biologically justified.

It has been documented that oral post-exercise amino acid supplementation had a synergistic effect on the contraction-induced escalation of muscle protein synthesis following an acute resistance exercise bout [35]. Treatment with amino acids has been shown to induce additive hypertrophy in response to continuous resistance training [36]. Recent human studies have shown that amino acids play a role in the phosphorylation of translational factors called eularyotic initiation factors, especially eIF4F and p70S6K, through an mTOR-mediated mechanism [37]. On the

other hand, several studies have not found benefits from protein supplementation [38, 39]. These studies utilized a single bout or short-term (10 days) ingestion to examine the rate of myofibrillar synthesis or protein synthesis [38]. In contrast, Godard et al. [39] tried to investigate the long-term supplementation of several amino acids and carbohydrate with resistance training. Unfortunately, they conducted the examination of total muscle cross-sectional areas by only magnetic resonance imaging, and did not perform a detailed morphological analysis (cross-sectional area of muscle fiber by biopsy sample). Since the evaluation of muscle cross-sectional area by magnetic resonance imaging appears to be influenced by the inner amount of adipose tissue, connective tissue, or water, it is unknown whether their protein supplementation actually failed to elicit positive effects on the morphometry of muscle fiber.

More recently, the administration of many essential amino acids tends to achieve a positive effect on muscle mass and protein synthesis both under normal conditions [2, 3, 40] and with resistance training [37]. Although a positive attenuating effect on sarcopenia has been observed in almost all studies utilizing many essential amino acids plus a high amount of leucine, supplementation with essential amino acids not enriched with leucine may fail to enhance muscle protein synthesis in the elderly. In addition, a higher amount of leucine should be supplemented along with large amounts of isoleucine and valine in order to avoid an imbalance of BCAA levels, as pointed out by Nicastro et al. [40] in their review.

7.3.2.2 Ursolic Acid

A water-insoluble pentacylic triterpenoid, ursolic acid is the major waxy component in apple peels. It is also found in many other edible plants. Interestingly, because it exerts beneficial effects in animal models of diabetes and hyperlipidemia [41], ursolic acid is thought to be the active component in a variety of folkloric antidiabetic herbal medicines [41]. As predicted by connectivity mapping, Kunkel et al. [42] found that ursolic acid reduced skeletal muscle atrophy in the setting of two-distinct atrophy-inducing stresses (fasting and muscle denervation). A major strength of the connectivity map is that it takes into account positive and negative changes in mRNA expression that together constitute an authentic mRNA expression signature. Thus, by querying the connective map with signatures of muscle atrophy, Kunkel et al. [42] were, in effect, querying with the reciprocal signature of muscle atrophy but also induced muscle hypertrophy.

Ursolic acid might increase muscle mass by inhibiting atrophy-associated skeletal muscle gene expression. Indeed, Kunkel et al. [42] found that acute ursolic acid treatment of fasted mice reduced atrogin-1 and MuRF1 mRNA levels in association with reduced muscle atrophy. Similarly, chronic ursolic acid treatment of unstressed mice reduced atrogin-1 and MuRF1 expression and induced muscle hypertrophy. Although ursolic acid increased skeletal muscle Akt phosphorylation in vivo, the experiments could not determine if it acted directly on skeletal muscle, how quickly it acted, and if the effect required IGF-I or insulin, which are always

present in healthy animals, even during fasting. To address these issues, Kunkel et al. [42] studied serum-starved skeletal myotubes and found that ursolic acid rapidly stimulated IGF-I receptor and insulin receptor activity, but only if IGF-I or insulin was also present. Taken together, their data suggest that ursolic acid first enhances the capacity of pre-existing IGF-I and insulin to activate skeletal muscle IGF-I receptors and insulin receptors, respectively. Importantly, ursolic acid alone was not sufficient to increase phosphorylation of the IGF-I receptor or insulin receptor. Rather, its effects also required IGF-I and insulin, respectively. This suggests that ursolic acid either facilitates hormone-mediated receptor autophosphorylation or inhibits receptor dephosphorylation. The latter possibility is supported by previous in vitro data showing that ursolic acid directly inhibits protein tyrosine phosphatase 1B, a tyrosine phosphatase that dephosphorylates (inactivates) the IGF-I and insulin receptors. Further research is needed to elucidate the effect of supplementation with ursolic acid in skeletal muscle and to attenuate muscle wasting (e.g., sarcopenia).

7.3.2.3 Antioxidant Supplementation

Free radicals are a highly reactive chemical species with a single unpaired electron in its outer orbit seeking to pair with another free electron. In particular, reactive oxygen species (ROS), deriving from oxidative metabolism, have higher reactivity than O_2. ROS are constantly generated in cells of aerobic organisms, in particular skeletal muscle, by the addition of a single electron to the oxygen molecule with subsequently damage of biological macromolecules (e.g., lipids). The interaction of ROS with normal cellular structures leads to potentially nonreversible modifications, with consequent cellular loss of function and death. ROS production has been shown to increase in skeletal muscle during aging. During the aging process, it is probable that increased levels of ROS lead to the modification of mitochondrial DNA and result in increases in myonuclear apoptosis.

In the case of investigations of diabetes, antioxidant supplementation seems to effectively prevent muscle atrophy [43]. The effect on cancer cachexia is partial although significant. In contrast, the data on antioxidant supplementation for mammalian sarcopenia are extremely limited and controversial, despite the clinical relevance and large interest (both from research and commercial points of view). Several studies have investigated the possibility of delaying the aging process by enhancing antioxidative capacity. For example, resveratrol, a natural polyphenol found in grapes, peanuts, and berries, has shown a protective effect against oxidative stress in skeletal muscle. Although most human studies analyze the relationship between dietary antioxidant supplementation and physical performance or muscle strength measures, the effect is still and largely controversial. As pointed out by a more recent review [44], there are currently no trials verifying the effects of antioxidant supplementation on sarcopenia (as identified by one of several the consensus definitions provided by international groups of experts). As proposed by Bonetto et al. [43], oxidative stress probably would behave as an additional factor

that would certainly amplify the wasting stimuli, but probably would not play a leading role in many other cases, and for which the effectiveness of antioxidant therapy was not demonstrated. Very intriguingly, a recent statement from the Society on Sarcopenia, Cachexia, and Wasting Disease does not mention antioxidant supplementation as a possible tool to manage sarcopenia in older persons [45].

7.3.3 Hormonal Supplementation

7.3.3.1 Testosterone

In males, levels of testosterone decrease by 1 % per year, and those of bioavailable testosterone by 2 % per year from age 30. In women, testosterone levels drop rapidly between the ages of 20 and 45. Testosterone increases muscle protein synthesis and its effects on muscle are modulated by several factors including genetic background, nutrition and exercise. Systemic reviews of the literature [46] have concluded that testosterone supplementation attenuates several sarcopenic symptoms including decreases in muscle mass and grip strength. For instance, a recent study of 6 months of supraphysiological dosage of testosterone in a randomized placebo-controlled trial reported increased leg lean body mass and leg and arm strength [47]. Although there are significant increases in strength among elderly males given high doses of testosterone, the potential risks may outweigh the benefits. Risks associated with testosterone therapy in older men include sleep apnea, thrombotic complications, and the increased risk of prostate cancer.

These side effects have driven the necessity for drugs that demonstrate improved therapeutic profiles. Novel, non-steroidal compounds, called selective androgen receptor modulators, have shown tissue-selective activity and improved pharmacokinetic properties. Whether these drugs are effective in treating sarcopenia has yet to be shown. Dehydroepiandrosterone (DHEA) is marketed as a nutritional supplement in the USA and is available over the counter. Unlike testosterone and estrogen, DHEA is a hormone precursor that is converted into sex hormones in specific target tissues. However, supplementation of DHEA in aged men and women results in an increase in bone density and testosterone and estradiol levels, but results in no changes in muscle size, strength, or function.

7.3.3.2 Ghrelin

Ghrelin, is a 28-amino acid peptide mainly produced by cells in the stomach, intestines, and hypothalamus. Ghrelin is a natural ligand for the growth hormone (GH)-secretagogue receptor that possesses a unique fatty acid modification, an n-octanoylation at Ser 3. Ghrelin plays a critical role in a variety of physiological processes, including the stimulation of GH secretion and regulation of energy

homeostasis by stimulating food intake and promoting adiposity via a GH-independent mechanism. In contrast, ghrelin inhibits the production of anorectic proinflammatory cytokines, including IL-1β, IL-6 and tumor necrosis factor (TNF)-α. Because of their combined anabolic effects on skeletal muscle and appetite, ghrelin and low molecular weight agonists of the ghrelin receptor are considered attractive candidates for the treatment of cachexia. For example, Nagaya et al. [48] gave human ghrelin (2 μg/Kg twice daily intravenously) for 3 weeks to cachexic patients with chronic obstructive pulmonary disease in an open-label study. After ghrelin therapy, significant increases from baseline measurements were observed for body weight, lean body mass, food intake, hand grip strength, maximal inspiratory pressure, and the Karnofsky performance score [48]. In another unblinded study, the same group demonstrated that treatment with human ghrelin (2 μg/Kg intravenously, twice daily for 3 weeks) significantly improved several parameters (e.g., lean body mass measured by Dual-energy X-ray Absorption and left ventricular ejection fraction) in 10 patients with chronic heart failure [49]. In a 1-year placebo-controlled study in healthy older adults over the age of 60 years given an oral ghrelin-mimetic (MK-677), an increase in appetite was observed [50]. The study did not show a significant increase in strength or function in the ghrelin-mimetic treatment group, when compared to the placebo group, however, a tendency was observed [50]. As pointed out in a recent review by Nass et al. [51], the use of this compound induces the potential deterioration of insulin sensitivity and development of diabetes mellitus in older adults with impaired glucose tolerance.

7.3.4 Pharmacological Approach

7.3.4.1 Myostatin Inhibition

Many researchers have conducted experiments to inhibit myostatin in models of muscle disorders such as Duchenne muscular dystrophy (DMD), amyotrophic lateral sclerosis, and cancer cachexia [3]. In addition, several investigators examined the effect of inhibiting myostatin to counteract sarcopenia using animals only. Lebrasseur et al. [52] reported several positive effects of 4 weeks of treatment with PF-354 (24 mg/Kg), a drug for myostatin inhibition, in aged mice. They showed that PF-354-treated mice exhibited significantly greater muscle mass (by 12 %), and increased performance such as treadmill time, distance to exhaustion, and habitual activity. Furthermore, PF-354-treated mice exhibited decreased levels of phosphorylated Smad3 and MuRF1 in aged muscle. More recently, Murphy et al. [53] showed, by way of once weekly injections, that a lower dose of PF-354 (10 mg/Kg) significantly increased the fiber cross-sectional area (by 12 %) and in situ force of tibialis anterior muscles (by 35 %) of aged mice (21-month-old). Blocking myostatin enhances muscle protein synthesis by potentially relieving the inhibition normally imposed on the Akt/mTOR signaling pathway by myostatin. These lines of evidence clearly highlight the therapeutic potential of antibody-directed inhibition of

myostatin for treating sarcopenia. However careful attention should be paid to the myostatin inhibition, as mice with null mutation of myostatin revealed to have impaired tendon structure and function.

7.3.4.2 Angiotensin-Converting Enzyme (ACE) Inhibitors

ACE inhibitors have long been used as a treatment in primary and secondary prevention in cardiovascular disease as well as secondary stroke prevention. It has now been suggested that ACE inhibitors may have a beneficial effect on skeletal muscle. ACE inhibitors may improve muscle function through improvements in endothelial function, metabolic function, anti-inflammatory effects, and angiogenesis thereby improving skeletal muscle blood flow. ACE inhibitors can increase mitochondrial numbers and IGF-I levels thereby helping to counter sarcopenia.

Observational studies have shown that the long-term use of ACE inhibitors was associated with a lower decline in muscle strength and walking speed in older hypertensive people and a greater lower limb lean muscle mass when compared with users of other antihypertensive agents. Several studies have shown that ACE inhibitors improved exercise capacity in both younger and older people with heart failure, but caused no improvement in grip strength. Few interventional studies using ACE inhibitors for physical function have been undertaken. One study looking at functionally impaired older people without heart failure has shown that ACE inhibitors increase 6-minute walking distance to a degree comparable to that achieved after 6 months of exercise training [54]. However, a study comparing the effects of nifedipine with ACE inhibitors in older people found no difference between treatments in muscle strength, walking distance, or functional performance [55]. It is possible that frailer subjects with slower walking speeds, who have a tendency to more cardiovascular problems, benefit more. Further evidence is required before recommending ACE inhibitors to counter the effects of sarcopenia. However, ACE inhibitors are associated with cardiovascular benefits and as older people frequently have underlying cardiovascular problems, these agents are already commonly prescribed.

7.3.4.3 Vitamin D

Vitamin D has been traditionally considered a key regulator of bone metabolism, and calcium and phosphorus homeostasis through negative feedback with the parathyroid hormone. Today, approximately 1 billion people, mostly elderly, worldwide have vitamin D deficiency. The prevalence of low vitamin D concentrations in subjects older than 65 years of age has been estimated at approximately 50 %, but this figure is highly variable because it is influenced by sociodemographic, clinical, therapeutic and environmental factors. Similarly, there is an age-dependent reduction found in vitamin D receptor expression in skeletal muscle. Prolonged vitamin D deficiency has been associated with severe muscle weakness, which improves with vitamin D supplementation.

A large body of evidence demonstrates that low vitamin D concentrations represent an independent risk factor for falls in the elderly [56]. Supplementation with vitamin D in double-blind randomized-controlled trials has been shown to increase muscle strength and performance and reduce the risk of falling in community-living elderly and nursing home residents with low vitamin D levels [57]. In contrast, several groups found no positive effect of vitamin D supplementation on fall event outcomes [58]. Cesari et al. [59] attributed these contradictory findings to the selection criteria adopted to recruit study populations, adherence to the intervention, or the extreme heterogeneity of cut-points defining the status of deficiency. A more comprehensive knowledge on vitamin D-related mechanisms may provide a very useful tool preventing muscle atrophy for older persons (sarcopenia). Figure 7.3 represents an overview of various effective strategies for attenuating sarcopenia.

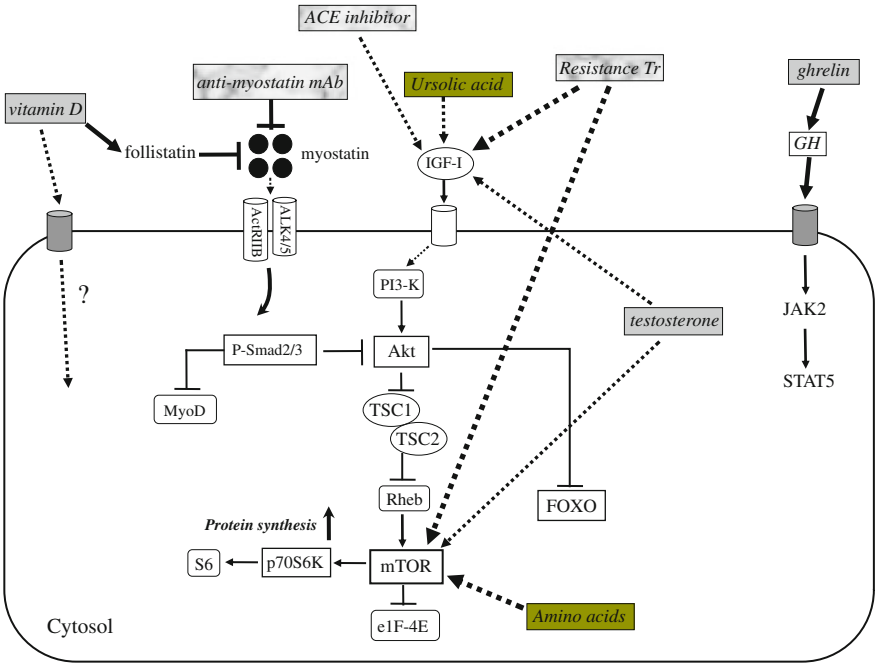

Fig. 7.3 Myostatin signals through the ActRIIB-ALK4/5 heterodimer activate Smad2/3 with blocking of MyoD transactivation in an autoregulatory feedback loop. Recent findings suggest that the myostatin-Smad pathway inhibits protein synthesis probably due to blocking the functional role of Akt. Treatment with an ACE inhibitor, ursolic acid, and testosterone upregulates the amount of IGF-I and then stimulates protein synthesis by activating the Akt/mTOR/p70S6K pathway. Resistance training also induces IGF-I expression and activates mTOR. In addition, supplementation with testosterone and amino acids enhances protein synthesis by stimulating mTOR. Abundant serum GH, which is induced by ghrelin, activates Janus kinase (JAK)2-signal transducer and activator of transcription (STAT)5 signaling to promote muscle-specific gene transcription necessary to hypertrophy. A recent finding [60] indicates that vitamin D enhances follistatin expression, and in turn blocks the functional role of myostatin in vitro. A direct role for vitamin D in muscle fiber remains to be elucidated

7.3.5 Another Candidate

7.3.5.1 Caloric Restriction (CR)

CR, which typically involves consuming 20–40 % fewer calories than normal, preserves mitochondrial health and attenuates sarcopenia. CR is recognized as the most robust intervention that retards both primary aging (natural age-related deterioration) and secondary aging (accelerated aging due to disease and negative lifestyle behaviors), thereby increasing lifespan in many species. While CR studies in primates and humans are largely ongoing, studies in rodents have consistently shown that CR extends maximum life span by up to 50 % and reduces the incidence of many age-associated diseases. These protective effects are likely attributable to the ability of CR to reduce the incidence of mitochondrial abnormalities (mitochondrial proton leakage) and attenuate oxidative stress. In rodents, CR appears to modulate mitochondrial efficiency, content, and function via decreased proton leakage, which is in turn is enabled by a shift to a less oxidative milieu. In terms of mitochondrial content and function, CR does not affect the gene expression, protein level, or activity of citrate synthase [61]. More recently, Lanza et al. [62] demonstrated that CR decreases whole-muscle protein synthesis and fractional synthesis rates of individual proteins in rodents. In addition, their analysis using representative transmission electron micrographs showed no attenuation of the reduction in mitochondrial density with aging. Taking these findings together, Lanza et al. [62] concluded that CR preserves mitochondrial function by protecting the integrity and function of existing cellular components rather than by increasing mitochondrial biogenesis. Furthermore, CR seems to counteract the age-related increases in proapoptotic signaling in skeletal muscle [63]. Noticeably, CR has been shown to modulate the majority of the apoptotic pathways involved in age-associated skeletal muscle loss, such as mitochondrion-, cytokine/receptor-, and Ca^{2+}/ER-stress-mediated signaling [63]. For instance, CR markedly inhibits increases in several mediators of the TNF-mediated pathway of apoptosis (TNF-α, TNF-receptor 1, cleaved caspase-3 and -8) possibly by enhancing production of a muscle-derived anabolic cytokine, IL-15, which competes with TNF-mediated signaling. In addition, the combination of CR with exercise training is proposed to counteract the apoptosis associated with sarcopenia more effectively.

How does CR modulate sarcopenia irrespective of mitochondrial function and apoptosis?

It is intriguing that many different studies have shown that PGC-1α is increased with CR in various organs such as brain, liver, heart, and brown and visceral adipose tissue [64]. Baker et al. showed a significant increase in PGC-1α in gastrocnemius muscle of rats after a 40 % CR diet beginning at 16 weeks of age [65]. It has become apparent that PGC-1α binds to and coactivates many transcription factors in addition to PPARγ, including most nuclear factors. Therefore, PGC-1α has various roles, such as in fatty acid oxidation, myokine secretion, the activation of autophagy, and neuromuscular junction (NMJ) gene induction, as well as

upregulation of mitochondrial biogenesis [66]. Indeed, Valdez et al. [67] demonstrated that lifelong CR significantly decreased the incidence of pre- and postsynaptic abnormalities (e.g., axonal swelling and synaptic detachment) in 24-month-old mice and the age-related loss of motor neurons probably due to PGC-1α induction. Since the level of basal autophagy in the skeletal muscle has been shown to be reduced with age [5, 31], normal function of autophagy by CR may attenuate the atrophy of muscle fiber with age. However, CR in mice did not modulate the level of several autophagy-linked molecules (Beclin-1, Atg9, LC3) at the protein level, except for Atg7 in sarcopenic muscles of rats [5]. It remains to be further elucidated whether CR activates autophagy signaling to inhibit muscle atrophy.

A more recent study [68] indicated that CR has no beneficial effect on health and survival in rhesus monkeys in contrast to many reports from studies using the same species [69, 70]. Further studies are needed to determine whether CR is effective in counteracting the age-related loss of muscle in human subjects and to what extent dietary intervention can be applied in human populations. Since excessive CR (over 50 %) may have a number of side effects (e.g., weakness, loss of stamina, osteoporosis, depression, anorexia nervosa, etc.), milder CR should be applied in the elderly. Figure 7.4 represents an overview of various adaptations in muscle fiber after CR.

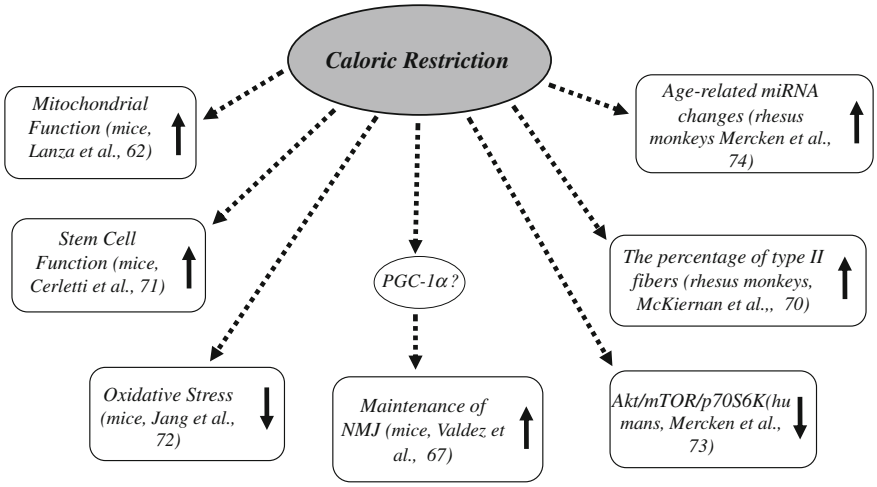

Fig. 7.4 Various adaptations by caloric restriction in mammalian skeletal muscle. CR affects multiple pathways and processes in the muscle fibers, as well as having systemic physiological effects [71–74]

7.4 Conclusions and Perspectives

The Akt/mTOR/p70S6K pathway and SRF-dependent signaling have been considered major contributors to protein synthesis and muscle-specific transcription, respectively.

Although studies using rodent muscles have indicated that atrogin-1 and MuRF contribute to the protein degradation in muscular wasting [7], these atrogenes would not regulate age-related muscle atrophy. In contrast, the fiber atrophy associated with sarcopenia as well as other muscle wasting diseases includes a marked autophagic defect and the activation of the myostatin-Smad pathway. Advances in our understanding of muscle biology have led to new approaches to the treatment of muscle wasting. For example, resistance training combined with amino acid containing supplements would be the best way to prevent age-related muscle wasting and weakness including sarcopenia. Supervised CR seems to possess a prominent counteracting role to muscle atrophy and mitochondrial dysfunction with age. Supplementation with ursolic acid and ghrelin are seemingly intriguing candidates in combating sarcopenia, although systematic and fundamental research into these treatments has not been conducted even in the rodent. These treatments will be tested in humans in the coming years and offer the possibility of treating sarcopenia/frailty.

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References

1. von Haehling S, Morley JE, Anker SD (2010) An overview of sarcopenia: facts and numbers on prevalence and clinical impact. J Cachexia Sarcopenia Muscle 1:129–133
2. Sakuma K, Yamaguchi A (2010) Molecular mechanisms in aging and current strategies to counteract sarcopenia. Curr Aging Sci 3:90–101
3. Sakuma K, Yamaguchi A (2011) Sarcopenia: molecular mechanisms and current therapeutic strategy. In: Perloft JW, Wong AH (eds) Cell aging. Nova Science Publisher, New York, pp 93–152
4. McKay BR, Ogborn DI, Bellamy LM, Tarnopolsky MA, Parise G (2012) Myostatin is associated with age-related human muscle stem cell dysfunction. FASEB J 25:2509–2521
5. Wohlgemuth SE, Seo AY, Marzetti E, Lees HA, Leeuwenburgh C (2010) Skeletal muscle autophagy and apoptosis during aging: effects of calorie restriction and life-long exercise. Exp Gerontol 45:138–148
6. Zhou J, Freeman TA, Ahmad F, Shang X, Mangano E, Gao E, Farber J, Wang Y, Ma XL, Woodgett J, Vagnozzi RJ, Lai H, Force T (2013) GSK-3α is a central regulator of age-related pathologies in mice. J Clin Invest 123:1821–1832
7. Sakuma K, Aoi W, Yamaguchi A (2015) Current understanding of sarcopenia: possible candidates modulating muscle mass. Pflügers Arch 467:213–229
8. Parkington JD, LeBrasseur NK, Siebert AP, Fielding RA (2004) Contraction-mediated mTOR, p70S6K, and ERK1/2 phosphorylation in aged skeletal muscle. J Appl Physiol 97:243–248

9. Kimball SR, O'Malley JP, Anthony JC, Crozier SJ, Jefferson LS (2004) Assessment of biomarkers of protein anabolism in skeletal muscle during the life span of the rat: sarcopenia despite elevated protein synthesis. Am J Physiol Endocrinol Metab 287:E772–E780
10. Léger B, Derave W, De Bock K, Hespel P, Russell AP (2008) Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation Res 11:163–175
11. Rahnert JA, Luo Q, Balog EM, Sokoloff AJ, Burkholder TJ (2011) Changes in growth-related kinases in head, neck and limb muscles with age. Exp Gerontol 46:282–291
12. Fry CS, Drummond MJ, Glynn EL, Dickinson JM, Gundermann DM, Timmerman KL, Walker DK, Dhanani S, Volpi E, Rasmussen BB (2011) Aging impairs contraction-induced human skeletal muscle mTORC1 signaling and protein synthesis. Skeletal Muscle 1:11
13. Mokalled MH, Johnson AN, Creemers EE, Olson EN (2012) MASTR directs MyoD-dependent satellite cell differentiation during skeletal muscle regeneration. Genes Dev 26:190–202
14. Guerci A, Lahoute C, Hébrard S, Collard L, Graindorge D, Favier M, Cagnard N, Batonnet-Pichon S, Précigout G, Garcia L, Tuil D, Daegelen D, Sotiropoulos A (2012) Srf-dependent paracrine signals produced by myofibers control satellite cell-mediated skeletal muscle hypertrophy. Cell Metab 15:25–37
15. Lamon S, Wallace MA, Léger B, Russell AP (2009) Regulation of STARS and its downstream targets suggest a novel pathway involved in human skeletal muscle hypertrophy and atrophy. J Physiol 587:1795–1803
16. Sakuma K, Akiho M, Nakashima H, Akima H, Yasuhara M (2008) Age-related reductions in expression of serum response factor and myocardin-related transcription factor a in mouse skeletal muscles. Biochim Biophys Acta Mol Basis Dis 1782:453–461
17. Bodine SC, Baehr LM (2014) Skeletal muscle atrophy and the E3 ubiquitin ligases, MuRF1 and MAFbx/atrogin-1. Am J Physiol Endocrinol Metab 5 Aug 2014. pii: ajpendo.00204.2014. [Epub ahead of print]
18. Edström E, Altun M, Hägglund M, Ulfhake B (2006) Atrogin-1/MAFbx and MuRF1 are downregulated in ageing-related loss of skeletal muscle. J Gerontol Series A Biol Sci Med Sci 61:663–674
19. Sandri M, Barberi L, Bijlsma AY, Blaauw B, Dyar KA, Milan G, Mammucari C, Meskers CG, Pallafacchina G, Paoli A, Pion D, Roceri M, Romanello V, Serrano AL, Toniolo L, Larsson L, Maier AB, Muñoz-Cánoves P, Musarò A, Pende M, Reggiani C, Rizzuto R, Schiaffino S (2013) Signaling pathways regulating muscle mass in ageing skeletal muscle. The role of IGF-1-Akt-mTOR-FoxO pathway. Biogerontology 14:303–323
20. Hwee DT, Baehr LM, Philp A, Baar K, Bodine SC (2014) Maintenance of muscle mass and load-induced growth in Muscle RING Finger 1 null mice with age. Aging Cell 13:92–101
21. Neel BA, Lin Y, Pessin JE (2013) Skeletal muscle autophagy: a new metabolic regulator. Trends Endocrinol Metabol 24:635–643
22. Demontis F, Perrimon N (2010) FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. Cell 143:813–825
23. McMullen CA, Ferry AL, Gamboa JL, Andrade FH, Dupont-Versteegden EE (2009) Age-related changes of cell death pathways in rat extraocular muscle. Exp Gerontol 44:420–425
24. Wenz T, Rossi SG, Rotundo RL, Spiegelman BM, Moraes CT (2009) Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging. Proc Natl Acad Sci USA 106:20405–20410
25. De Palma C, Morisi F, Cheli S, Pambianco S, Cappello V, Vezzoli M, Rovere-Querini P, Moggio M, Ripolone M, Francolini M, Sandri M, Clementi E (2012) Autophagy as a new therapeutic target in Duchenne muscular dystrophy. Cell Death Dis 3:e418
26. Vainshtein A, Grumati P, Sandri M, Bonaldo P (2014) Sakeletal muscle, autophagy, and physical activity: the ménage á trois of metabolic regulation in health and disease. J Mol Med 92:127–137
27. Sakuma K, Yamaguchi A (2012) Sarcopenia and cachexia: the adaptations of negative regulators of skeletal muscle mass. J Cachexia Sarcopenia Muscle 3:77–94

28. Carlson ME, Hsu M, Conboy IM (2008) Imbalance between pSmad3 and Notch induces CDK inhibitors is old muscle stem cells. Nature 454:528–532
29. Fiatarone MA, O'Neil EF, Ryan ND, Clements KM, Solares GR, Nelson ME, Roberts SB, Kehayias JJ, Lipsitz LA, Evans WJ (1994) Exercise training and nutritional supplementation for physical frailty in very elderly people. N Engl J Med 330:1769–1775
30. McCartney N, Hicks AL, Martin J, Webber CE (1996) A longituidinal trial of weight training in the elderly: continued improvements in year 2. J Gerontol A Biol Sci Med Sci 51:B425–B433
31. Sakuma K, Aoi W, Yamaguchi A (2014) The intriguing regulators of muscle mass in sarcopenia and muscular dystrophy. Front Aging Neurosci 6:230
32. Mayhew DL, Kim JS, Cross JM, Bamman MM (2009) Translational signaling responses preceding resistance training-mediated myofibers hypertrophy in young and old humans. J Appl Physiol 107:1655–1662
33. Kosek DJ, Kim JS, Petrella JK, Cross JM, Bamman MM (2006) Efficacy of 3 day/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. J Appl Physiol 101:531–544
34. Timmerman KL, Volpi E (2008) Amino acid metabolism and regulatory effects in aging. Curr Opin Clin Nutr Metab Care 11:45–49
35. Walker DK, Dickinson JM, Timmerman KL, Drummond MJ, Reidy PT, Fry CS, Gundermann DM, Rasmussen BB (2011) Exercise, amino acids, and aging in the control of human muscle protein synthesis. Med Sci Sports Exerc 43:2249–2258
36. Esmarck B, Andersen JL, Olsen S, Richter EA, Mizuno M, Kjaer M (2005) Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. J Physiol 567:301–311
37. Drummond MJ, Dreyer HC, Pennings B, Fry CS, Dhanani S, Dillon EL, Sheffield-Moore M, Volpi E, Rasmussen BB (2008) Skeletal muscle protein anabolic response to resistance exercise and essential amino acids is delayed with aging. J Appl Physiol 104:1452–1461
38. Walrand S, Short KR, Bigelow ML, Sweatt AJ, Hutson SM, Nair KS (2008) Functional impact of high protein intake on healthy elderly people. Am J Physiol Endocrinol Metab 295: E921–E928
39. Godard MP, Williamson DL, Trappe SW (2002) Oral amino-acid provision does not affect muscle strength or size gains in older men. Med Sci Sports Exerc 34:1126–1131
40. Nicastro H, Artioli GG, Dos Santos Costa A, Sollis MY, Da Luz CR, Blachier F Lancha AH Jr (2011) An overview of the therapeutic effects of leucine supplementation on skeletal muscle under atrophic conditions. Amino Acids 40:287–300
41. Wang ZH, Hsu CC, Huang CN, Yin MC (2009) Anti-glycative effects of oleanolic acid and ursolic acid in kidney of diabetic mice. Eur J Pharmacol 628:255–260
42. Kunkel SD, Suneja M, Ebert SM, Bongers KS, Fox DK, Malmberg SE, Alipour F, Shields RK, Adams CM (2011) mRNA expression signatures of human skeletal muscle atrophy identify a natural compound that increases muscle mass. Cell Metab 13:627–638
43. Bonetto A, Penna F, Muscaritoli M, Minero VG, Fanelli FR, Baccino FM, Costelli P (2009) Are antioxidants useful for treating skeletal muscle atrophy? Free Radic Biol Med 47:906–916
44. Cerullo F, Gambassi G, Cesari M (2012) Rationale for antioxidant supplementation in sarcopenia. J Aging Res 2012:Article ID 316943, p 8
45. Morley J.E, Abbatecola A.M, Argiles JM, Baracos V, Bauer J, Bhasin S, Cederholm T, Coats AJ, Cummings SR, Evans WJ, Fearon K, Ferrucci L, Fielding RA, Guralnik JM, Harris,T.B, Inui A, Kalantar-Zadeh K, Kirwan BA, Mantovani G, Muscaritoli M, Newman AB, Rossi-Fanelli F, Rosano GM, Roubenoff R, Schambelan M, Sokol GH, Storer TW, Vellas B, von Haehling S, Yeh SS, Anker SD; Society on Sarcopenia, Cachexia and Wasting Disorders Trialist Workshop (2011) Sarcopenia with limited mobility: an international consensus. J Am Med Dir Assoc 12:403–409
46. Bhasin S, Calof O, Storer TW, Lee ML, Mazer NA, Jasuja R, Montori VM, Gao W, Dalton JT (2006) Drug insight: testosterone and selective androgen receptor modulators as anabolic therapies for physical dysfunction in chronic illness and ageing. Nature Clin Pract Endocrinol Metab 2:146–159

47. Sinha-Hikim I, Cornford M, Gaytan H, Lee ML, Bhasin S (2006) Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. J Clin Endocrinol Metab 91:3024–3033
48. Nagaya N, Itoh T, Murakami S, Oya H, Uematsu M, Miyatake K, Kangawa K (2005) Treatment of cachexia with ghrelin in patients with COPD. Chest 128:1187–1193
49. Nagaya N, Moriya J, Yasumura Y, Uematsu M, Ono F, Shimizu W, Ueno K, Kitakaze M, Miyatake K, Kangawa K (2004) Effects of ghrelin administration on left ventricular function, exercise capacity, and muscle wasting in patients with chronic heart failure. Circulation 110:3674–3679
50. Bach MA, Rockwood K, Zetterberg C, Thamsborg G, Hébert R, Devogelaer JP, Christiansen JS, Rizzoli R, Ochsner JL, Beisaw N, Gluck O, Yu L, Schwab T, Farrington J, Taylor AM, Ng J, Fuh V, MK 0677 Hip fracture study group (2004) The effects of MK-0677, an oral growth hormone secretagogue, in patients with hip fracture. J Am Geriatr Soc 52:516–52
51. Nass R, Gaylinn BD, Thorner MO (2011) The ghrelin axis in disease: potential therapeutic indications. Mol Cell Endocrinol 340:106–110
52. Lebrasseur NK, SchelhornTM, Bernardo BL, Cosgrove PG, Loria P, Brown TA (2009) Myostatin inhibition enhances the effects on performance and metabolic outcomes in aged mice. J Gerontol A Biol Sci Med Sci 64:940–948
53. Murphy KT, Koopman R, Naim T, Léger B, Trieu J, Ibebunjo C, Lynch GS (2010) Antibody-directed myostatin inhibition in 21-mo-old mice reveals novel roles for myostatin signaling in skeletal muscle structure and function. FASEB J 24:4433–4442
54. Sumukadas D, Witham MD, Struthers AD, Mcmurdo MET (2007) Effect of perindopril on physical function in elderly people with functional impairment: a randomized controlled trial. CMAJ 177:867–874
55. Bunout D, Barrera G, De La Maza MP, Leiva L, Backhouse C, Hirsch S (2009) Effects of enalapril or nifedipine on muscle strength or functional capacity in elderly subjects. A double blind trial. J Renin Angiotensin Aldosterone Syst 10:77–84
56. Snijder MB, Van Schoor NM, Pluijm SM, Van Dam RM, Visser M, Lips P (2006) Vitamin D status in relation to one-year risk of recurrent falling in older men and women. J Clin Endocrinol Metab 91:2980–2985
57. Annweiler C, Schott AM, Berrut G, Fantino B, Beauchet O (2009) Vitamin D-related changes in physical performance: a systemic review. J Nutr Health Aging 13:893–898
58. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, Nicholson GC (2010) Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. JAMA 291:1815–1822
59. Cesari M, Incalzi RA, Zamboni V, Pahor M (2011) Vitamin D hormone: a multitude of actions potentially influencing the physical function decline in older persons. Geriatr Gerontol Int 11:133–142
60. Garcia LA, King KK, Ferrini MG, Norris KC, Artaza JN (2011) 1,25(OH)2vitamin D3 stimulates myogenic differentiation by inhibiting cell proliferation and modulating the expression of promyogenic growth factors and myostatin in C2C12 skeletal muscle cells. Endocrinology 152:2976–2986
61. Hancock CR, Han DH, Higashida K, Kim SH, Holloszy JO (2011) Does calorie restriction induce mitochondrial biogenesis? FASEB J 25:785–791
62. Lanza IR, Zabielski P, Klaus KA, Morse DM, Heppelmann CJ, Bergen HR 3rd, Dasari S, Walrand S, Short KR, Johnson ML, Robinson MM, Schimke JM, Jakaitis DR, Asmann YW, Sun Z, Nair KS (2012) Chronic caloric restriction preserves mitochondrial function in senescence without increasing mitochondrial biogenesis. Cell Metab 16:777–788
63. Dirks AJ, Leeuwenburgh C (2004) Aging and lifelong calorie restriction result in adaptations of skeletal muscle apoptosis repressor, apoptosis-inducing factor, X-linked inhibitor of apoptosis, caspase-3, and caspase-12. Free Radic Biol Med 36:27–39
64. Gouspillou G, Hepple RT (2013) Facts and controversies in our understanding of how caloric restriction impacts the mitochondrion. Biochem Pharmacol 48:1075–1084

65. Baker DJ, Betik AC, Krause DJ, Hepple RT (2006) No decline in skeletal muscle oxidative capacity with aging in long-term calorically restricted rats: effects are independent of mitochondrial DNA integrity. J Gerontol A Biol Sci Med Sci 61:675–684
66. Chan MC, Arany Z (2014) The many roles of PGC-1α in muscle–recent developments. Metabolism 63:441–451
67. Valdez G, Tapia JC, Kang H, Clemenson GD Jr, Gage FH, Lichtman JW, Sanes JR (2010) Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. Proc Natl Acad Sci USA 107:14863–14868
68. Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, Longo DL, Allison DB, Young JE, Bryant M, Ward WF, Qi W, Ingram DK, de Cabo R (2012) Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature 489:318–322
69. McKiernan SH, Colman RJ, Lopez M, Beasley TM, Aiken JM, Anderson RM, Weindruch R (2011) Caloric restrictin delays aging-induced cellular phenotypes in rhesus monkey skeletal muscle. Exp Gerontol 46:23–29
70. McKiernan SH, Colman RJ, Aiken E, Evans TD, Beasley TM, Aiken JM, Weindruch R, Anderson RM (2012) Cellular adaptation contributes to calorie restriction-induced preservation of skeletal muscle in aged rhesus monkeys. Exp Gerontol 47:229–236
71. Cerletti M, Jang YC, Finley LWS, Haigis MC, Wagers AJ (2012) Short-term calorie restriction enhances skeletal muscle stem cell function. Cell Stem Cell 10:515–519
72. Jang YC, Liu Y, Hayworth CR, Bhattacharya A, Lustgarten MS, Muller FL, Chaudhuri A, Qi W, Li Y, Huang J-Y, Verdin E, Richardson A, Van Remmen H (2012) Dietary restriction attenuates age-associated muscle atrophy by lowering oxidative stress in mice even in complete absence of CuZnSOD. Aging Cell 11:770–782
73. Mercken EM, Crosby SD, Lamming DW, JeBailey L, Krzysik-Walker S, Villareal DT, Capri M, Franceschi C, Zhang Y, Becker K, Sabatini DM, de Cabo R, Fontana L (2012) Calorie restriction in humans inhibits the PI3 K/AKT pathway and induces a younger transcription profile. Aging Cell 12:645–651
74. Mercken EM, Majounie E, Ding J, Guo R, Kim J, Bernier M, Mattison J, Cookson MR, Gorospe M, de Cabo R, Abdelmohsen K (2013) Age-associated miRNA alterations in skeletal muscle from rhesus monkeys reversed by caloric restriction. Aging 5:692–703

Chapter 8
The Role of Functional Foods and Their Bioactive Components in Bone Health

Bahram H. Arjmandi and Sarah A. Johnson

Abstract Osteoporosis is a chronic disorder characterized by a loss of bone mass and quality leading to an increased risk of fragility fractures. It afflicts millions of men and women worldwide and as such is a major public health problem. Certain lifestyle factors, including nutrition, are known to reduce the risk of developing osteoporosis and therefore play an important role in bone health. Epigenetics refers to heritable changes in gene expression that occur without changing the underlying DNA sequence. Environmental factors such as nutrition can lead to epigenetic changes which consequently may influence bone health. For instance, nutritional status during pregnancy or other critical periods of development may lead to epigenetic modifications that negatively influence gene expression leading to osteoporosis later in life. There is evidence to support epigenetic involvement in bone metabolism and the pathogenesis of osteoporosis; however, the evidence is limited and its involvement in the development of osteoporosis is not well understood and largely remains unknown. In addition, there is quite a paucity of evidence to date regarding the relationship between epigenetics, nutrition, and bone health and is therefore even less understood. This chapter describes the existing evidence regarding functional foods and their bioactive components on the prevention and reversal of bone loss in animal models and humans. The research conducted in this area to date has not investigated the underlying mechanisms by which these foods and their bioactive components modulate bone metabolism with respect to

B.H. Arjmandi (✉)
Department of Nutrition, Food and Exercise Sciences, Florida State University, 412 Sandels Building, Tallahassee, FL 32306, USA
e-mail: barjmandi@fsu.edu

S.A. Johnson
Department of Nutrition, Food and Exercise Sciences, Florida State University, 428 Sandels Building, Tallahassee, FL 32306, USA
e-mail: sba07@my.fsu.edu

B.H. Arjmandi · S.A. Johnson
Center for Advancing Exercise and Nutrition Research on Aging, Florida State University, Tallahassee, FL 32306, USA

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epigenetics. However, many of the findings can lead one to speculate about a possible involvement of epigenetics and sets the stage for future research to take this into consideration.

8.1 Introduction

Osteoporosis is a chronic and debilitating skeletal disorder characterized by decreased bone mass and microstructural deterioration resulting in an increased tendency to develop fragility fractures [1, 2]. Postmenopausal osteoporosis is the most common cause of bone loss and occurs, in part, due to an abrupt cessation of ovarian hormone production which leads to an imbalance between bone resorption and bone formation [3, 4]. Osteoporosis and its related bone fractures are a major public health concern as they are associated with increased morbidity and mortality, poor quality of life, and a large economic burden [5, 6]. In the United States (U.S.) alone, more than 44 million people either have or are at risk of osteoporosis [7]. Due to the growing population of individuals ≥50 years of age in the U.S., the prevalence and incidence of osteoporosis is likely to continue to increase with affected individuals being at a greater risk of falls and fractures, therefore increasing morbidity and mortality [5]. The economic burden associated with osteoporosis is estimated to increase to approximately $474 billion over the next few decades [6]. Hence, the morbidity and mortality associated with osteoporosis as well as the growing costs of its associated medical care illustrate the importance of identifying safe, efficacious, and cost-effective ways to prevent its occurrence.

With regard to the prevention and treatment of osteoporosis, certain lifestyle factors, including nutrition, have been shown to play an important role in maintaining and/or improving bone health. In fact, several lines of evidence have demonstrated the impact of essential nutrients and non-essential nutrients from foods, e.g. plant foods rich in phytochemicals, or the lack thereof, on overall skeletal health. It has been suggested and demonstrated that genetic factors are involved in the development of osteoporosis. Epigenetics refers to modifications to DNA without changing the underlying DNA sequence, such as DNA methylation, histone modifications, and microRNAs, which lead to heritable changes in gene expression. Environmental factors, including nutrition, can lead to epigenetic changes which consequently may influence nutrition and subsequently bone health [8, 9]. Although the involvement of epigenetics in bone health is hypothesized, its role in the development of osteoporosis is not well-established and largely remains unknown. Furthermore, the relationship between epigenetics, nutrition, and bone health is even less understood and research in this area is minimal at best. This chapter presents and discusses the findings from preclinical and clinical studies regarding the effects various functional foods and their bioactive components on bone health. Additionally, evidence to support possible epigenetic involvement in the ability of various functional foods and their bioactive components to modulate bone health will be discussed.

8.2 Low Bone Mass and Osteoporosis

As the aging population continues to rise, there is an increased concern for the health-related needs of older people. Approximately 85 % of older Americans suffer from one or more nutrition-related chronic disorders including age-related bone loss and osteoporosis. According to the latest report by the National Osteoporosis Foundation [10], it is estimated that a total of 54 million American adults aged 50 and older have either osteoporosis or low bone mass. In fact, in 2010 approximately 10.2 million older adults suffered from osteoporosis and it is predicted that that by 2015, close to three million fractures will occur as a result of osteoporosis in the U.S. with a direct economic burden of more than $25 billion. Although the prevalence of age-related bone loss is greater in women than in men, male osteoporosis is also a major public health concern. In fact, approximately two million men in the U.S. currently have osteoporosis and another 16.1 million are at risk of developing osteoporosis. Additionally, men account for one third of all hip fractures and one half of all symptomatic vertebral fractures which result in major orthopedic problems [11].

One of the causes for male osteoporosis is hypogonadism [12, 13] which can occur as a result of aging. On average, men 65 years of age or older experience an approximate 25 % reduction in serum testosterone levels which may lead to decreased bone mineral density (BMD) [14]. Although male osteoporosis has been increasingly recognized in recent years, there is a gap in the understanding of its etiology and the drug treatment efficacy in comparison with women. Aside from drug therapies, diet and lifestyle also play an important role in maintaining healthy bone in both men and women. In reference to functional foods and their bioactive components, we and other investigators have shown the bone-protective effects of soy protein and its isoflavones and dried plum in both men and women. Nonetheless, it should be acknowledged that there is a paucity of studies in men and animal models of male osteoporosis to form a consensus. Though one can assume that anything that prevents or builds bone in women should also be effective in men, including maintaining a low level of estrogen, further studies are needed to confirm this view. Therefore, for the purpose of this book chapter, we direct our discussion mostly towards the effects of treatments and or intervention on female osteoporosis.

Meanwhile, it is emphasized that bone integrity and bone health in both in women and men are greatly affected by nutrition and lifestyle factors at any stage of the lifecycle. Postmenopausal women are at the greatest risk of developing osteoporosis because of sudden cessation in ovarian hormone production. Diminished estrogen levels associated with menopause result in an initial phase of rapid bone loss followed by a period of slower deterioration of the skeleton. This rapid phase of bone loss occurs within the first five to ten years following the cessation of menses or surgical removal of the ovaries. Such bone loss can result in a considerable reduction of bone mass and increased risk of fracture [15, 16].

It goes without saying that prevention is the most effective way of combating osteoporosis in both men and women. Evidence suggests that, especially during the

first three or four decades of life, appropriate public health strategies to prevent bone fragility include adequate lifetime calcium intake, maintaining an appropriate body weight, performing regular weight-bearing physical activity, avoiding smoking and alcohol abuse, and adopting healthy dietary practices [15].

8.3 Epigenetics and Bone Health

Epigenetics influences the development of age-related diseases including osteoporosis and osteoarthritis [17]. Although positive modulation of epigenetic processes by pharmacological agents or naturally occurring compounds is much desired in order to prevent and/or intervene certain diseases such as osteoporosis, for practical purposes we may be limited to the use of biomarkers in serum or urine samples for monitoring the effectiveness of a potential therapeutic agent. However, there are studies suggesting that bone metabolism may be modulated via epigenetic regulations such as DNA methylation, histone modification, and microRNAs. For instance, methylation of CpG islands of promoter and regulatory regions has been shown to repress gene expression of key modulators of bone metabolism. This was evidenced in a study by Reppe et al. [18] who demonstrated that serum sclerostin levels not only correlate with the incidence of fractures in postmenopausal women but can be related to epigenetic mechanisms. Sclerostin, which is encoded by *SOST*, is found in human bone and is synthesized by osteocytes. When they studied DNA methylation upstream of *SOST*, the researchers noted an association between the DNA methylation assessed in bone biopsy samples and a number of genes, such as *ABCA8*, *MEPE* and *ACSL3*, implicated with higher BMD values. Another example of this was demonstrated by Delgado-Calle et al. [19] who reported that DNA methylation of the genes responsible for expression of receptor activator of nuclear factor-Kappa B ligand (RANKL) and osteoprotegerin (OPG), *TNFSF11* and *TNFRSF11B,* respectively, leads to repression of their expression. RANKL is a molecule which stimulates osteoclast formation, activation, and survival and OPG is a molecule that binds to RANKL thereby preventing it from binding to its receptor and having these effects on osteoclastogenesis. In another study by their group, Delgado-Calle et al. [20] showed that the degree of CpG island methylation was inversely associated with alkaline phosphatase (ALP) gene expression in human osteoblasts and is a key regulator of during the transition of osteoblasts to osteocytes through gene silencing. It has also been shown that DNA methylation can be modulated by environmental factors, which can be beneficial or harmful depending on which genes are repressed as a result. Arnsdorf et al. [21] demonstrated that mechanical stimulation is capable of reducing DNA methylation, thereby leading to increased gene expression of *collagen I*, *osteocalcin*, and *osteopontin* in murine-derived bone marrow progenitor cells. This is an important finding as it suggests that lifestyle modifications such as exercise and dietary factors are likely to exert some of their beneficial effects on bone health through epigenetic regulations.

Histone modifications, such as acetylation and deacetylation, are another type of epigenetic modification that regulates bone metabolism. There is evidence that histone deacetylases (HDACs) are beneficial to bone health by promoting bone development and maintenance. HDAC inhibitors, such as valproate and suberoylanilide hydroxamic acid (SAHA), are widely used clinically to treat cancer and epilepsy. They have been studied in order to better understand the influence of histone hyperacetylation on bone metabolism. However, their use has produced differential effects including both bone building and bone loss [17, 22]. McGee-Lawrence and associates [22] demonstrated that treating C57BL/6 J mice with the HDAC inhibitor SAHA caused a reduction in the number of osteoblasts on trabecular surfaces leading to trabecular bone loss. This loss occurred even though there was a simultaneous increase in osteoblast activity of preexisting mature osteoblasts suggesting the deleterious effects were on immature osteoblasts. Additionally, consistent with the decline in osteoblast number and osteoblast surface they noted a reduction in serum levels of procollagen type 1 amino-terminal propeptide, a bone formation marker secreted by osteoblasts, as well as decreased gene expression of type 1 collagen, osteocalcin, and osteopontin. There is also evidence to suggest that environmental factors can lead to disturbances in the epigenetic regulation of bone development and bone health. For instance, using a rat model of fetal alcohol syndrome, Leu et al. [23] showed that ethanol exposure mimicking that of the human third trimester caused long-term suppression of osteogenesis and adipogenesis in bone marrow-derived mesenchymal stem cells and reductions changes in the gene expression of ALP, osteocalcin, runt-related transcription factor 2 (Runx2), adipocyte fatty-acid-binding protein (*aP2*), and peroxisome proliferator-activated receptor gamma (*PPARγ*), leading to blunted development of trabecular bone, smaller body sizes and bodyweights compared with control animals. These effects were due, in part, to altered trimethylation at histone 3 lysine 27, suggesting that epigenetic modification is one of the mechanisms leading to the adverse effects of alcohol on bone development and the increased risk for osteoporosis later in life.

MicroRNAs are non-coding RNAs that regulate gene expression through epigenetic modifications including gene silencing and mRNA degradation. MicroRNAs have been shown to play a role in the differentiation of osteoblasts and osteoclasts and have been associated with the development of osteoporosis [17]. For example, in glucocorticoid-treated osteoblast cultures, microRNA-29a gain of function has been shown to enhance WNT/β-catenin signaling through HDAC signaling regulation leading to improved osteoblast differentiation [24]. In humans, Wang et al. [25] profiled the expression of microRNAs in peripheral blood mononuclear cells taken from postmenopausal women with low and high BMD, which indicated that microRNA-133a was significantly increased in those with low BMD compared with those with high BMD and that microRNA-133a was correlated with osteoclast-related gene expression although not significantly. This finding is of relevance to postmenopausal osteoporosis since microRNA-133a has been demonstrated to be involved in osteoblastogenesis and may serve as a biomarker for postmenopausal osteoporosis.

8.4 The Role of Nutrition in Epigenetic Regulation of Bone Health

As mentioned earlier, although therapies are available in terms of anti-resorptive and anabolic agents, these therapies are not always effective and are not free of side effects and contraindications. Thus, identifying non-pharmacologic treatment modalities, such as functional foods and their bioactive components, that can prevent and/or alleviate the negative effects of osteoporosis, including the development of fractures, is crucial. Functional foods and their bioactive components have been shown to prevent and reverse bone loss through decreasing bone resorption and the rate of bone turnover as well as through enhancing the rate of bone formation. However, the mechanisms underlying how they affect bone metabolism are poorly understood and cannot be elaborated on beyond their effects on certain mRNA levels of transcriptional factors know to influence bone. We postulate that several of these mechanisms are mediated through epigenetic regulation. Additionally, although the overall impact of the nutritional status of mothers has been known to affect the well-being of their offspring for centuries, including bone development and long-term health, in more recent years there have been observations suggesting that epigenetic regulation and modifications play a role. However, as to what extent these modulations will be via epigenetic regulation remains to be illustrated.

8.5 Evidence for Bone-Protective Properties of Foods and Their Bioactive Components

In general, consumption of a diet rich in fruits and vegetables has been shown to exert protective effects on bone health [26], in part, through antioxidative and anti-inflammatory mechanisms. These protective effects have been primarily attributed to their polyphenolic content [27, 28]. Phenolic compounds, including flavonoids, exhibit strong antioxidant and anti-inflammatory properties that have important implications for skeletal health [26, 29, 30]. Some foods that are rich in phenolic compounds and have been investigated for their effectiveness in improving bone health include dried plums, blueberries, soy, flaxseed, and grapes.

8.5.1 Dried Plums

Dried plums (*Prunus domestica*) have been ranked as having one of the highest oxygen radical absorbance capacities (ORAC) among the commonly consumed fruits and vegetables, likely due to the fact that they are a rich source of polyphenolic compounds, including chlorogenic acid and neochlorogenic acid, which

are potent antioxidants. Additionally, they are rich in nutrients including vitamin K, potassium, magnesium, and boron [31]. All of the abovementioned nutrients and non-nutrients and their associated physiological functions are known to be important for bone health. For these reasons, dried plums have been investigated to determine if they would have beneficial effects on bone health, and their bone-protective effects have been well-established in animal models and to some degree in humans by our laboratory as well as others.

Because the incidence and prevalence of bone loss and osteoporosis is greater in women than in men, much emphasis has been placed on research in this population, as well as animal models of postmenopausal bone loss. As such, there is ample evidence to support the role of dried plum on bone health in the female rat model of osteoporosis. Our laboratory [32] was the first to report the effects of dried plum on bone health. Using an ovariectomized rat model of postmenopausal osteoporosis, 90-day-old female Sprague-Dawley rats were divided into four groups: sham-operated (Sham), ovariectomized (Ovx), Ovx + 5 % (low dose, LD) dried plums, and Ovx + 25 % (high dose, HD) dried plums. As expected, ovariectomy led to significant declines in BMD of the 4th lumbar vertebrae and femurs as well as a decrease in tibial trabecular bone area. The HD-dried plum diet completely prevented this bone loss. Additionally, dried plum dose-dependently increased circulating insulin-like growth factor-I (IGF-I) levels, while not affecting tartrate-resistant acid phosphatase (TRAP5b) levels. This suggested that dried plum exerted its bone-protective effects by increasing the rate of bone formation and not through inhibiting bone resorption. In 2005, our laboratory [33] sought to determine whether dried plums were able to restore bone mass after bone loss has occurred. Using an ovariectomized rat model of postmenopausal osteoporosis, 90-day-old female Sprague-Dawley rats were divided into five groups: Sham, Ovx control, Ovx + 17β-estradiol (E_2), Ovx + 5 % dried plum (LD), Ovx + 15 % dried plum (MD), and Ovx + 25 % dried plum (HD) for 60 days. All doses of dried plum were effective in restoring femoral and tibial BMD, while only the HD-dried plum diet was able to restore lumbar BMD. In terms of biomechanical properties, all doses of dried plums led to a 6.9 and 6.0 % increase in overall yield and ultimate force of the femur although none reached statistical significance. The improvements noted in biomechanical properties can, in part, be attributed to improvements in BMD and microarchitectural properties (primarily in the HD group) including bone volume/total volume (BV/TV), connectivity density, trabecular number (Tb.N), trabecular separation (Tb.S), and structure model index (SMI) (See Fig. 8.1). To our knowledge this is the first time that it has been shown that an agent, drug or nutrient, can reverse the loss of trabecular microstructures. In 2010, our laboratory reported [34] that among several functional foods and bioactive components (i.e. dried plum, figs, dates, raisins, blueberries, dried plum polyphenols, fructooligosaccharides, and β-hydroxy-β-methylbutyrate), 5 % fructooligosaccharides +7.5 % dried plum was the most efficacious in reversing loss of BMD in the right femur and fourth lumbar spine, fourth lumbar spine calcium loss, and trabecular separation in Ovx Sprague-Dawley rats. None of the treatments altered serum or urinary markers of bone turnover. The findings of this study suggest that the addition of fructooligosaccharides to a lower dose of dried plum than was previously effective

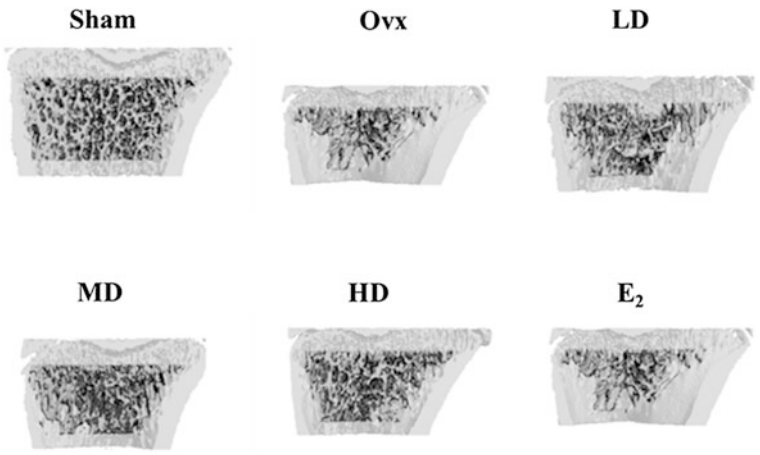

Fig. 8.1 Three-dimensional view of representative proximal tibia samples from each treatment group. Sham, sham-operated; *Ovx*, ovariectomized; *LD*, low dose dried plum; *MD*, medium dose dried plum; *HD*, high dose dried plum; E_2, 17β-estradiol

helps to improve its efficacy with respect to promoting bone health. In a similar study conducted by our laboratory, Johnson et al. [35] found that the combination of a soy-based diet with either dried plum, fructooligosaccharides, or both significantly improved whole body BMD and femoral, while only the combination of a soy-based diet, 7.5 % dried plum, and 5 % fructooligosaccharides had the most pronounced effect on lumbar BMD. All interventions were noted to improve biomechanical properties of bone as demonstrated by increased ultimate load. These improvements were suggested to be due, in part, to their ability to enhance bone formation and reduce bone resorption as shown by increased alkaline phosphatase and decreased urinary deoxypyridinoline (Dpd).

In 2012, Rendina et al. [36] reported that feeding a high dose of dried plum (25 % w/w) for four weeks to female ovariectomized C57BL/6 J mice prevented the loss of BMD and bone mineral content (BMC) of the spine and trabecular microarchitectural properties of the vertebra and proximal tibia, resulting in greater bone strength and stiffness in the vertebra. Additionally, medium (15 % w/w) and high doses of dried plum restored myeloid and lymphoid levels to that of the sham-operated mice and suppressed ex vivo concanavalin A stimulated lymphocyte tumor necrosis factor-alpha production in splenocytes. This suggests that dried plum may exert its bone-protective effects through mediating immune cell populations and activation. In a later study, Rendina et al. [37] reported the results of comparing the osteoprotective effects of dried plum with other fruits (i.e. dried apple, apricot, grape, and mango) in ovariectomized C57BL/6 mice over an eight-week period. Their findings indicated that when compared with the other fruits, dried plum had superior anabolic effects on trabecular bone microarchitectural properties of the vertebra and was able to prevent tibial bone loss, as well as

restoration of trabecular biomechanical properties of the spine including bone strength and stiffness. Additionally, dried plum was superior in its ability to enhance plasma glutathione peroxidase activity, to down-regulate osteoclast differentiation through attenuation of *Nfatc1* gene expression and to up-regulate osteoblast activity through enhancing *Col1a1* mRNA levels and perhaps by suppressing Ovx-induced apoptosis by decreasing *Bak1* gene expression. In a recent study published by the same laboratory, Smith et al. [38] compared the effects of dried plum supplementation to treatment with parathyroid hormone (PTH). As to be expected, 6 weeks of treatment with the two high doses of dried plum (15 and 25 % w/w) restored whole body and femoral BMD to that of the Sham group and improved trabecular bone volume and cortical thickness. Systemic blood biomarkers of bone metabolism (i.e. *N*-terminal procollagen type 1 (PINP) and Dpd were reduced indicating a reduction in bone turnover. With respect to dynamic bone histomorphometric analysis of the tibial metaphysis, dried plum restored the Ovx-induced increase in cancellous bone formation rate (BFR) and mineralizing surface (MS/BS) to that of the Sham group. Dried plum also upregulated gene expression of bone morphogenetic protein 2 (*Bmp2*), a regulator of osteogenesis, and insulin-like growth factor-I (*Igf-I*) while downregulating nuclear factor T cell activator 1 (*Nfatc1*), a transcription factor involved in the regulation of osteoclast differentiation. Compared with that of the effects of PTH on bone, dried plum reduced the rate of bone turnover rather than increasing the rate of bone formation. Although in that study the mechanical properties of bone were not assessed, it can be suggested that the quality of bone in dried plum-fed animals was superior to that of PTH treatment. Nonetheless, this is speculative based on the rate of bone turnover and further studies are needed to confirm this notion.

In 2003, Mühlbauer et al. [39] were the first to report the ability of dried plums to inhibit bone resorption in male rats (Wistar Hanlbm) as assessed by measuring the urinary excretion of tritium-labeled tetracycline ([^{3}H]-Tc) released from bones of 9-week-old rats prelabeled with tritiated tetracycline. In 2006, our laboratory [40] investigated the extent to which dried plum would reverse bone loss in gonadal hormone deficient male rats. In order to do this, 6-month-old male Sprague-Dawley rats were either Sham or orchidectomized (Orx) and divided into five groups: Sham, Orx control, Orx + 5 % dried plum (LD), Orx + 15 % dried plum (MD), or Orx + 25 % dried plum (HD) for 90 days. The results showed that MD and HD dried plum prevented the Orx-induced loss of BMD of the whole body, femur, and lumbar spine, the decrease in biomechanical properties including cortical bone ultimate load as well as compressive force and stiffness of the trabecular bone within the vertebrae, and deterioration of trabecular bone microarchitectural properties in the distal femur and vertebral body. In terms of biochemical markers, serum levels of IGF-I were increased while urinary excretion of Dpd and bone mRNA levels of RANKL and OPG were reduced by all doses of dried plum. In a follow-up study published in 2007 by our group, Bu et al. [41] reported the results of a study in which the extent to which dried plum would reverse bone loss in osteopenic Orx rats was investigated in addition to comparing its effects with that of PTH. The findings indicated that dried plum is able to reverse Orx-induced bone loss in terms of increased density and

enhanced microarchitectural properties. The effects of dried plum on increasing vertebral trabecular bone volume (BV/TV) and number and decreasing trabecular separation were comparable to PTH. Although dried plum led to significant improvements in vertebral and femoral BMD and cortical thickness, the extent was not as great as PTH. To determine whether supplementation of the diet with dried plum would reverse aged-related bone loss, Halloran et al. [42] supplemented the diets of six-month-old (adult) and eighteen-month-old (old) male mice with 0, 15, and 25 % dried plum by weight for six months. Their results showed that dried plum at a dose of 25 % led to a gain in cancellous bone volume which exceeded baseline by approximately 50 % in adult mice and 40 % in old mice, whereas only adult mice receiving dried plum at a dose of 15 % gained bone. This study demonstrated that supplementation with dried plum can restore bone that has already been lost as a result of aging. In a follow-up study, Smith et al. [43] investigated the effects of dried plum supplementation on bone structural properties as well as indices of bone formation and resorption after four and twelve weeks of treatment in a mouse model of age-related osteoporosis to understand the bone metabolic changes occurring during early supplementation. Their findings suggest that supplementation with dried plum (25 % w/w) initially suppressed the rate of bone turnover (at 4 weeks) and followed by a normalization of bone turnover (at 12 weeks), leading to increased bone mass and microarchitectural properties.

In 2002, our laboratory published the first clinical study looking at dried plums and bone health [44]. This three-month study investigated the role of 100 g dried plums versus 75 g dried apples in improving biochemical markers of bone turnover in postmenopausal women. The results demonstrated that the group who consumed dried plums had significantly improved serum levels of biochemical markers of bone formation [i.e. IGF-I and bone-specific alkaline phosphatase (BAP)], whereas there we no changes in the control group or in serum [i.e. TRAP5b) and urinary (i.e. Ddp and helical peptide (HP)] biochemical markers of bone resorption. At the time of this study, the results suggested that dried plum had positive effects on bone formation without influencing bone resorption. In a follow-up study published in 2011 [45], we reported the findings of a 12-month comparative control design in which 160 osteopenic, postmenopausal women were randomized to receive either 100 g dried plums versus 75 g dried apples in order to determine the extent to which dried plum would reverse bone loss. The BMD of the ulna and spine of women consuming dried plum were significantly higher than the group consuming the control at 12 months. Additionally, dried plum consumption led to significant decreases in biochemical markers of bone turnover (i.e. BAP at 12 months) and TRAP5b (at 3 months remained unchanged throughout the study). Our 1-year study findings with reference to BAP and TRAP5b are in contrast to our earlier 3-month findings. To investigate possible mechanisms of action to explain the findings of our 1-year study, we later measured serum levels of RANKL, OPG, and sclerostin. Although dried plum consumption led to increases in RANKL (+1.99 vs. 18.33 % in control) and OPG (+4.87 vs. −2.15 % in control) and decreases in sclerostin (−1.12 vs. +3.78 %), they did not reach statistical significance. Nonetheless, biochemical markers of bone turnover are not always reliable and are therefore are

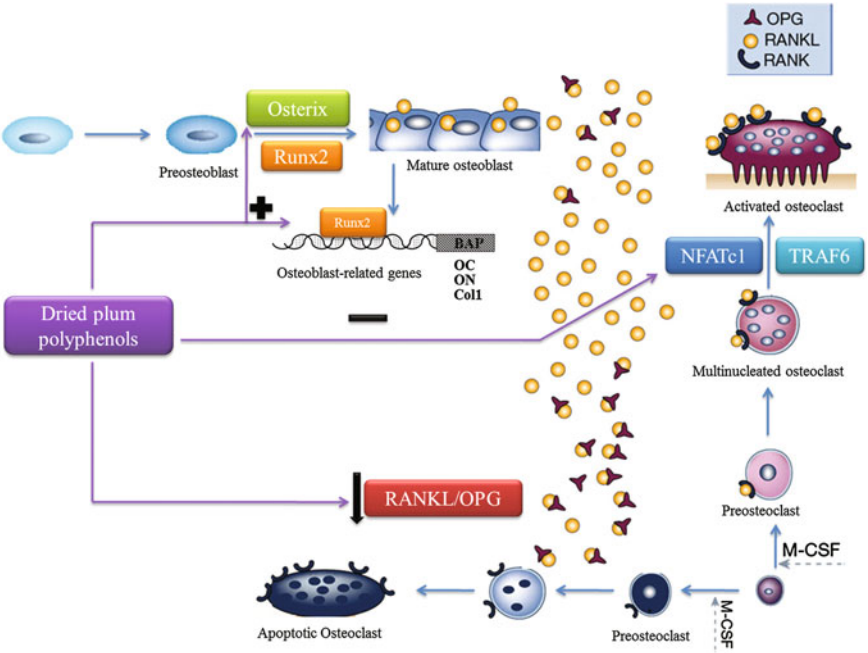

Diagram 8.1 Proposed mechanisms of action of dried plum polyphenols on bone metabolism

not clinically relevant and is the reason that they are not used in the diagnosis of osteoporosis. Hence, the results of these studies with respect to biochemical markers of bone turnover should be interpreted with caution and greater value should be placed in the resulting BMD values in the ulna and spine. In short, because dried plum has been shown to increase serum ALP, decrease serum sclerostin and favorably tilt the ratio of OPG to RANKL, it is suggested that the positive effect of dried plum on bone is through epigenetic regulations of bone metabolism. Although this notion is speculative and direct evidence is needed, it can be indirectly supported as all of the aforementioned molecules seem to exert epigenetic influences on osteoblast and osteoclast cells. Diagram 8.1 depicts the probable mechanisms of action by which dried plum or its bioactive components exert their effects on bone.

8.5.2 Blueberries

Emerging evidence indicates that blueberries (*Vaccinium*) have strong free radical scavenging capacities due to their polyphenolic composition [46, 47]. Blueberries contain flavonoids (i.e. anthocyanins, catechin, epichatechin, quercetin, kaempferol, and myrecetin) as well as phenolic acids (i.e. gallic acid, p-hydroxybenzoic acid,

chlorogenic, p-coumaric, caffeic, ferulic, and ellagic acids), and stilbenes (i.e. pterostilbene) [48–50]. In the U.S., the most common species of blueberries include lowbush "wild" blueberries (*Vaccinium angustifolium* Aiton) which are grown in the wild, highbush "cultivated" blueberries (*Vaccinium corymbosum* L.) which are commercially grown on plantations and are bred for their production and characteristics, and rabbiteye blueberries (*Vaccinium ashei* Reade) which are grown in the southern region of the U.S. [51, 52]. Although the total polyphenolic content vary greatly among species and cultivars of blueberries, all blueberries are rich sources of phenolic compounds with an average content of 300 mg/100 g fresh weight [48]. On a dry weight basis, total phenolic content is about 2,500 mg/100 g and is one of the highest among fruits and vegetables [52]. Phenolic compounds have been shown to have biological activity and have high antioxidant capacity [49, 51, 53], and blueberry extract ranks as one of the highest in antioxidant capacity in comparison with other fruits and reference compounds such as vitamin C [52]. Total antioxidant capacity, as measured by ORAC, may range from 13.9 to 45.9 μmol Trolox equivalents (TE)/g of fresh berries and these values vary among species and cultivars of the *Vaccinium* species [52, 53]. The composition and interaction of polyphenols present in blueberries make them an excellent source of stable free radical scavengers [47, 54]. With respect to bone, Garrett et al. [55] demonstrated that free radicals generated in the bone microenvironment increase osteoclast formation and bone resorption, thereby resulting in bone loss and hence, antioxidants and antioxidant-rich foods, such as blueberries, may inhibit this activity.

Using six-month old female Sprague-Dawley rats, our laboratory [56] demonstrated that treatment of Ovx rats with 5 % blueberry w/w for 100 days prevented the loss of whole body BMD and somewhat prevented the loss of tibial and femoral BMD when compared to Sham and Ovx control rats. Blueberry-treated Ovx rats were found to have lower levels of biochemical marker of bone turnover including mRNA levels of alkaline phosphatase, collagen type I, and TRAP5b when compared to Ovx control rats (See Fig. 8.2). The findings of this study suggest that the bone-protective effects of blueberries may be through the suppression of bone

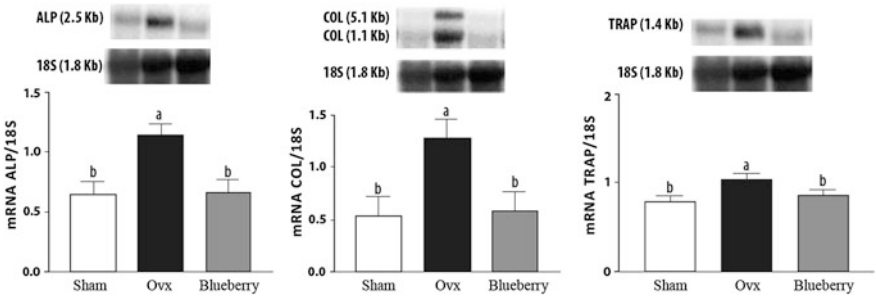

Fig. 8.2 Effects of ovariectomy (*Ovx*) and blueberry on femoral mRNA levels of alkaline phosphatase (*ALP*), collagen type I (*COL*), and tartrate-resistant acid phosphatase-5b (*TRAP*). Bars are mean ± standard error of the mean (*SEM*). Values that do not share the same superscript letters are significantly ($P < 0.05$) different from each other. n = 4 samples per group

resorption. Conversely, in a later study, when 7.5 % blueberry was combined with 2 % fructooligosaccharides, Arjmandi et al. [34] saw no effect on ovariectomy-induced bone loss, bone calcium, or biochemical markers of bone turnover in three-month old female Sprague-Dawley rats.

In a 2010 report, Chen et al. [57] indicated that blueberry-fed (10 %) young weanling male and female Sprague-Dawley rats had increased bone mass. The authors suggested these effects were through an uncoupling effect of blueberries on osteoblastogenesis and osteoclastogenesis such that osteoblast differentiation was increased while osteoclast differentiation was decreased, as well as an increase in osteoblast progenitors in bone marrow. Additionally, it was suggested that the observed bone-protective effects of blueberries were related to the high levels of polyphenol metabolites found in the serum following their consumption and that they may act through the p38/mitogen-activated protein kinase/β-catenin canonical Wnt signaling cascade. A later study published by the same laboratory in 2011 [58] reported that feeding a diet supplemented with 10 % blueberries to pre-pubertal rats throughout development or for a short period of time (between postnatal day 20 and 34) prevented ovariectomy-induced bone loss in adult life of female Sprague-Dawley rats. The authors suggested that this effect was, in part, due to the ability of blueberries to increase myosin expression through the regulation of the runt domain-containing transcription factor 2 (Runx2) gene, thereby suppressing cellular senescence of osteoblasts and their precursors, mesenchymal stromal cells. In a later study published in 2012 by Zhang et al. [59], feeding pre-pubertal rats a diet supplemented with 10 % blueberries immediately for as little as 14 days was shown to prevent ovariectomy-induced effects on bone later in life including bone loss and decreases in collagen type I, as well alterations in the expression of markers of osteoblastic cell senescence including Sirt1, p16/p21, and collagenase. The authors suggest that the ability of blueberries to prevent ovariectomy-induced osteoblastic cell senescence and subsequent bone loss involves the prevention of collagen degradation. In 2013, Zhang et al. [60] reported the dose-dependent ability of a blueberry-supplemented diet on BMD and BMC in young rats without effecting growth. Their findings indicated that these effects were through the inhibition of bone resorption as evidenced by decreased RANKL/OPG ratio, TRAP5b, and PPARγ expression.

Altogether, these findings support a role for blueberry consumption and supplementation in the prevention of bone loss when consumed early in life before bone loss has occurred, as well as the prevention and reversal of bone loss later in life. Nonetheless, due to the fact that all studies evaluating the bone-protective effects of blueberries to date were conducted using in vitro and in vivo models, these findings cannot be extrapolated to humans but do warrant future clinical studies to confirm these effects in humans.

8.5.3 Soy, Flaxseed, Grapes, and Phytoestrogens

Estrogens (i.e. estradiol, estriol, and estrone) are hormones important for growth, differentiation, and normal functioning of several target tissues, including reproductive tissues such as the mammary gland, uterus, ovary, testis, and prostate as well as other non-reproductive tissues such as bone [61]. Estrogens exert their physiological effects through binding to an intranuclear binding protein within the target cells known as estrogen receptors (ER), thereby causing a conformational change that enables the receptor to bind with the estrogen response element (ERE) and subsequently induce changes in gene expression [61–63]. There are at least two subtypes of the estrogen receptor—ERα and ERβ [61, 62] which share considerable homology but differ in the *C*-terminal ligand-binding domain and in the *N*-terminal transactivation domain [64]. In addition to estrogens, selective ER modulators (SERMs) are able to bind to ER and may function as agonists or antagonists depending on subtype of ER in which they bind, as well as the cellular environment [62]. Examples of SERMs include tamoxifen, raloxifene, and naturally occurring phytoestrogens such as isoflavones, lignans, and coumestans present in certain foods such as soy and flaxseed [61–65].

8.5.3.1 Soy

Isoflavones, including genistein, daidzein, and glycitein, are a naturally occurring subclass of flavonoids found in soybeans and are often referred to as SERMs due to their preferential binding to ERβ, although they are able to bind with ERα as well [66]. However, as mentioned above, binding of SERMs including phytoestrogens is not enough to predict how they will interact with ER and ERE and subsequently either activate or not activate gene transcription in vivo and they may therefore function as agonists or antagonists [63, 66]. Phytoestrogens are non-steroidal plant compounds estrogen-like structures found in many fruits, vegetables, and grains. Hence, food sources rich in phytoestrogens may provide postmenopausal women with yet an additional practical and safe alternative therapy. Additionally, since estrogen therapy may not be feasible in men, SERMs or SERM-like compounds which exert estrogen-like effects on bone without affecting other tissues could be of benefit to men as well.

Among the early studies using ovariectomized Sprague-Dawley rats was a study by our laboratory which indicated that soy protein has positive effects on bone. Our findings [67] demonstrated that replacing casein with soy protein as the protein source in the diet prevented bone loss such that vertebral BMD was similar to that of the estrogen-treated group. Nonspecific markers of bone formation (i.e. serum alkaline phosphatase activity) and bone resorption (i.e. serum TRAP5b activity) were higher in ovariectomized rats consuming soy protein than in sham-operated control rats. To determine whether soy protein itself or its isoflavones exert beneficial effects on bone, we conducted a study using 95-day-old Sprague-Dawley

rats [68]. Similar to our earlier findings [67], ovariectomy led to an increase in bone turnover as indicated by higher serum alkaline phosphatase activity, serum IGF-I and insulin-like growth factor binding protein 3 (IGFBP3) concentrations, and urinary hydroxyproline. These increases were not ameliorated by either soy with normal or reduced isoflavone content. Ovariectomy led to an increased rate of bone formation as indicated by histomorphometry and this was not ameliorated by either of the soy diets. Although it appears that the bone-protective effects of soy protein are related to its non-protein constituents, i.e. isoflavones, the effects of other components such as saponins and phytic acid, cannot be ruled out. The results from these studies indicate that the beneficial effects of soy protein are due, in part, to enhanced bone formation rather than suppression of bone resorption. In a subsequent study, we found that soy protein led to improvements in tibial BMD and BMC in ovariectomized rats although it was unable to restore bone loss to that of the sham-operated control group [69]. When combined with fructooligosaccharides, a soy protein-based diet was most effective in reversing the loss of microarchitectural parameters such as tibial trabecula number, separation, and thickness while separately each is more effective than the combination in improving BMD of the whole body, tibiae, and lumbar vertebrae [70]. Conversely, the combination of soy protein with fructooligosaccharides and dried plum effectively improved lumbar BMD [35] while the combination of genistein and fructooligosaccharides was more effective than either component alone in preventing the loss of BMD of the whole body, right femur, and fourth lumbar [71].

Although several clinical studies and meta-analyses [72–74] of the currently published clinical studies have demonstrated the efficacy of soy and its isoflavones in improving biomarkers of bone metabolism and bone density and strength, reported findings have been inconsistent with some studies indicating either a modest [75–77] or no effect [78, 79]. We have reported [80] that the addition of 40 g soy protein daily to the diets of postmenopausal women led to increased serum IGF-I and a reduction in urinary Dpd excretion and the impact was the greatest in women not on HRT. In a subsequent yearlong clinical trial, we found that the addition of 25 g per day of soy protein (delivering 60 mg soy isoflavones per day) to the diets of postmenopausal women did not show differential effects when compared with control [81]. Meta-analyses of existing clinical trials consistently have indicated that soy isoflavones are effective in improving BMD of the lumbar spine and reducing urinary Dpd excretion in postmenopausal women when consumed for at least six months [72–74].

The orchidectomized rat model has been characterized as an appropriate and practical model for studying age-related bone loss in men even though the pathogenesis of male osteoporosis may not be entirely due to androgen deficiency [82, 83]. In thirteen-month-old orchidectomized male rats, soy protein with and without its isoflavones, and to a lesser degree soy isoflavones, combined with casein reduced the loss of whole body BMD while all treatments improved trabecular bone histomorphometric properties of the proximal tibia [84]. In a subsequent study using thirteen-month-old orchidectomized male rats [85], soy protein containing 1,200 mg/kg diet isoflavones, and to a lesser extent soy protein

containing 600 mg/kg diet isoflavones, reduced the loss of trabecular bone volume, number, and separation of the femoral neck, in part, due to increased gene expression of ALP, collagen type I, and osteocalcin. In younger (95-day-old) orchidectomized male rats, soy protein regardless of its isoflavone content was unable to prevent the loss of femoral BMD or BMC but did improve bone quality as indicated by increased yield force. However, soy protein was able to prevent the orchidectomy-induced reduction in bone length, which implies that soy may have beneficial effects on bone development [86]. In humans, the findings are limited to the results of two clinical studies. We demonstrated [87] that soy protein did not have an effect on markers of bone turnover (i.e. serum alkaline phosphatase, BAP, and urinary Dpd) in 46 men, while Newton et al. [76] reported a modest preservation of spine BMD in older men and women. Overall, the current evidence does not strongly support the use of soy for the treatment of bone loss in men.

The ultimate measure of success of a therapeutic or dietary intervention should be based on a reduction in the incidence and prevalence of fractures. Few studies have evaluated the effects of soy or its isoflavones on fracture prevention or healing. In a study previously conducted by our laboratory (unpublished data), we investigated the effect of dietary vitamin E and soy isoflavones on the outcome of bone fracture repair in ovariectomized rats. Twelve-month-old female Sprague-Dawley rats were ovariectomized and received a standard diet for a period of 120 days to mimic ovarian hormone deficiency-associated osteoporosis. Thereafter, the bone repair model was created in both fibulae of each rat by osteotomy. Immediately after fracture induction, rats were placed on treatment with one group of ovariectomized rats receiving 525 mg/kg diet vitamin E plus 1,000 isoflavones mg/kg diet. One hundred days after osteotomy, rats were sacrificed to evaluate the outcome of bone repair by bend testing. We found that the combination of isoflavones and vitamin E can improve the material properties of fractured bone. Kolios et al. [88] reported the findings of their study which demonstrated that estrogen and equol, but not genistein, were able to improve metaphyseal fracture healing in the ovariectomized rat model of osteoporosis through improved elasticity of callus formation, biomechanical properties, and trabecular structure. Although it is too early to claim that soy or its isoflavones enhance the fracture healing processes, the osteopenic rat model can be considered a valuable animal model for investigating the effects of treatments under these conditions.

Early exposure to soy and its isoflavones has also been shown to have an impact on bone health outcomes. In order to determine whether early exposure to soy isoflavones would result in improve BMD, biomechanical and microarchitectural properties in young adulthood, Kaludjerovic and Ward [89] exposed male and female CD-1 mice to daidzein, genistein, daizein plus genistein, or the synthetic hormone diethylstilbestrol from postnatal days one to five and followed them until four months of age. Their findings indicated that females but not males in all treatment groups had higher BMD of the lumbar vertebrae than control and vertebrae were more resistant to compression fractures in the daidzein and diethylstilbestrol groups. Additionally, treatment with daizein and genistein led to greater trabecular connectivity and trabecular thickness, respectively, than control.

In a subsequent study with a similar study design, Kaludjerovic and Ward [90] found that daidzein plus genistein provided protection against ovariectomy-induced bone loss but not orchidectomy-induced bone loss. Conversely, Gutowska et al. [91] demonstrated that pre- and postnatal exposure to soy isoflavones increased ERβ expression in osteocytes of the femur, led to higher and lower serum concentrations of estradiol and calcium, respectively, and higher magnesium and fluoride content in the bones. There are a number of reports indicating that there is placental transfer of phytoestrogens from mother to the fetus [92, 93]. With respect to epigenetics, it is also possible that epigenetic modifications may affect the ability of phytoestrogens to be efficiently transferred from mother to infant through placental transfer. Future studies should investigate the effects of maternal transfer of soy isoflavones on bone growth and development as well as the long-term effects on bone loss later in life due to hormone deficiency.

8.5.3.2 Flaxseed

Among the edible plant foods, flaxseed is the richest source of lignans which are reported to have both weak estrogenic and anti-estrogenic activities [94]. It is also the richest source of secoisolariciresinol diglycoside, a mammalian lignin precursor, which is converted to the lignans enterodiol and enterolactone by colonic bacteria [95, 96]. The polyunsaturated fatty acid content of flaxseed, particularly α-linolenic acid (18:3n-3), may result in greater bone formation rats and may decrease the rate of bone resorption by inhibiting the biosynthesis of prostaglandins [97]. Lignans present in flaxseed also possess antioxidant properties [94]. Oxygen-derived free radicals, which are formed by a number of phagocytes including monocytes, macrophages, and neutrophils, have been reported to increase in chronic inflammatory diseases, aging, and osteoporosis. In vivo and in vitro findings indicate that free radicals generated in the bone environment enhance osteoclast formation and bone resorption [55]. Hence, it has been postulated that flaxseed would reduce the rapid rate of bone loss experienced in ovarian hormone deficiency, in part, by enhancing antioxidant status. The early evaluation of flaxseed, flaxseed oil, or its lignans has found that flaxseed and its bioactive components are neither beneficial nor harmful to growing male and female animals [98–100] while other studies have demonstrated beneficial effects on bone development [98, 101, 102]. However, feeding flaxseed or its lignans to growing female rats appear to have beneficial effects on bone strength although these benefits did not persist in adulthood [98, 102]. Flaxseed and its bioactive components have been shown to have preserve bone tissue in ovariectomized rats [103–106]. In addition to these findings, Sacco et al. have reported [103–105] the superior bone-protective effects of flaxseed on ovariectomized rats when combined with low-dose estrogen therapy versus flaxseed, estrogen, or basal diet. There is a paucity of studies investigating the bone-protective effects of flaxseed and its bioactive components in humans. When studied in postmenopausal women, it was demonstrated by two separate laboratories that flaxseed has no effects on biomarkers of bone metabolism [107] or BMD [108]. Whether longer-term or

dose-dependent, studies using whole flaxseed or its bioactive components, e.g. lignans or flaxseed oil, would exert a positive influence on BMD in postmenopausal women remains to be explored. Additionally, whether the animal findings in growing animals would translate to humans also remains to be determined. Furthermore, it is possible that the addition of flaxseed to the diets of pregnant animals or humans would result in improved bone development and bone health over the life course. Nonetheless, this is speculative and requires investigation.

8.5.3.3 Grapes

Grapes are an excellent source of polyphenols including flavans, anthocyanins, catechins, flavonols, and stilbenes (e.g., resveratrol). Whole grapes, grape seeds, and their bioactive components have been shown to have bone-protective properties. There are studies that link moderate alcohol consumption to improved bone mineral density and lower risk of hip fracture in humans [109]. The findings of epidemiological studies suggest that the beneficial effects of alcohol consumption, particularly wine, may be due to other components found in wine besides ethanol [110, 111]. Red wine has a high content of phenolic compounds, e.g. flavonoids such as resveratrol, which may favorably influence bone metabolism. Resveratrol, a flavonoid found naturally in the skins of most grape cultivars, has been reported [112] to antagonize the dioxin-induced inhibition of osteogenesis in bone forming culture. The influence of resveratrol on bone may, in part, be due to its ability to bind and activate ERs in bone [113]. Feeding resveratrol to spontaneously hypertensive stroke-prone ovariectomized rats has also been shown to prevent ovariectomy-induced decreases in femoral strength [114]. These beneficial effects, in part, may be associated with the ability of resveratrol in enhancing bone formation [115]. In an ovariectomized rat model of osteoporosis, Lin et al. [116] demonstrated that feeding resveratrol for 90 days resulted in greater BMD and ALP and lower TRAP5b than those of the other groups. The current evidence supports a possible role of grapes and their bioactive components, as well as processed grape products such as wine, in protecting skeletal health.

8.6 Conclusion

Osteoporosis-related fractures are a major cause of morbidity and mortality in the U.S. and worldwide, are very costly to treat, and as such are a public health concern. Preventive strategies such as diet and nutrition are more important than ever in reducing the morbidity and mortality associated with osteoporosis, as well as the economic burden associated with its prevalence. We know that genetics plays a role in the development of osteoporosis; however, emerging evidence is suggesting that epigenetics, or heritable changes in gene expression, may not only play a role in bone health outcomes but may itself be influenced by external environmental factors

such as nutrition. Additionally, these epigenetic modifications may influence critical periods of bone development such as in utero and during childhood and adolescence, as well as the development of bone loss and osteoporosis later in life. It will be important to conduct research studies in the future that are designed to evaluate the influence of nutritional interventions, such as functional foods and their bioactive components, on epigenetic modifications and the impact of these modifications on bone health outcomes. The findings of such studies will provide insight into the involvement of epigenetics on the effectiveness of nutrition interventions and will facilitate the development of more efficacious interventions. With respect to human bone health outcomes, the development of biomarkers of epigenetic modifications in various bodily fluids such as blood and urine will be useful for determining individuals who are at a greater risk of bone loss and osteoporosis. Additionally, this will facilitate the translation of preclinical findings to clinical trials and later to public health interventions aimed at promoting bone health.

References

1. Raisz LG (2005) Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest 115:3318–3325
2. Kanis JA, Melton LJ, Christiansen C, Johnston CC, Khaltaev N (1994) The diagnosis of osteoporosis. J Bone Miner Res 9:1137–1141
3. Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society (2010) Menopause 17:25–54; quiz 55-26
4. Pacifici R (1996) Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res 11:1043–1051
5. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A (2007) Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. J Bone Miner Res 22:465–475
6. Looker AC, Borrud LG, Dawson-Hughes B, Shepherd JA, Wright NC (2012) Osteoporosis or low bone mass at the femur neck or lumbar spine in older adults: United States, 2005–2008. NCHS Data Brief 93:1–8
7. Osteoporosis handout on health (2011). http://www.niams.nih.gov/Health_Info/Bone/Osteoporosis/osteoporosis_ff.asp
8. Delgado-Calle J, Garmilla P, Riancho JA (2012) Do epigenetic marks govern bone mass and homeostasis? Curr Genomics 13:252–263
9. Harvey N, Dennison E, Cooper C (2014) Osteoporosis: a lifecourse approach. J Bone Miner Res 29:1917–1925
10. Wright NC, Looker AC, Saag KG, Curtis JR, Delzell ES, Randall S, Dawson-Hughes B (2014) The recent prevalence of osteoporosis and low bone mass in the United States based on bone mineral density at the femoral neck or lumbar spine. J Bone Miner Res 29(11):2520–2526
11. Johnell O, Kanis JA (2006) An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int 17:1726–1733
12. Becker C (2008) Pathophysiology and clinical manifestations of osteoporosis. Clin Cornerstone 9:42–47, discussion 48–50
13. Szulc P, Munoz F, Claustrat B, Garnero P, Marchand F, Duboeuf F, Delmas PD (2001) Bioavailable estradiol may be an important determinant of osteoporosis in men: the MINOS study. J Clin Endocrinol Metab 86:192–199

14. Orwoll E, Lambert LC, Marshall LM, Phipps K, Blank J, Barrett-Connor E, Cauley J, Ensrud K, Cummings S (2006) Testosterone and estradiol among older men. J Clin Endocrinol Metab 91:1336–1344
15. NIH Consensus Development Panel on Osteoporosis Prevention Da, and Therapy: Osteoporosis prevention, diagnosis, and therapy. JAMA (2001) 285:785–795
16. Kalu DN (1991) The ovariectomized rat model of postmenopausal bone loss. Bone Miner 15:175–191
17. Vrtačnik P, Marc J, Ostanek B (2014) Epigenetic mechanisms in bone. Clin Chem Lab Med 52:589–608
18. Reppe S, Noer A, Grimholt RM, Halldórsson BV, Medina-Gomez C, Gautvik VT et al (2014) Methylation of bone SOST, its mRNA, and serum sclerostin levels correlate strongly with fracture risk in postmenopausal women. J Bone Miner Res doi:10.1002/jbmr.2342. [Epub ahead of print]
19. Delgado-Calle J, Sañudo C, Fernández AF, García-Renedo R, Fraga MF, Riancho JA (2012) Role of DNA methylation in the regulation of the RANKL-OPG system in human bone. Epigenetics 7:83–91
20. Delgado-Calle J, Sañudo C, Sánchez-Verde L, García-Renedo RJ, Arozamena J, Riancho JA (2011) Epigenetic regulation of alkaline phosphatase in human cells of the osteoblastic lineage. Bone 49:830–838
21. Arnsdorf EJ, Tummala P, Castillo AB, Zhang F, Jacobs CR (2010) The epigenetic mechanism of mechanically induced osteogenic differentiation. J Biomech 43:2881–2886
22. McGee-Lawrence ME, McCleary-Wheeler AL, Secreto FJ, Razidlo DF, Zhang M, Stensgard BA, Li X, Stein GS, Lian JB, Westendorf JJ (2011) Suberoylanilide hydroxamic acid (SAHA; vorinostat) causes bone loss by inhibiting immature osteoblasts. Bone 48:1117–1126
23. Leu YW, Chu PY, Chen CM, Yeh KT, Liu YM, Lee YH, Kuo ST, Hsiao SH (2014) Early life ethanol exposure causes long-lasting disturbances in rat mesenchymal stem cells via epigenetic modifications. Biochem Biophys Res Commun 453(3):338–344
24. Ko JY, Chuang PC, Chen MW, Ke HC, Wu SL, Chang YH, Chen YS, Wang FS (2013) MicroRNA-29a ameliorates glucocorticoid-induced suppression of osteoblast differentiation by regulating β-catenin acetylation. Bone 57:468–475
25. Wang Y, Li L, Moore BT, Peng XH, Fang X, Lappe JM, Recker RR, Xiao P (2012) MiR-133a in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. PLoS ONE 7:e34641
26. Weaver CM, Alekel DL, Ward WE, Ronis MJ (2012) Flavonoid intake and bone health. J Nutr Gerontol Geriatr 31:239–253
27. Sellappan S, Akoh CC, Krewer G (2002) Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. J Agric Food Chem 50:2432–2438
28. Fukumoto LR, Mazza G (2000) Assessing antioxidant and prooxidant activities of phenolic compounds. J Agric Food Chem 48:3597–3604
29. Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20:933–956
30. Manthey JA, Buslig B (1996) Flavonoids in the living system. In: Proceedings of an American chemical society symposium on advances in experimental medicine and biology, vol 439 p 278
31. Hooshmand S, Arjmandi BH (2009) Viewpoint: dried plum, an emerging functional food that may effectively improve bone health. Ageing Res Rev 8:122–127
32. Arjmandi BH, Lucas EA, Juma S, Soliman A, Stoecker BJ, Khalil DA, Smith BJ, Wang C (2001) Dried plums prevent ovariectomy-induced bone loss in rats. J Am Nutraceutical Assoc 4:50–56
33. Deyhim F, Stoecker BJ, Brusewitz GH, Devareddy L, Arjmandi BH (2005) Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. Menopause 12:755–762

34. Arjmandi BH, Johnson CD, Campbell SC, Hooshmand S, Chai SC, Akhter MP (2010) Combining fructooligosaccharide and dried plum has the greatest effect on restoring bone mineral density among select functional foods and bioactive compounds. J Med Food 13:312–319
35. Johnson CD, Lucas EA, Hooshmand S, Campbell S, Akhter MP, Arjmandi BH (2011) Addition of fructooligosaccharides and dried plum to soy-based diets reverses bone loss in the ovariectomized rat. Evid Based Complement Alternat Med 2011:836267
36. Rendina E, Lim YF, Marlow D, Wang Y, Clarke SL, Kuvibidila S, Lucas EA, Smith BJ (2012) Dietary supplementation with dried plum prevents ovariectomy-induced bone loss while modulating the immune response in C57BL/6 J mice. J Nutr Biochem 23:60–68
37. Rendina E, Hembree KD, Davis MR, Marlow D, Clarke SL, Halloran BP, Lucas EA, Smith BJ (2013) Dried plum's unique capacity to reverse bone loss and alter bone metabolism in postmenopausal osteoporosis model. PLoS One 8:e60569
38. Smith BJ, Bu SY, Wang Y, Rendina E, Lim YF, Marlow D, Clarke SL, Cullen DM, Lucas EA (2014) A comparative study of the bone metabolic response to dried plum supplementation and PTH treatment in adult, osteopenic ovariectomized rat. Bone 58:151–159
39. Mühlbauer RC, Lozano A, Reinli A, Wetli H (2003) Various selected vegetables, fruits, mushrooms and red wine residue inhibit bone resorption in rats. J Nutr 133:3592–3597
40. Franklin M, Bu SY, Lerner MR, Lancaster EA, Bellmer D, Marlow D, Lightfoot SA, Arjmandi BH, Brackett DJ, Lucas EA, Smith BJ (2006) Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone 39:1331–1342
41. Bu SY, Lucas EA, Franklin M, Marlow D, Brackett DJ, Boldrin EA, Devareddy L, Arjmandi BH, Smith BJ (2007) Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. Osteoporos Int 18:931–942
42. Halloran BP, Wronski TJ, VonHerzen DC, Chu V, Xia X, Pingel JE, Williams AA, Smith BJ (2010) Dietary dried plum increases bone mass in adult and aged male mice. J Nutr 140:1781–1787
43. Smith BJ, Graef JL, Wronski TJ, Rendina E, Williams AA, Clark KA, Clarke SL, Lucas EA, Halloran BP (2014) Effects of dried plum supplementation on bone metabolism in adult C57BL/6 male mice. Calcif Tissue Int 94:442–453
44. Arjmandi BH, Khalil DA, Lucas EA, Georgis A, Stoecker BJ, Hardin C, Payton ME, Wild RA (2002) Dried plums improve indices of bone formation in postmenopausal women. J Womens Health Gend Based Med 11:61–68
45. Hooshmand S, Chai SC, Saadat RL, Payton ME, Brummel-Smith K, Arjmandi BH (2011) Comparative effects of dried plum and dried apple on bone in postmenopausal women. Br J Nutr 106:923–930
46. Banfi G, Iorio EL, Corsi MM (2008) Oxidative stress, free radicals and bone remodeling. Clin Chem Lab Med 46:1550–1555
47. Prior R, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner N, Ehlenfeldt M, Kalt W, Krewer G, Mainland C (1998) Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of vaccinium species. J Agric Food Chem 46:2686–2693
48. Sellappan S, Akoh C, Krewer G (2002) Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. J Agric Food Chem 50:2432–2438
49. Sellappan S, Akoh CC, Krewer G (2002) Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries 50
50. Moze S, Polak T, Gasperlin L, Koron D, Vanzo A, Abram V (2011) Phenolics in Slovenian bilberries (Vaccinium myrtillus L.) and blueberries (Vaccinium corymbosum L.). J Agric Food Chem 59:6998–7004
51. Kalt W, Ryan DAJ, Duy JC, Prior RL, Ehlenfeldt MK, Kloet SPV (2001) Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (vaccinium section cyanococcus spp.) J Agric Food Chem 49 (10):4761–4767

52. Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner N, Ehlenfeldt M, Kalt W, Gerald K, Mainland CM (1998) Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of vaccinium species J Agric Food Chem 46 (7):2686–2693
53. Ehlenfeldt MK, Prior RL (2001) Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry J Agric Food Chem 49(5):2222–2227
54. Pulido R, Bravo L, Saura-Calixto F (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agric Food Chem 48:3396–3402
55. Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR (1990) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 85:632–639
56. Devareddy L, Hooshmand S, Collins JK, Lucas EA, Chai SC, Arjmandi BH (2008) Blueberry prevents bone loss in ovariectomized rat model of postmenopausal osteoporosis. J Nutr Biochem 19:694–699
57. Chen JR, Lazarenko OP, Wu X, Kang J, Blackburn ML, Shankar K, Badger TM, Ronis MJ (2010) Dietary-induced serum phenolic acids promote bone growth via p38 MAPK/β-catenin canonical Wnt signaling. J Bone Miner Res 25:2399–2411
58. Zhang J, Lazarenko OP, Blackburn ML, Shankar K, Badger TM, Ronis MJ, Chen JR (2011) Feeding blueberry diets in early life prevent senescence of osteoblasts and bone loss in ovariectomized adult female rats. PLoS One 6:e24486
59. Zhang J, Lazarenko OP, Blackburn ML, Badger TM, Ronis MJ, Chen JR (2013) Blueberry consumption prevents loss of collagen in bone matrix and inhibits senescence pathways in osteoblastic cells. Age (Dordr) 35:807–820
60. Zhang J, Lazarenko OP, Kang J, Blackburn ML, Ronis MJ, Badger TM, Chen JR (2013) Feeding blueberry diets to young rats dose-dependently inhibits bone resorption through suppression of RANKL in stromal cells. PLoS One 8:e70438
61. Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138:863–870
62. Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engström O, Ljunggren J, Gustafsson JA, Carlquist M (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J 18:4608–4618
63. Kostelac D, Rechkemmer G, Briviba K (2003) Phytoestrogens modulate binding response of estrogen receptors alpha and beta to the estrogen response element. J Agric Food Chem 51:7632–7635
64. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263
65. Teede HJ, Dalais FS, McGrath BP (2004) Dietary soy containing phytoestrogens does not have detectable estrogenic effects on hepatic protein synthesis in postmenopausal women. Am J Clin Nutr 79:396–401
66. Messina M, McCaskill-Stevens W, Lampe JW (2006) Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. J Natl Cancer Inst 98:1275–1284
67. Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewica-Sapuntzakis M, Gou P (1996) Dietary soy protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 126:161–167
68. Arjmandi BH, Birnbaum R, Goyal NV, Getlinger MJ, Juma S, Alekel L, Hasler CM, Drum ML, Hollis BW, Kukreja SC (1998) Bone-sparing effect of soy protein in ovarian hormone-deficient rats is related to its isoflavone content. Am J Clin Nutr 68:1364S–1368S

69. Devareddy L, Khalil DA, Smith BJ, Lucas EA, Soung DY, Marlow DD, Arjmandi BH (2006) Soy moderately improves microstructural properties without affecting bone mass in an ovariectomized rat model of osteoporosis. Bone 38:686–693
70. Devareddy L, Khalil DA, Korlagunta K, Hooshmand S, Bellmer DD, Arjmandi BH (2006) The effects of fructo-oligosaccharides in combination with soy protein on bone in osteopenic ovariectomized rats. Menopause 13:692–699
71. Hooshmand S, Juma S, Arjmandi BH (2010) Combination of genistin and fructooligosaccharides prevents bone loss in ovarian hormone deficiency. J Med Food 13:320–325
72. Ma DF, Qin LQ, Wang PY, Katoh R (2008) Soy isoflavone intake increases bone mineral density in the spine of menopausal women: meta-analysis of randomized controlled trials. Clin Nutr 27:57–64
73. Ma DF, Qin LQ, Wang PY, Katoh R (2008) Soy isoflavone intake inhibits bone resorption and stimulates bone formation in menopausal women: meta-analysis of randomized controlled trials. Eur J Clin Nutr 62:155–161
74. Taku K, Melby MK, Nishi N, Omori T, Kurzer MS (2011) Soy isoflavones for osteoporosis: an evidence-based approach. Maturitas 70:333–338
75. Shedd-Wise KM, Alekel DL, Hofmann H, Hanson KB, Schiferl DJ, Hanson LN, Van Loan MD (2011) The soy isoflavones for reducing bone loss study: 3-yr effects on pQCT bone mineral density and strength measures in postmenopausal women. J Clin Densitom 14:47–57
76. Newton KM, LaCroix AZ, Levy L, Li SS, Qu P, Potter JD, Lampe JW (2006) Soy protein and bone mineral density in older men and women: a randomized trial. Maturitas 55:270–277
77. Alekel DL, Van Loan MD, Koehler KJ, Hanson LN, Stewart JW, Hanson KB, Kurzer MS, Peterson CT (2010) The soy isoflavones for reducing bone loss (SIRBL) study: a 3-y randomized controlled trial in postmenopausal women. Am J Clin Nutr 91:218–230
78. Tai TY, Tsai KS, Tu ST, Wu JS, Chang CI, Chen CL, Shaw NS, Peng HY, Wang SY, Wu CH (2012) The effect of soy isoflavone on bone mineral density in postmenopausal Taiwanese women with bone loss: a 2-year randomized double-blind placebo-controlled study. Osteoporos Int 23:1571–1580
79. Levis S, Strickman-Stein N, Ganjei-Azar P, Xu P (2011) Doerge DR, Krischer J: Soy isoflavones in the prevention of menopausal bone loss and menopausal symptoms: a randomized, double-blind trial. Arch Intern Med 171:1363–1369
80. Arjmandi BH, Khalil DA, Smith BJ, Lucas EA, Juma S, Payton ME, Wild RA (2003) Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion. J Clin Endocrinol Metab 88:1048–1054
81. Arjmandi BH, Lucas EA, Khalil DA, Devareddy L, Smith BJ, McDonald J, Arquitt AB, Payton ME, Mason C (2005) One year soy protein supplementation has positive effects on bone formation markers but not bone density in postmenopausal women. Nutr J 4:8
82. Erben RG, Eberle J, Stahr K, Goldberg M (2000) Androgen deficiency induces high turnover osteopenia in aged male rats: a sequential histomorphometric study. J Bone Miner Res 15:1085–1098
83. Vanderschueren D, Vandenput L, Boonen S, Van Herck E, Swinnen JV, Bouillon R (2000) An aged rat model of partial androgen deficiency: prevention of both loss of bone and lean body mass by low-dose androgen replacement. Endocrinology 141:1642–1647
84. Khalil DA, Lucas EA, Smith BJ, Soung DY, Devareddy L, Juma S, Akhter MP, Recker R, Arjmandi BH (2005) Soy isoflavones may protect against orchidectomy-induced bone loss in aged male rats. Calcif Tissue Int 76:56–62
85. Soung DY, Devareddy L, Khalil DA, Hooshmand S, Patade A, Lucas EA, Arjmandi BH (2006) Soy affects trabecular microarchitecture and favorably alters select bone-specific gene expressions in a male rat model of osteoporosis. Calcif Tissue Int 78:385–391
86. Juma SS, Ezzat-Zadeh Z, Khalil DA, Hooshmand S, Akhter M, Arjmandi BH (2012) Soy protein with or without isoflavones failed to preserve bone density in gonadal hormone-deficient male rat model of osteoporosis. Nutr Res 32:694–700

87. Khalil DA, Lucas EA, Juma S, Smith BJ, Payton ME, Arjmandi BH (2002) Soy protein supplementation increases serum insulin-like growth factor-I in young and old men but does not affect markers of bone metabolism. J Nutr 132:2605–2608
88. Kolios L, Sehmisch S, Daub F, Rack T, Tezval M, Stuermer KM, Stuermer EK (2009) Equol but not genistein improves early metaphyseal fracture healing in osteoporotic rats. Planta Med 75:459–465
89. Kaludjerovic J, Ward WE (2009) Neonatal exposure to daidzein, genistein, or the combination modulates bone development in female CD-1 mice. J Nutr 139:467–473
90. Kaludjerovic J, Ward WE (2010) Neonatal administration of isoflavones attenuates deterioration of bone tissue in female but not male mice. J Nutr 140:766–772
91. Gutowska I, Baranowska-Bosiacka I, Noceń I, Piotrowska K, Marchlewicz M, Wiernicki I, Chlubek D, Wiszniewska B (2012) Soy isoflavones administered pre- and postnatally may affect the ERα and ERβ expression and elements' content in bones of mature male rats. Hum Exp Toxicol 31:346–354
92. Foster WG, Chan S, Platt L, Hughes CL (2002) Detection of phytoestrogens in samples of second trimester human amniotic fluid. Toxicol Lett 129:199–205
93. Adlercreutz H, Yamada T, Wähälä K, Watanabe S (1999) Maternal and neonatal phytoestrogens in Japanese women during birth. Am J Obstet Gynecol 180:737–743
94. Touré A, Xueming X (2010) Flaxseed lignans: source, biosynthesis, metabolism, antioxidant activity, bio-active components, and health benefits. Compr Rev Food Sci Food Saf 9:261–269
95. Thompson LU, Robb P, Serraino M, Cheung F (1991) Mammalian lignan production from various foods. Nutr Cancer 16:43–52
96. Collins BM, McLachlan JA, Arnold SF (1997) The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. Steroids 62:365–372
97. Watkins BA, Li Y, Lippman HE, Feng S (2003) Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism. Prostaglandins Leukot Essent Fatty Acids 68:387–398
98. Ward WE, Yuan YV, Cheung AM, Thompson LU (2001) Exposure to flaxseed and its purified lignan reduces bone strength in young but not older male rats. J Toxicol Environ Health A 63:53–65
99. Ward WE, Yuan YV, Cheung AM, Thompson LU (2001) Exposure to purified lignan from flaxseed (Linum usitatissimum) alters bone development in female rats. Br J Nutr 86:499–505
100. Cohen SL, Ward WE (2005) Flaxseed oil and bone development in growing male and female mice. J Toxicol Environ Health A 68:1861–1870
101. Lau BY, Fajardo VA, McMeekin L, Sacco SM, Ward WE, Roy BD, Peters SJ, Leblanc PJ (2010) Influence of high-fat diet from differential dietary sources on bone mineral density, bone strength, and bone fatty acid composition in rats. Appl Physiol Nutr Metab 35:598–606
102. Lukas R, Gigliotti JC, Smith BJ, Altman S, Tou JC (2011) Consumption of different sources of omega-3 polyunsaturated fatty acids by growing female rats affects long bone mass and microarchitecture. Bone 49:455–462
103. Sacco SM, Jiang JM, Reza-López S, Ma DW, Thompson LU, Ward WE (2009) Flaxseed combined with low-dose estrogen therapy preserves bone tissue in ovariectomized rats. Menopause 16:545–554
104. Sacco SM, Jiang JM, Reza-Lopez S, Ma DW, Thompson LU, Ward WE (2009) Flaxseed does not antagonize the effect of ultra-low-dose estrogen therapy on bone mineral density and biomechanical bone strength in ovariectomized rats. J Toxicol Environ Health A 72:1209–1216
105. Sacco SM, Chen J, Ganss B, Thompson LU, Ward WE (2014) Flaxseed enhances the beneficial effect of low-dose estrogen therapy at reducing bone turnover and preserving bone microarchitecture in ovariectomized rats. Appl Physiol Nutr Metab 39:801–810
106. Boulbaroud S, Mesfioui A, Arfaoui A, Ouichou A (2008) el-Hessni A: Preventive effects of flaxseed and sesame oil on bone loss in ovariectomized rats. Pak J Biol Sci 11:1696–1701

107. Lucas EA, Wild RD, Hammond LJ, Khalil DA, Juma S, Daggy BP, Stoecker BJ, Arjmandi BH (2002) Flaxseed improves lipid profile without altering biomarkers of bone metabolism in postmenopausal women. J Clin Endocrinol Metab 87:1527–1532
108. Dodin S, Lemay A, Jacques H, Légaré F, Forest JC, Mâsse B (2005) The effects of flaxseed dietary supplement on lipid profile, bone mineral density, and symptoms in menopausal women: a randomized, double-blind, wheat germ placebo-controlled clinical trial. J Clin Endocrinol Metab 90:1390–1397
109. Berg KM, Kunins HV, Jackson JL, Nahvi S, Chaudhry A, Harris KA, Malik R, Arnsten JH (2008) Association between alcohol consumption and both osteoporotic fracture and bone density. Am J Med 121:406–418
110. Fairweather-Tait SJ, Skinner J, Guile GR, Cassidy A, Spector TD, MacGregor AJ (2011) Diet and bone mineral density study in postmenopausal women from the TwinsUK registry shows a negative association with a traditional English dietary pattern and a positive association with wine. Am J Clin Nutr 94:1371–1375
111. Tucker KL, Jugdaohsingh R, Powell JJ, Qiao N, Hannan MT, Sripanyakorn S, Cupples LA, Kiel DP (2009) Effects of beer, wine, and liquor intakes on bone mineral density in older men and women. Am J Clin Nutr 89:1188–1196
112. Singh SU, Casper RF, Fritz PC, Sukhu B, Ganss B, Girard B, Savouret JF, Tenenbaum HC (2000) Inhibition of dioxin effects on bone formation in vitro by a newly described aryl hydrocarbon receptor antagonist, resveratrol. J Endocrinol 167:183–195
113. Gehm BD, McAndrews JM, Chien PY, Jameson JL (1997) Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci USA 94:14138–14143
114. Mizutani K, Ikeda K, Kawai Y, Yamori Y (2000) Resveratrol attenuates ovariectomy-induced hypertension and bone loss in stroke-prone spontaneously hypertensive rats. J Nutr Sci Vitaminol (Tokyo) 46:78–83
115. Mizutani K, Ikeda K, Kawai Y, Yamori Y (1998) Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 253:859–863
116. Lin Q, Huang YM, Xiao BX, Ren GF (2005) Effects of resveratrol on bone mineral density in ovarectomized rats. Int J Biomed Sci 1:76–81

Chapter 9
Nutritional Interventions for Cardiovascular Aging and Age-Related Cardiovascular Diseases

Ken Shinmura

Abstract Both morbidity and mortality of cardiovascular diseases (CVDs) increase with age. The increased prevalence of cardiovascular risk factors with age and cardiovascular aging contribute to the association between aging and CVDs. Most developed countries would benefit from the development of novel therapeutics to control cardiovascular aging because they are confronted with an aged society. Dietary restriction (DR) including caloric restriction (CR) and alternate-day fasting is an established nutritional intervention with scientifically proved anti-aging effects. Recent experimental and clinical investigations demonstrate that DR exerts pleiotropic effects on the cardiovascular system. CR prevents the progression of atherosclerosis and vascular aging via direct and indirect mechanisms. CR prevents cardiac senescence by attenuating oxidative damage and enhancing cardiac autophagy, leading to improved cardiac function in aged animals. DR improves myocardial ischemic tolerance in rodents of all ages. DR counteracts age-associated changes in autonomic nerve function. CR may mitigate metabolic cardiomyopathy associated with obesity and type 2 diabetes mellitus. The mechanisms underlying the beneficial cardiovascular effects of DR are multifaceted, but considerable progress has been made in the past decade toward their understanding. Recent investigations reveal that DR triggers an active defense response against stressful conditions. At the center of this response are cardiovascular protective signals, which include the mammalian target of rapamycin, AMP-activated kinase, sirtuins, and endothelial nitric oxide synthase. They form a network with both positive and negative feedbacks. Therefore, DR and CR mimetics that can replicate the effects of CR are promising interventions for regulating cardiovascular aging and managing patients with CVDs.

K. Shinmura (✉)
Department of Cardiology, Keio University School of Medicine,
35 Shinanomachi,
160-8582 Shinjuku-ku, Tokyo, Japan
e-mail: shimmura@z5.keio.jp

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9.1 Nutritional Interventions for Cardiovascular Aging and Age-Related Cardiovascular Diseases

9.1.1 Dietary Restriction Is an Established Anti-aging Nutritional Intervention

Numerous experimental interventions designed to regulate the aging process have been attempted. To date, an established intervention that has been consistently shown to reduce the rate of aging and to increase both mean and maximal lifespan in various species is lifelong dietary restriction (DR) [1–6]. Caloric restriction (CR) is the most evaluated method among various types of DR. In addition to remarkable lifespan extension, CR profoundly affects age-related physiological and pathophysiological alterations [1–6]. The effect of CR is clearly distinct from that of malnutrition and starvation. In general, there is no evidence of malnutrition or cachexia in animals treated with standard CR regimens if caloric intake is restricted to 40–70 % of ad libitum (AL) quantities [1–6]. CR decreases oncogenesis and apoptosis, and some experimental studies suggest that CR attenuates the incidence of dementia and neurodegenerative diseases [1–7]. CR is also anticipated to reduce cardiovascular morbidity and mortality by conferring pleiotropic cardiovascular protection [3–6].

The exact mechanisms by which CR extends lifespan have not been fully evaluated, but mounting evidence demonstrates that a reduction in oxidative stress contributes, at least in part, to the anti-aging effects of CR [1, 3–6, 8, 9]. CR attenuates the age-associated increase in mitochondrial reactive oxygen species (ROS) production, lipid peroxidation, protein oxidation, and oxidative damage to mitochondrial DNA in various organs. Several studies have reported that CR significantly decreases oxidative damage in the aged heart [4]. The levels of 8-oxo-2′-deoxyguanosine were lower in cardiac mitochondria obtained from rats receiving CR than in those from AL-fed controls [4].

Although it is well accepted that oxidative stress is involved in the aging process and the anti-aging effect of CR is closely related to a reduction in ROS-induced cellular damage, there are still very little data that demonstrate relationships between oxidative stress and the aging process in humans or larger animals. An increased baseline level of oxidative damage to DNA is associated with age and several age-related diseases including cardiovascular diseases (CVDs) [4]. Additionally, higher levels of protein carbonyls are observed with increased age in healthy human subjects [4]. In contrast, a recent investigation from Comprehensive Assessment of the Long-term Effect of Reducing Intake of Energy (CALERIE) demonstrated that DNA damage was reduced from baseline after 6 months in individuals assigned to CR but not in control subjects [10]. Although the effects of CR on lifespan in non-human primates are inconsistent, reductions in age-associated oxidative damage by CR have been observed in non-human primates [11]. These results suggest that the oxidative stress theory of aging would apply to humans, at least in part.

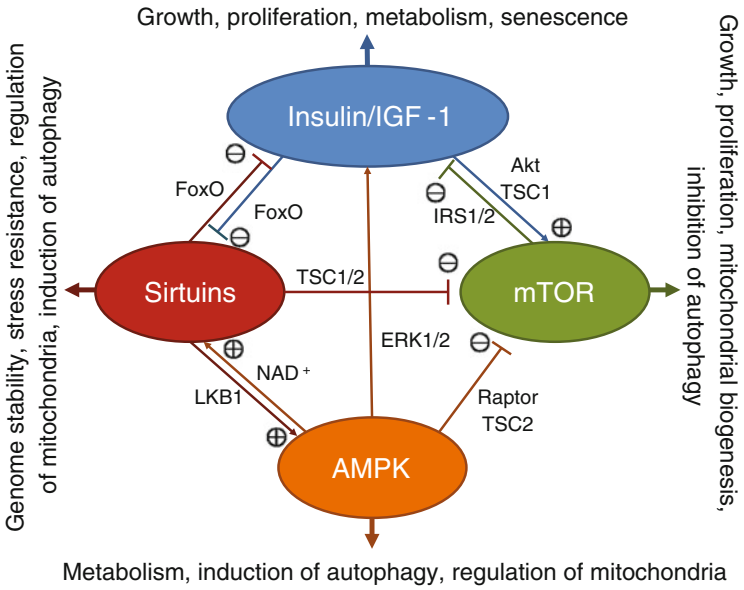

Fig. 9.1 Four types of intracellular signaling pathways mediating the effects of caloric restriction (CR) form a network. *Insulin/IGF-1* insulin/insulin-like growth factor-1, *FoxO* Forkhead box protein O, *IRS1/2* insulin receptor substrate 1/2, *mTOR* mammalian target of rapamycin, *ERK1/2* extracellular signal-regulated kinase 1/2, *LKB1* liver kinase B1, *NAD*$^+$ Nicotinamide adenine dinucleotide, *AMPK* AMP-activated protein kinase, *TSC* tuberous sclerosis complex

Considerable progress has been made in the past decade toward understanding why CR works. Recent investigations reveal that CR triggers an active defense response that evolved to promote survival during stressful conditions (Fig. 9.1) [5, 12]. At the center of this response are so-called longevity regulatory pathways, which include insulin/insulin-like growth factor 1, the mammalian target of rapamycin (mTOR), AMP-activated kinase (AMPK), and nicotinamide adenine dinucleotide $(NAD)^+$-dependent deacetylases, which are also called sirtuins. These signaling pathways form a network with both positive and negative feedbacks [5, 12]. They also play an important role in the development of CR-induced cardiovascular protection as described below.

9.1.2 Caloric Restriction and Alternate-Day Fasting (or Intermittent Fasting)

Rodents receiving CR are generally fed 50–70 % of their average caloric intake and are not able to eat freely throughout the protocol. Food is given once a day at a fixed time, just before the 12-h dark cycle. Alternative-day fasting (ADF) or intermittent

fasting (IF) is another protocol of DR wherein individuals are subjected to various periods of fasting [13]. In general, rodents are fed AL only every other day and subjected to a 24-h fasting period except for free water drinking. Based on animal experiments, ADF also results in prolonged lifespan, reduced metabolic risk factors for diabetes and CVDs, and reduced prevalence of age-related diseases, similar to those observed with CR [13]. Therefore, ADF and CR regimens appear to affect similar intracellular signaling pathways that lead to the preferable outcomes. However, CR results in greater body weight (BW) loss compared to ADF. Recent investigations also revealed several differences between the effects of CR and those of ADF [14, 15], strongly suggesting that a distinct signaling pathway is involved in the effects of CR and ADF.

9.1.3 Possible Indications of Dietary Restriction for Cardiovascular Diseases

The prevalence of left ventricular hypertrophy (LVH), atrial fibrillation (AF), and congestive heart failure (CHF) increases dramatically with aging [16]. The prevalence of LVH also increases with elevated blood pressure (BP) and increased body mass index (BMI). LVH evaluated by either echocardiography or electrocardiography has been shown to be associated with increased risks for coronary heart disease, sudden death, stroke, and overall cardiovascular events [16]. In the Framingham study, a history of AF without identified cause (lone AF) was present in 16.8 % of men and 6 % of women with AF at a mean age of 70 years [16]. During long-term follow-up, individuals with lone AF suffered more than 4 times as many strokes (most were cerebral embolism) as control subjects. The morbidity of CHF increases with aging, as does mortality [16, 17]. Approximately half of older patients with CHF show normal left ventricular (LV) systolic but abnormal LV diastolic function [17, 18]. The development of LV diastolic dysfunction and increased prevalence of AF associated with advanced age are clearly related to the physiological decline of cardiac function due to cardiovascular aging. Myocardial infarction (MI) is the most common cause of CHF associated with systolic dysfunction. The incidence of MI also increases with age because advanced age is a risk factor for the development of atherosclerosis [17]. Elderly patients are not only more likely to experience a MI than young patients but are also more likely to develop CHF following a MI [17].

The poor outcomes of CVDs in the elderly can be explained, at least in part, by cardiac aging both at the cellular and organ levels. Thus, improved understanding of cardiovascular aging and the development of novel therapeutics to control cardiovascular aging will benefit most developed countries that are confronted with an aging or aged society. Therefore, DR attracted attention for the purpose of controlling cardiovascular aging in these countries.

To evaluate the possible indications for DR in age-related CVDs, a discussion of the effects of DR on atherosclerosis and vascular aging is warranted. Atherosclerosis and vascular aging are not independent of each other and each contributes specific components to what is presently referred to as clinical vascular disease [19]. Therefore, aging blood vessels provide the milieu in which atherosclerosis can flourish. DR can prevent both atherosclerosis and vascular aging [3, 6].

Next, LVH and CHF are representative diseases associated with cardiac aging [16]. Thus, the prevention of CHF associated with LVH, most of which is characterized as heart failure with preserved ejection fraction (HFpEF), is regarded as a good indication for DR.

Since the hormesis theory, implying a favorable adaptive response to a sublethal degree of stress, is one of the strong hypotheses by which DR may elicit various preferable effects [1, 3, 5], acute coronary syndrome (ACS) and ischemic heart disease (IHD) may be additional target diseases for the clinical application of DR. In fact, mounting evidence demonstrates that animals receiving CR exhibit powerful resistance against various acute stress stimuli [1, 3, 5].

In addition to population aging, overeating and obesity are common health problems in developed countries. The accumulation of visceral adipose tissue associated with overeating and obesity causes metabolic syndrome and type 2 diabetes mellitus (T2DM), resulting in an increase in morbidity and mortality from atherosclerotic diseases [20, 21]. Mounting evidence indicates that overeating and obesity accelerate the aging process, whereas DR retards the aging process. Therefore, CVDs associated with metabolic disorders should be the best indication for DR.

CR can attenuate the incidence of neurodegenerative diseases in animal models of Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, Parkinson's disease, and spinal muscular atrophy [7]. These results strongly suggest that DR can prevent the development of genetic and/or degenerative diseases regardless of aging and obesity. Thus, this author speculates that DR may retard the development of genetic cardiomyopathy such as mitochondrial myopathy. However, no experimental evaluation has been reported, and this issue remains unsolved.

9.2 Nutritional Interventions to Protect Against Vascular Aging and Atherosclerosis

9.2.1 Evidence from Human Studies

9.2.1.1 Effects of Caloric Restriction on Vascular Aging and Atherosclerosis

Mounting evidence supports the concept that "a man is as old as his arteries" [19]. Increased thickening and stiffness of large arteries and endothelial dysfunction in apparently healthy elderly persons, along with the ensuing increase in systolic BP

and pulse pressure, precede clinical disease and predict a higher risk for developing clinical atherosclerosis, ACS and stroke [19]. Some of these vascular changes that occur with aging in apparently healthy people have been observed in hypertensive patients at an earlier age and are more remarkable than those in apparently healthy subjects. Such age-related vascular changes can be referred to as vascular aging. When stated in this context, vascular aging becomes a risk factor for eventual clinical disease manifestation.

Atherosclerosis and the subsequent cardiovascular complications, such as ACS, IHD, and stroke, are major causes of death worldwide. The risk factors for atherosclerosis include hypertension, T2DM, higher levels of serum total and low-density lipoprotein (LDL) cholesterol, and smoking. Aging is also an important risk factor for atherosclerosis and persists as an independent contributor when all other known factors are controlled. Premature or accelerated vascular aging can be promoted by cardiovascular risk factors, and cellular senescence is also observed in patients with atherosclerosis [19]. Therefore, atherosclerosis is a disease of both organismal aging and cellular senescence.

CR has been shown to retard vascular aging in both rodents and humans [3, 6, 10, 11, 22–25]. The mechanisms underlying the beneficial cardiovascular effects of CR are undoubtedly multifaceted, including improvements in systemic risk factors for atherosclerosis such as dyslipidemia and insulin resistance, BP reduction and exertion of direct anti-aging effects on the vasculature [3, 6].

Phase 1 of CALERIE consisted of 3 pilot studies to determine whether an investigation of the effects of long-term CR in free-living humans was feasible and to obtain preliminary data on the adaptive responses to CR [10, 24]. In the Pennington Phase 1 CALERIE study, 6 months of CR resulted in a 10 % decrease in BW, significant decreases in core body temperature, 24-h energy expenditure, and triiodothyronine, body fat mass, visceral adipose tissue, subcutaneous adipose tissue, fat cell size, intrahepatic fat, and fasting insulin; and an improvement in insulin sensitivity. In the Washington University Phase 1 CALERIE study, 12 months of CR resulted in an 11 % decrease in BW. Total body fat mass, visceral fat mass, subcutaneous abdominal fat mass, leptin, fasting insulin, and glucose and insulin areas under the oral glucose tolerance curve decreased significantly, while the insulin sensitivity index increased in response to CR. In the Tufts Phase 1 CALERIE study, 12 months of CR resulted in a 10 % decrease in BW, body fat mass, visceral adipose tissue, subcutaneous adipose tissue, serum LDL-cholesterol levels, fasting insulin, and an improvement in insulin sensitivity. These 3 independent studies revealed that CR improves insulin sensitivity and lowers serum triglyceride levels in obese individuals, suggesting anti-atherosclerotic effects of CR in humans [24].

All participants in Phase 1 of CALERIE were initially overweight but were classified in the upper normal BMI range to moderately overweight at the end of the study period [24]. Notably, Phase 1 demonstrated that CR is feasible in free-living humans and led to the implementation of Phase 2 of CALERIE. In Phase 2, the participants were healthy men from 21 to 50 years old and women from 21 to

47 years old; each subject's BMI was between 22 and 28 kg/m^2 [26]. Finally, 220 participants were randomized in a 2:1 ratio between the CR and control (AL) groups, and 218 started the assigned intervention, which is ongoing. The intervention involves a 25 % decrease in calorie intake for 2 years. Primary outcomes are resting metabolic rate and core temperature, and secondary outcomes address oxyradical formation, cardiovascular risk markers, insulin sensitivity and secretion, immune function, neuroendocrine function, quality of life, and cognitive function [26].

The members of the Caloric Restriction Society (CRS) restrict their food intake by their own intention. Most CRS members are men, and only 4 of those we have studied are women. Fontana et al. measured risk factors for atherosclerosis in 18 CRS members, with an average age of 50 ± 10 years and who had been practicing CR for an average of 6 years, and 18 healthy age-matched individuals eating typical American diets [23]. Calorie intakes in the CRS group were lower by 40 % than those in the control group. Average BMI was 19.6 ± 1.9 kg/m^2 in the CRS group and 25.9 ± 3.2 kg/m^2 in the control group. Total body fat averaged 6.7 % in the CRS men and 22.4 % in the control group men. Total and LDL cholesterol and triglyceride levels were markedly lower, while high-density lipoprotein cholesterol levels were higher in the CRS than in the control group. Fasting plasma insulin and glucose levels were also significantly lower in the CRS group. Two of the most remarkable findings in the CRS members were their extremely low BP and thin carotid intima–media thickness measured by carotid echography [23]. In addition, the CRS members exhibited lower levels of chronic inflammation, as reflected by significantly lower circulating levels of C-reactive protein and tumor necrosis factor-α (TNF-α) than the healthy age-matched controls [27].

9.2.1.2 Effects of Alternate-Day Fasting on Vascular Aging and Atherosclerosis

Compared with the protocol that is used in experimental animal studies, the ADF protocol recently used in human clinical studies is mild [28]. ADF consists of an AL feed day alternated with a 25 % energy intake fast day. Although the observational duration has been 12 weeks at most, ADF was effective for weight loss (−6.5 %) and improving the atherosclerotic risk factors in normal weight and overweight adults [28]. Although the long-term effects of ADF on vascular aging and atherosclerosis remain unclear, this ADF protocol seems to be promising for treating overweight patients and preventing the development of T2DM in patients with CVDs.

9.2.2 Evidence from Experimental Animal Studies

Mounting evidence demonstrates that increased production of ROS leads to endothelial dysfunction with aging in laboratory animals [3, 6, 11]. One of the

consequences of increased oxidative stress with aging is a functional inactivation of nitric oxide (NO) by high levels of superoxide production. Impaired NO bioavailability due to age-related oxidative stress in the coronary circulation and other vascular beds results in severe impairments of flow/shear-induced vasodilation [3, 6, 11]. In addition to maintaining normal organ blood flow, NO derived from endothelial cells confers vasoprotective and cardioprotective effects, including inhibition of platelet aggregation, inhibited adhesion of inflammatory cells to endothelial cells, disruption of pro-inflammatory cytokine-induced signaling pathways, inhibition of apoptosis, preservation of endothelial progenitor cell function and regulation of tissue energy metabolism [3, 6, 11]. The impairment of NO bioavailability is aggravated by an age-related decline in endothelial NO synthase (eNOS) expression, reduced availability of tetrahydrobiopterin, decreased availability of intracellular L-arginine, and imbalance between eNOS and inducible NO synthase (iNOS). Age-related impairment of NO bioavailability is likely to promote vascular inflammation and atherogenesis and lead to cellular energetic imbalance [3, 6]. In addition to inactivating NO and causing oxidative macromolecular damage, ROS play important roles in the pathophysiological signaling pathways of endothelial and smooth muscle cells [3, 6]. In particular, oxidative stress and the subsequent activation of redox-sensitive signaling pathways are thought to be implicated in the inflammatory process in aged vasculature. Most adverse consequences of oxidative stress are mediated through production of the highly reactive oxidant peroxynitrite, the reaction product of NO and superoxide.

CR upregulates and activates eNOS, increases NO bioavailability, and improves endothelial dysfunction in aged animals and humans [3, 6, 11, 24, 29]. Furthermore, attenuation of the age-associated increase in oxidative stress is also thought to contribute to the anti-aging action of CR [3, 4, 6, 11]. In fact, recent studies clearly demonstrated that CR effectively attenuates the vascular production of ROS in aged rodents [3, 4, 6, 11]. The mechanisms by which CR attenuates vascular production of ROS have not yet been completely clarified. However, recent investigations have indicated that NF-E2-related factor 2 (Nrf2) is involved in regulation of the aging process and may contribute to the protective effects of CR in mammals [6, 25]. Nrf2 is a transcriptional factor that binds to the antioxidant response element of target genes and increases the transcription of a variety of proteins involved in oxidative stress resistance and detoxification of ROS. In aged rodents, there is a significant decline in Nrf2 transcriptional activity, resulting in the promotion of cellular oxidative stress [6, 25]. In contrast, CR is associated with the induction of Nrf2-regulated genes in the vasculature [6, 25].

Abundant experimental and clinical evidence indicates that aging is associated with chronic low-grade inflammation that predisposes the vasculature to atherosclerosis. Recent investigations have uncovered important cross-talk among inflammation, ROS generation, and endothelial dysfunction in the pathogenesis of vascular aging [3, 6]. Among these factors, activation of nuclear factor-κB (NF-κB)

plays a key role in endothelial activation and pro-inflammatory gene expression with aging [3, 6, 30]. In humans, plasma levels of several inflammatory markers (TNF-α, soluble vascular cell adhesion molecule-1, soluble E-selectin, interleukin [IL]-6, IL-18 and monocyte chemotactic protein-1), most of which are regulated by NF-κB, are positively correlated with age and are independent of other cardiovascular risk factors. In this regard, it is significant than CR attenuates vascular NF-κB induction and endothelial activation in aged rats [30]. CR can also disrupt other pro-inflammatory signaling pathways, including c-Jun N-terminal kinase (JNK), p38 kinase, and activator protein-1 DNA-binding activity [3, 6].

The sirtuin family has been shown to play an important role in various aspects of CR [3–6, 12]. Cohen et al. demonstrated that Sirt1 protein levels increase in various tissues of rats subjected to CR, including the brain, visceral fat pads, kidney and liver [31], while Sirt1 is expressed abundantly in the cardiovascular system and is induced by CR [3, 4]. Pharmacological activation of Sirt1 by resveratrol or Sirt1 overexpression confers significant anti-oxidative and anti-inflammatory effects, probably leading to attenuated cellular senescence [3, 4, 6, 12, 32]. Moreover, endothelial-specific overexpression of Sirt1 effectively attenuates the development of atherosclerosis in apolipoprotein (Apo)-E-deficient mice [33]. CR may regulate both eNOS activity and expression through Sirt1 activation. Recent studies demonstrated that Sirt1 and eNOS co-localize in endothelial cells and that Sirt1 deacetylates eNOS, stimulating eNOS activity and increasing NO production in endothelial cells [32]. Sirt1 overexpression or Sirt1 activators were shown to induce eNOS expression in endothelial cells. With respect to upstream molecules that activate Sirt1 in endothelial cells, several observations suggest that circulating factors play a key role in the phenotypic responses due to CR [22, 34]. Neuroendocrine mediators present in the circulation reach endothelial cells and elicit a variety of responses to CR. Recently, Csiszar et al. treated human endothelial cells with sera obtained from *Macaca mulatta* with long-term CR and found that circulating factors induced by CR activate angiogenic processes without Nrf2 activation [34]. The involvement of Sirt1 in this response was not evaluated in this study. The actual circulating factors by which vascular protection is mediated during CR are presently unknown.

Figure 9.2 provides a summary of possible mechanisms by which CR confers vascular protection.

In contrast, there have been very few investigations that evaluate the effect of ADF on vascular aging and atherosclerosis in rodents. Rodents and monkeys maintained on ADF exhibit enhanced insulin sensitivity [13]. Rodents treated with ADF show decreases in resting BP and heart rate (HR) [13, 35]. These results suggest that ADF also prevents vascular aging and atherosclerosis. Further investigations are needed to compare the effect of ADF with that of CR on vascular aging and atherosclerosis.

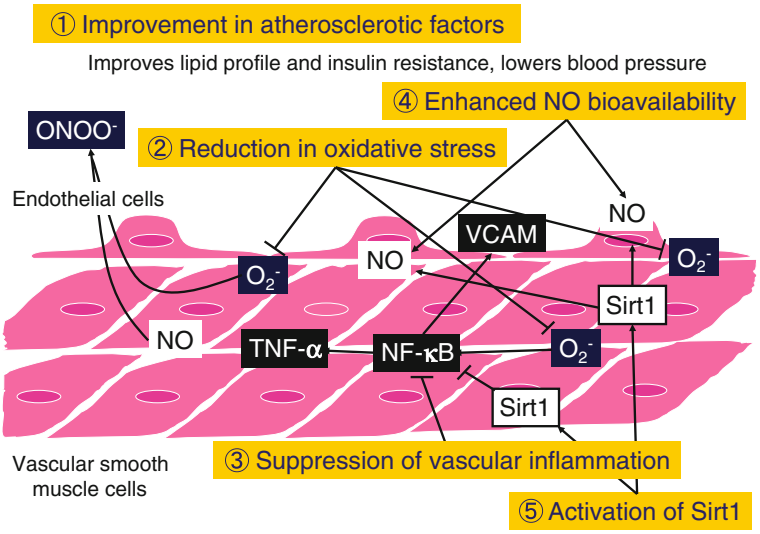

Fig. 9.2 Possible mechanisms by which CR antagonizes vascular aging and atherosclerosis. *NO* nitric oxide, *ONOO*$^-$ peroxynitrite, *VCAM* vascular cell adhesion molecule, O_2^- superoxide, *TNF-α* tumor necrosis factor-α, *NF-κB* nuclear factor-κB

9.3 Nutritional Interventions to Protect Against Cardiac Aging

9.3.1 Impaired Cardiac Function Associated with Advanced Age

The heart exhibits a continuum of the structural, functional, and molecular alterations that occur with aging. These age-associated alterations seem to have relevance to the sharp increases in LVH, AF, and CHF prevalence among the elderly [16].

9.3.1.1 Structural Changes

In cross-sectional studies of subjects without hypertension or clinically apparent CVD, the LV wall thickness reportedly increases with aging in both sexes [16]. In elderly patients without apparent CVD, cardiomyocyte enlargement is observed at autopsy and is associated with a robust decrease in the estimated myocyte number, although it has been demonstrated that overall LV mass is not increased, compared to that in younger patients [16]. An increase in the amount of collagen is observed in the aged myocardium and is associated with an increase in collagen cross-linking [16]. In addition, the accumulation of lipofuscin (a brown granular pigment that

consists of cross-linked lipids and proteins produced during lysosomal digestion), amyloid (an insoluble fibrous protein that aggregates with a specific structure traits), and other metabolic wastes are observed in the aged myocardium.

9.3.1.2 Functional and Molecular Changes

In general, parameters of LV systolic performance at rest are preserved during aging without apparent CVD [16]. The maximum ejection fraction during exhaustive upright exercise decreases with aging in healthy subjects [16]. This age-associated decrease may be explained by the decrease in coronary flow reserve due to coronary arteriosclerosis and the impaired response in contractile function and HR to β-adrenergic stimulation during exercise in the elderly.

In contrast, the aged heart exhibits impaired LV diastolic function [3, 16, 17, 36, 37]. The age-associated impairment in LV diastolic function is complicated. Senescent cardiomyocytes are characterized by prolonged relaxation, diminished contraction velocity, decreased β-adrenergic response, and increased myocardial stiffness [3, 37]. This impairment in LV diastolic function contributes to the increased incidence of HFpEF and AF in the elderly. There is ample evidence from studies using senescent rats implicating slowed cardiac relaxation and altered Ca^{2+} handling in the impaired diastolic function [3, 37]. In particular, impaired sarcoplasmic reticulum calcium ATPase (SERCA) activity, which is mainly responsible for controlling $[Ca^{2+}]_i$ by taking up Ca^{2+} into the sarcoplasmic reticulum (SR) during relaxation, contributes to the abnormalities in cardiac relaxation [8, 37]. The decrease in SR Ca^{2+} uptake during relaxation, which results in prolonged contraction, is associated with decreased SERCA2 content and activity in experimental models of senescence [8, 37]. SERCA2a protein levels have been reported to be significantly decreased in the senescent human myocardium [36].

In addition, the accumulation of myocardial collagen and extracellular matrix increases with aging and also contributes to myocardial stiffness and cardiac diastolic dysfunction [16, 17, 37]. Furthermore, an increased size of cardiomyocytes contributes to the LV diastolic dysfunction associated with aging [17]. Myocyte hypertrophy is associated with changes in the cytoskeletal proteins that can alter the microtubule architecture and heighten the organization of sarcomeres within individual myocytes. Increased collagen volume fraction, larger cardiomyocyte diameter, and higher resting cardiomyocyte tension have been correlated with LV diastolic stiffness [17, 37]. With aging, the myosin heavy chain isoform shifts from α to β in the rodent heart [37]. Lieber et al. [38] demonstrated that α- and β-tubulin were significantly increased and desmin was decreased in aged rats, and this finding may explain the cardiac dysfunction observed with aging. Post-translational modification of myofilament proteins including titin may play an important role in diastolic heart failure associated with aging [8].

Half of the elderly patients with CHF exhibit the phenotype of HFpEF [18]. Aging, female sex, and a history of high BP are clinically established risk factors for the development of HFpEF. Recent studies have revealed that the prognosis of

HFpEF is similar to that associated with LV systolic dysfunction [18]. However, therapeutic strategies aimed at improving LV diastolic dysfunction have not been established yet. Thus, nutritional interventions that can retard cardiac aging to manage HFpEF are promising in the clinical setting.

9.3.2 Effects of Dietary Restriction on Cardiac Senescence and Age-Associated Left Ventricular Diastolic Dysfunction

9.3.2.1 Rodent Caloric Restriction Experiments

The impact of long-term CR on cardiac senescence was first reported in 1997 [39]. Taffet et al. [39] reported that long-term CR improves age-associated changes in late diastolic function in mice.

Using Fischer-344 rats, we investigated the impact of long-term CR, which started at the age of 8 months and continued until the age of 30 months, on cardiac senescence and the effects of CR on cardiac diastolic dysfunction associated with aging in particular [8]. Our results demonstrated that long-term CR improves diastolic function in the senescent myocardium by ameliorating the age-associated deterioration in myocyte relaxation. Furthermore, our results indicated that attenuation of the decrease in SERCA2 with aging and enhanced autophagic flux are associated with functional improvements in the aged heart. In addition, a decreased size of cardiomyocytes may contribute to the amelioration of LV diastolic dysfunction in rats receiving CR. Our results indicated that cardiomyocyte responsiveness to Ca^{2+} estimated from the relationship between Ca^{2+} transient and myocyte shortening is similar between isolated myocytes obtained from AL- and CR-fed rat hearts. This finding supports our hypothesis that inhibiting the SERCA2 expression decline is a major factor in preserving LV diastolic function by CR. However, it is also possible that CR affects age-associated alterations in cytoskeletal proteins, leading to the improvement in LV diastolic function.

The accumulation of myocardial collagen and extracellular matrix increases with aging, contributing to increased cardiac fibrosis, myocardial stiffness, and cardiac diastolic dysfunction. Dhahbi et al. [40] demonstrated that long-term CR reduces the myocardial collagen and extracellular matrix content and attenuates cardiac fibrosis associated with aging. Thus, CR-induced changes in cardiac connective tissue may contribute, in part, to the amelioration of diastolic function, especially late diastolic function, as observed by Taffet et al. [39]. In contrast, we could not find a significant decrease in cardiac fibrosis in CR-fed rat hearts [8]. Our results suggested that long-term CR ameliorates the age-associated deterioration of early diastolic function by maintaining the function of the SR. In this regard, our findings differ from those of Taffet et al. [39] because those authors found no improvement in early diastolic cardiac function in mice. However, our results are consistent with

those of Seymour et al. [41], who reported that CR improves cardiac remodeling and diastolic dysfunction in Dahl-SS rats.

More recently, another group reported the effect of long-term CR on cardiac senescence and evaluated LV function in Fischer 344 rats at an earlier age. Compared to AL-fed rats, 24-month-old CR-fed rats had reduced levels of cardiac and aortic fibrosis, increased density of cardiomyocytes that were smaller in size, attenuated diastolic dysfunction, and normal systolic function and arterioventricular coupling [14]. Tachycardic response to dobutamine was also intact in 24-month-old rats receiving CR, and aortic stiffness was reduced. Adjustment for BW differences through ratiometric or allometric scaling did not affect the overall pattern of differences between the AL- and CR-fed rats. However, the attenuation of morphological and functional age-associated changes in the 24-month-old rats receiving CR either was not observed or was smaller in the 29-month-old rats receiving CR [14]. Taken together with our results [8], these results indicate that CR prevents age-associated LV dysfunction by postponing cardiac senescence.

Surprisingly, Yan et al. recently reported that short-term CR may reverse age-associated LV dysfunction [42]. When short-term (2 months) CR was initiated after age-associated LV dysfunction developed in 20-month-old mice, the decrease in cardiac function, and increases in LV weight, myocardial fibrosis and apoptosis were reversed and LV function became comparative to that of young mice or mice where CR was initiated at a young age. The authors also found that short-term CR prevents alterations in cytoskeletal proteins, which contribute to aging cardiomyopathy, but not the decrease in cardiomyocyte number associated with advanced age [42]. Nevertheless, CR has great therapeutic implications if instituting this intervention even later in life can rapidly correct aging cardiomyopathy. Table 9.1 provides a summary of the articles reported so far.

Table 9.1 The effect of caloric restriction (CR) on left ventricular (LV) function and cardiac aging

Authors	Taffet et al. [39]	Shinmura et al. [8]	Ahmet et al. [14]	Yan et al. [42]
Species and strain	B6D2-F1 hybrid mouse	Fischer-344 rat	Fischer-344 rat	129/Sv mouse
Age (at the time of observation)	30–35 month old	30 month old	24 month old	22 month old
Duration of CR	28–33 months	22 months	22 months	2 months
LV systolic function	Unchanged	Unchanged	Improved	Improved
LV diastolic function	Improved	Improved	Improved	Not described
Cardiac fibrosis	Not examined	Unchanged	Decrease	Decrease
Cardiomyocyte size	Not examined	Decrease	Decrease	Decrease
Cardiomyocyte apoptosis	Not examined	Decrease	Not examined	Decrease
Total cardiomyocyte number	Not examined	Not examined	Unchanged	Unchanged

9.3.2.2 Mechanisms by Which Caloric Restriction Retards Cardiac Aging

The mechanisms by which long-term CR retards cellular senescence and attenuates the physiological decline of organ function have not been fully elucidated. Aging occurs, in part, as a result of the accumulation of oxidative damage caused by oxidative free radicals that are generated continuously during the course of metabolic processes [4]. In contrast, CR decreases the age-associated accumulation of oxidative damage to lipids, proteins, and DNA. We found that the expression of protein carbonyls is less in CR-fed rat hearts compared with AL-fed rat hearts [4, 8]. Thus, it is possible that long-term CR retards cellular senescence and ameliorates age-related functional decline by attenuating oxidative damage in the aged heart. However, there is still no direct evidence that the attenuation of oxidative damage is the primary means by which CR prevents cardiac senescence.

Another possible mechanism by which long-term CR retards cardiac senescence is the enhancement of autophagy [4, 8]. Autophagy under basal conditions plays a housekeeping role in the turnover of cytoplasmic constituents. Thus, enhanced autophagy during CR is considered protective because it degrades and removes damaged organelles and accumulated protein aggregates. Inuzuka et al. [43] demonstrated that the suppression of phosphoinositide 3-kinase (PI3 K) preserves cardiac function and attenuates the expression of senescence markers associated with enhanced autophagy. Temporal inhibition of autophagy in tamoxifen-treated $Atg5^{flox/flox}$; $MerCreMer^{+}$ mice leads to LV hypertrophy, LV dilatation, and contractile dysfunction [44]. Because autophagy is not inhibited but is only somewhat imperfect in the aged heart, the accumulation of impaired SR and mitochondria is sublethal and may result in diastolic dysfunction only. Impaired autophagy in the aged heart may contribute, in part, to the accumulation of lipofuscin, further inhibiting autophagy. We found that long-term CR attenuates the accumulation of lipofuscin [8], suggesting that long-term CR disrupts this cycle in the aged heart. In addition, we demonstrated that enhanced autophagy is associated with suppression of the mTOR pathway in the heart [8]. The activation of mTOR exerts a negative regulatory effect on the induction of autophagy [12]. Rapamycin, an inhibitor of mTOR, has been shown to regress existing cardiac hypertrophy induced by pressure overload [45]. A recent proteome analysis revealed that either 10-weeks of CR or rapamycin treatment improved protein turnover in aged hearts [46]. It is plausible that the inhibition of mTOR signaling during CR plays a key role in the development of CR-induced cardioprotection.

Recent investigations strongly support the hypothesis that enhanced autophagy is essential for the cardiac adaptive response to prolonged CR [47–49]. Chen et al. [47] observed reduced cardiac function during CR in AMPKα2 knockout (KO) mice, which was associated with increased markers of oxidative stress, endoplasmic reticulum stress and myocyte apoptosis. In addition, CR down-regulated the

expression of ATP5g2, reduced ATP content, and failed to accelerate cardiac autophagic flux in AMPKα2 KO mice. Similarly, Zheng et al. recently reported that AMPK inhibition by the AMPK kinase-dead transgene significantly accentuates CR-induced changes in myocardial contractile function and intracellular Ca^{2+} handling, and negates CR-induced changes in autophagy and autophagy signaling, although it does not affect them under AL feeding [49]. Taken together, these findings indicate an indispensable role for AMPK in the maintenance of cardiac homeostasis under prolonged CR, mainly by up-regulating cardiac autophagic influx. Zhang et al. [48] demonstrated that disruption of the Akt2 gene alleviates CR-induced changes in cardiac contractile function through the regulation of autophagy. Prolonged CR activates both AMPK and Akt2, and they counteract each other in the effect on mTOR signaling. Therefore, Akt2 knockout augments CR-induced responses in mTOR signaling.

Figure 9.3 provides a summary of the effects of CR on cardiac aging.

9.3.2.3 Rodent Alternate-Day Fasting Experiments

Although the protective effect of CR against age-associated cardiac dysfunction has been established in rodents, the effect of ADF on cardiac aging and cardiac function remains controversial. Castello et al. reported the effects of long-term ADF, from 2 months until 24 months of age, on cardiac fibrosis and oxidative stress associated with advanced age in rats [50]. ADF significantly attenuated cardiac oxidative damage, the expression of pro-inflammatory cytokines, NF-κB DNA binding capacity, and cardiac fibrosis, compared to those observed in AL-fed 24-month-old rats. The authors further found that ADF attenuates cardiomyocyte hypertrophy in aged rats, probably by suppressing extracellular signal-regulated kinases 1 and 2 and PI3Kγ activation and restoring suppressor of cytokine signaling 3 and signal transducer and activator of transcription 3 activity, changes which are associated with advanced age [51].

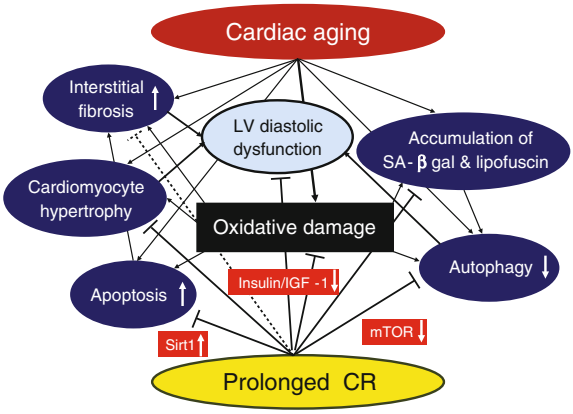

Fig. 9.3 Possible mechanisms by which CR retards cardiac aging and improves cardiac function. *LV* left ventricular, *SA-βgal* senescence-associated β-galactosidase

In contrast, a report by Ahmet et al. [15] seems unexpected because the authors concluded that ADF has a deleterious effect on cardiac function. In this study, ADF was started at 4 months and continued until 10 months of age. ADF resulted in a 9 % reduction of cardiomyocyte diameter and a 3-fold increase in interstitial myocardial fibrosis. Pressure-volume loop analyses revealed a stiff heart during diastole in ADF-treated rats, whereas combined dobutamine and volume overloading examinations showed a significant reduction in LV diastolic compliance and lack of increase in systolic pump function. The mechanism by which ADF may result in the development of diastolic dysfunction with a diminished cardiac reserve remains unsolved, but the authors speculate that the lack of important nutrients or the activation of the renin-angiotensin-aldosterone system may play a role.

9.3.2.4 Human Caloric Restriction Experiment

Meyer et al. demonstrated that CR is beneficial for LV diastolic function in humans, because the E/A ratio was greater in the CR group than in the group fed a Western diet, with no significant differences in LV systolic function between them [27]. The authors speculated that the beneficial effect of CR on LV diastolic function is due to decreases in systolic BP and systemic inflammation during CR. Although this finding raises the possibility of the clinical application of CR to treat LV diastolic dysfunction, it seems too premature to conclude that CR improves age-associated LV diastolic dysfunction in humans. Meyer et al. evaluated LV function by echocardiography in subjects with an average age of 53 years, most of the individuals practicing CR are middle-aged [27]. However, LV diastolic dysfunction is detected and causes significant health problems at an advanced age.

Although ADF effectively reduces BW even in humans, additional studies evaluating the effect of ADF on cardiovascular functions are warranted before clinical applications of ADF in humans.

9.4 Nutritional Interventions to Protect Against Acute Coronary Syndrome and Ischemic Heart Disease

9.4.1 Changes in Myocardial Ischemic Tolerance with Advanced Age

Increasing evidence demonstrates that the hearts of senescent animals are more susceptible to ischemia than those of young animals [3]. Several clinical investigations have also indicated that morbidity and mortality after MI are higher in the elderly [17]. The decreased number of myocytes in aged hearts may contribute to the observed impaired tolerance to ischemia. Additionally, development of cardiac hypertrophy and the increase in interstitial matrix may adversely affect oxygen

delivery to cardiomyocytes during ischemia. Enhanced inflammatory responses to ischemia/reperfusion injury, high levels of ROS production in the presence of weaker antioxidant defenses during ischemia/reperfusion, and weakening of aerobic metabolism efficiency in the post-ischemic myocardium, have also been reported in aged animals [3]. Tani et al. [52] demonstrated that the increased vulnerability to ischemia in the aged heart is associated with an enhanced increase in intracellular Na^+ content during ischemia, which probably results in increased intracellular Ca^{2+} content through Na^+/Ca^{2+} exchange during early reperfusion. Other investigators have reported that an age-related decrease in Ca^{2+} regulatory functions at the SR level [36], including attenuated expression of SERCA2 protein, may contribute to increased damage after ischemia/reperfusion in aged hearts.

Furthermore, recent investigations have demonstrated that the cardioprotective effect of ischemic preconditioning (PC) is impaired in middle-aged and aged animals [53]. Clinical investigations also support the concept that ischemic PC is impaired in the elderly [53]. Because senescence is associated with a disturbance of the innate adaptive response of tissues against various stresses, loss of ischemic PC in aged hearts may reflect the physiological deterioration of this adaptive response.

9.4.2 Improvement in Ischemic Tolerance with Dietary Restriction

9.4.2.1 Effects of Caloric Restriction on Myocardial Ischemia/Reperfusion Injury

Lifelong CR significantly attenuates myocardial oxidative stress and the inflammatory response associated with aging [1, 4–6]. Accordingly, some investigators including our laboratory have evaluated the effect of long-term CR on the degree of myocardial ischemia/reperfusion injury and the development of ischemic PC [3, 54–63]. In 2001, Broderick et al. demonstrated that CR for 8 months improves recovery of cardiac function after 25 min of ischemia in working rat hearts [55]. The authors also reported that the cardioprotective effect of CR is associated with an improvement in mitochondrial respiration. However, they did not evaluate whether CR improves ischemic tolerance in aged hearts. Abete et al. [54] demonstrated that CR can restore the cardioprotective effect of ischemic PC in aged rat hearts. CR was started at the age of 12 months, and hearts were subjected to 20 min of global ischemia followed by 40 min of reperfusion at 24 months of age. CR did not improve the recovery of LV function after ischemia/reperfusion but dramatically restored the PC effect in senescent hearts. Long et al. [58] reported that CR for 6 months restores the PC effect in middle-aged rat hearts. However, in their report, recovery of cardiac output and aortic flow after 25 min ischemia followed by 30 min reperfusion was reduced in CR-fed versus AL-fed rat hearts.

We subjected 6-month-old rats to 6 months of CR to address whether CR improves myocardial ischemic tolerance and restores the development of ischemic PC in middle-aged rats [61]. Six months of CR improved the recovery of LV function after ischemia/reperfusion and attenuated infarct size in 12-month-old rat hearts. In addition, CR restored the ischemic PC effect in middle-aged hearts. Together with those of other recent reports (Table 9.2), our results establish the potential of CR intervention aimed at attenuating myocardial damage to reduce ischemic stress in humans.

Clearly, the use of short-term CR is much easier to incorporate into clinical practice than lifelong CR. Thus, we then evaluated the effect of short-term CR on the degree of myocardial ischemia/reperfusion injury and the development of ischemic PC in young and aged animals [60]. Six-month-old (young) and

Table 9.2 The effects of CR on myocardial ischemia/reperfusion (I/R) injury and ischemic preconditioning

Species and strain	Age	Duration of CR	Effects of CR	Authors
Fischer-344 rat	12 month old	11.5 months	Attenuated myocardial inflammation after I/R in vivo	Chandrasekar et al. [56]
Wister rat	10 month old	8 months	Reduced myocardial I/R injury ex vivo	Broderick et al. [55]
Wister rat	6 month old	5 months	Restored the effect of ischemic preconditioning in aged hearts ex vivo	Abete et al. [54]
	24 month old	12 months		
Sprague Dawley rat	20 month old	8 months	Restored the effect of ischemic preconditioning, but reduced cardiac function after I/R ex vivo	Long et al. [58]
Fischer-344 rat	4 month old	5 weeks	Reduced myocardial I/R injury ex vivo in both ages of rats	Shinmura et al. [60]
	24 month old	5 weeks		
C57BL6 mouse	4 month old	5 weeks	Reduced myocardial I/R injury ex vivo	Shinmura et al. [62]
Fischer-344 rat	12 month old	6 months	Reduced myocardial I/R injury and restored the effect of ischemic preconditioning ex vivo	Shinmura et al. [61]
B6D2F1 mouse	30 month old	6 months	Reduced myocardial I/R injury ex vivo	Edwards et al. [57]
C57BL6 mouse	6 month old	3 months	Reduced myocardial I/R injury ex vivo	Shinmura et al. [3]
C57BL6 mouse	4 month old	5 weeks	Reduced myocardial I/R injury ex vivo	Sung et al. [63]
C57BL6 mouse	12 month old	3 months	Reduced myocardial I/R injury ex vivo	Peart et al. [59]

24-month-old (aged) Fischer-344 male rats were randomly divided into 2 groups: AL rats were fed AL, whereas CR rats were fed 90 % of the AL caloric intake for 2 weeks followed by 65 % of the caloric intake for 2 weeks. Short-term CR attenuated both reversible (recovery of LV function) and irreversible (lactate dehydrogenase and creatine kinase leakage) damage in both young and aged hearts but short-term CR did not restore ischemic PC in aged hearts. The highlight of our study was the discovery that short-term CR improves myocardial ischemic tolerance regardless of biological age [60]. Thus, short-term CR is a nutritional induction of PC.

Although several investigations demonstrate that a single nonlethal fasting attenuates myocardial ischemia/reperfusion injury in rodents [64], it remains undetermined whether ADF reduces myocardial ischemia/reperfusion injury.

9.4.2.2 Effects of Dietary Restriction on Myocardial Infarction and Post-infarct Left Ventricular Remodeling

The effects of DR on infarct size after permanent coronary ligation and post-infarct LV remodeling were evaluated in vivo. After 3 months of ADF or AL feeding, MI was induced by permanent coronary ligation in 5-month-old rats. Infarct size evaluated by triphenyltetrazolium chloride staining 24 h after MI was significantly smaller in ADF-fed rats [65]. Furthermore, post-infarct LV remodeling, MI expansion, and LV function evaluated by echocardiography 10 weeks after MI were significantly improved in ADF-fed rats. Therefore, ADF may protect myocardium from ischemic injury and attenuate post-infarct LV remodeling, probably via anti-apoptotic and anti-inflammatory mechanisms. In contrast, the same group reported that 3 months of CR prior to permanent coronary ligation failed to protect the hearts from ischemic injury and attenuate post-infarct LV remodeling in 5-month-old rats [14].

When ADF was started 2 weeks after permanent coronary ligation, the survival of rats after MI markedly improved [66]. In addition, ADF attenuated post-infarct LV remodeling and improved LV function after MI. Molecular studies revealed the upregulation of angiogenic factors including hypoxia-inducible factor 1α, brain-derived neurotrophic factor, and vascular endothelial growth factor in the hearts under fasting conditions. These results suggest that the protective effect of ADF against post-infarct LV remodeling is stronger than that of CR, but the clinical application of ADF may be limited because the practice of reperfusion therapy is common for large MI in clinical settings.

9.4.3 Mechanisms by Which Dietary Restriction Improve Myocardial Ischemic Tolerance

Identifying the molecular mechanisms through which CR exerts cardioprotection against myocardial ischemia has great potential for establishing a novel therapeutic strategy to manage IHD patients.

The protective effect of short-term CR was not abrogated by the administration of 2 distinct ATP-sensitive K^+ channel blockers [60], suggesting that different mechanisms underlie the cardioprotection afforded by ischemic PC and that afforded by CR. Thus, we focused on the adiponectin–AMPK signaling pathway, which may be activated during CR [60]. Using the adiponectin antisense transgenic mouse, we have demonstrated that short-term CR confers cardioprotection against ischemia/reperfusion injury and that the increase in circulating adiponectin levels, especially the increase in the high molecular weight form of adiponectin, associated with CR is necessary for the cardioprotective effects of CR [62]. Furthermore, subsequent activation of AMPK plays an obligatory role in CR-induced cardioprotection. AMPK also plays an important role in regulating energy balance in the myocardium: the activation of AMPK during ischemia/reperfusion can reduce ischemia-induced necrosis and apoptosis. An increase in the cellular AMP/ATP ratio is a major regulator of AMPK activity, but adiponectin has also been reported to activate AMPK. Most of the beneficial effects of adiponectin are reportedly mediated by the AMPK-associated signaling pathway. CR may induce mild energy substrate shortages. It seems reasonable to assume that short-term CR switches off ATP-consuming pathways and switches on ATP-generating pathways through activation of the adiponectin–AMPK signaling pathway in the myocardium.

Serum adiponectin levels increased with 6 months of CR to the same extent as with short-term CR [61]. However, expression levels of either phosphorylated AMPK or phosphorylated acetyl-CoA carboxylase (ACC) did not increase after 6 months of CR [61], suggesting that AMPK and ACC were not activated in the hearts of rats receiving prolonged CR. We speculate that the transient activation of AMPK-associated signaling is necessary for the induction of a cardioprotected phenotype during prolonged CR, as discussed above. However, our results suggest that different mechanisms are involved in the cardioprotection afforded by prolonged versus short-term CR.

CR enhances autophagy in aged hearts [8]. Enhanced autophagy during reperfusion is reported to be detrimental to cardiomyocytes, whereas enhanced autophagy during ischemia is protective against myocardial damage [67]. Thus, the activation of AMPK and suppression of mTOR signaling during CR may contribute to, at least in part, CR-induced cardioprotection against ischemia/reperfusion injury.

As described above, CR confers vascular protection through Sirt1 activation [3, 6, 22]. In the heart, we found that prolonged CR significantly increases Sirt1 and decreases acetyl-histone H3 in the nuclear fraction [61], suggesting that the activated form of Sirt1 increases in the hearts of CR-fed rats. Sadoshima and his colleagues clearly demonstrated that cardiac Sirt1 protects myocardium from

ischemia/reperfusion injury by upregulating the antioxidants, manganese superoxide dismutase and thioredoxin 1, and an anti-apoptotic protein, Bcl-xL, and downregulating the proapoptotic molecules, Bax and cleaved caspase-3, via deacetylation of the transcriptional factor, FoxO1 [68]. The authors also indicated that cardiomyocyte autophagy is activated during nutrient starvation through Sirt1-FoxO1-dependent mechanisms. However, direct evidence demonstrating that Sirt1 is involved in the cardioprotective effect of CR was lacking. Accordingly, we made cardiac-specific Sirt1-deficient mice and found that cardiac Sirt1 is essential for CR-induced cardioprotection against ischemia/reperfusion injury [69].

Long-term CR retards cardiac senescence and ameliorates age-related functional decline probably by attenuating oxidative damage in the aged heart [4, 8]. However, it has not yet been clarified how long-term CR attenuates oxidative damage in the heart. Because 3 of 7 sirtuin family members target mitochondria, it is plausible that sirtuins directly attenuate ROS production in the mitochondria. However, the effect of CR on mitochondrial ROS production in the heart remains unclear. In our experiments, the enzymatic activity of the electron transport chain (ETC), baseline mitochondrial respiration, and mitochondrial H_2O_2 production in the hearts of middle-aged (12-month-old) rats treated with 6 months of CR were comparable to those in middle-aged rats fed AL [70]. The only difference was that CR attenuated maximal H_2O_2 production in mitochondria; this was assessed by adding rotenone (complex I inhibitor) in the presence of pyruvate/malate. We speculated that the difference in mitochondrial function between AL and CR becomes more remarkable under stressful conditions, such as ischemia/reperfusion. As expected, CR preserved state 3 respiration and increased the respiratory control index in the presence of pyruvate/malate in the ischemic-reperfused heart [70]. These findings suggest that the mitochondria in the heart of CR-fed animals are well coupled during the ischemia/reperfusion sequence. Mitochondria obtained from ischemic-reperfused hearts of CR-fed rats produced less H_2O_2 in the presence of pyruvate/malate [70], suggesting that the mitochondria produce less ROS during early reperfusion. Preserved mitochondrial respiration with attenuated H_2O_2 production in the heart of CR-fed rats subjected to ischemia/reperfusion strongly suggested that the mitochondria are more resistant to ischemia/reperfusion injury.

For the mechanism by which CR attenuates mitochondrial ROS production during ischemia/reperfusion, we speculate that post-translational modification of specific mitochondrial proteins during CR plays an essential role because mitochondrial proteins belonging to complex I and complex III of the ETC were significantly deacetylated during CR in a sirtuin-dependent manner [70]. However, we could not identify which sirtuin was responsible for the deacetylation of the specific mitochondrial proteins. Further investigation is needed to determine which sirtuin is responsible for the effect of CR on mitochondrial protein deacetylation and function.

In addition to sirtuins, we noticed the specific role of NO as a mediator of CR-induced cardiovascular protection [3, 71]. The interaction between Sirt1 and eNOS seems to be essential for the development of vascular protection afforded by CR [3, 6, 32]. Increasing evidence demonstrates that NO, either endogenous or exogenous, represents one of the most important defenses against myocardial ischemia/

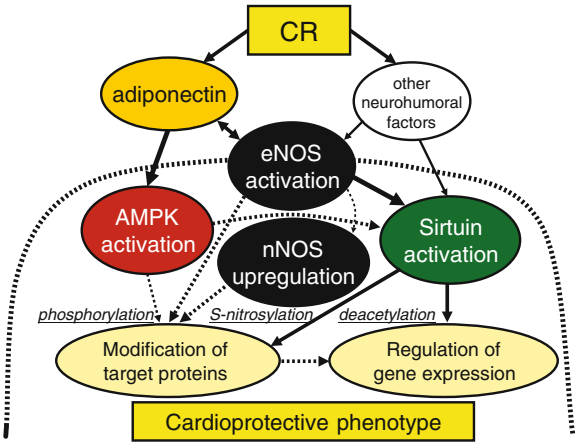

Fig. 9.4 Possible mechanisms by which short-term and prolonged CR confer cardioprotection against ischemia/reperfusion injury. *eNOS* endothelial nitric oxide synthase, *nNOS* neuronal nitric oxide synthase

reperfusion injury [3]. Furthermore, NO appears to be a common mediator of the protection afforded by a wide array of seemingly unrelated pharmacological and non-pharmacological interventions in the cardiovascular system [3]. Therefore, the increased NO bioavailability as a result of CR must play a key role in the development of CR-induced cardiovascular protection. Using eNOS-deficient mice, we demonstrated that eNOS is essential for CR-induced cardioprotection against ischemia/reperfusion injury and the close interaction between eNOS and Sirt1 during the development of CR-induced cardioprotection [3, 71]. NO derived from eNOS is necessary for Sirt1 activation. Our results were in agreement with those of a previous report by Nisoli et al. [29], who demonstrated that CR enhances mitochondrial biogenesis by inducing eNOS. Although eNOS plays an important role in the regulation of BP during CR, the essential role of eNOS in CR-induced cardioprotection is independent of its effect on BP [71]. We speculate that eNOS may play a dual role, as a trigger and mediator, in the development of CR-induced cardioprotection as it does in the development of late PC.

Figure 9.4 provides a summary of possible mechanisms by which short-term and long-term CR confer cardioprotection against ischemia/reperfusion injury.

9.5 Effects of Dietary Restriction on the Autonomic Nerve System

Age-associated decline in autonomic nerve function is partially responsible for the increase in arrhythmias and syncope with advanced age [72]. Several studies have demonstrated the effect of DR on autonomic nerve function in rodents or humans [35, 72].

To evaluate the effect of DR on beat-to-beat HR variability (HRV) and diastolic BP variability (DPV), Sprague Dawley rats implanted with telemetric transmitters were subjected to AL, ADF, or CR feeding at 3 months of age [35]. Both ADF and CR produced increases in the low frequency (LF) and the high frequency (HF) components of HRV spectra within 1 month. ADF decreased the LF and LF/HF of the DPV spectra, but CR did not. These results suggest that DR produces decreased sympathetic nerve activity and augments parasympathetic tone. The effect on parasympathetic tone seems to be stronger from ADF than that from CR.

Stein et al. [72] evaluated HRV in 22 individuals following CR aged 35–82 years and 20 age-matched controls eating Western diets (WD). Individuals in the experimental group continued a self-imposed CR diet for 3–15 years. All HRV values were significantly higher in the CR group than those in the WD group. HRV data in the CR group were comparable to published norms for healthy individuals 20 years younger than each individual's age. These results strongly suggest that CR decreases sympathetic tone, increases parasympathetic tone, and restores circadian rhythm variability. In conclusion, DR may have direct systemic effects that counteract the expected age-associated changes in autonomic nerve function in humans.

9.6 Nutritional Interventions to Protect Against Metabolic Cardiomyopathy Associated with Obesity and Type 2 Diabetes Mellitus

Overeating and obesity are huge health problems in developed counties. Obesity-associated metabolic disorders, such as metabolic syndrome and T2DM, accelerate atherosclerosis and vascular aging, resulting in the development of IHD, stroke, peripheral artery disease, and other CVDs [20, 21]. Furthermore, metabolic disorders cause cardiac hypertrophy, HFpEF, and pulmonary artery hypertension. Thus, the effect of DR on cardiac dysfunction associated with metabolic disorders is of great clinical interest.

Ob/ob mice manifest marked obesity, insulin resistance, cardiac hypertrophy, mitochondrial dysfunction, and ectopic lipid accumulation due to leptin deficiency. Sloan et al. [73] compared the effect of CR on myocardial metabolism and function in 4-week-old ob/ob mice to that of leptin supplementation. Although CR normalized BW and glucose tolerance, fat mass and circulating lipid levels remained increased in ob/ob mice receiving CR. Palmitate oxidation in the heart remained elevated in CR-fed ob/ob mice and was normalized by intraperitoneal or intracerebroventricular leptin administration. These results suggest that impaired hypothalamic leptin signaling leads to an increase in myocardial fatty acid oxidation despite CR.

In addition, the beneficial effects of CR were impaired in ob/ob mice when CR was started from middle age. AlGhatrif et al. [74] reported that CR restored LV diastolic function, reversed myocardial steatosis, and improved insulin sensitivity when it was started at 2 months of age in ob/ob mice, whereas none of these changes were observed when CR was started at 6–7 months of age. However, CR decreased cardiac oxidative stress and normalized NOS activity, suggesting that some beneficial effects of CR remained in the older mice.

Nagata and her colleagues [75] characterized DahlS.Z-Lepr(fa)/Lepr(fa)(DS/obese) rats as a new animal model of metabolic syndrome. DS/obese rats develop hypertension and manifest LV remodeling and diastolic dysfunction as well as increased cardiac oxidative stress and inflammation. Four weeks of CR reduced BW, body fat content, and BP in DS/obese rats but did not affect BP in DS/lean rats, which were control homozygous lean littermates [75]. Furthermore, CR attenuated LV hypertrophy fibrosis and diastolic dysfunction in DS/obese rats and was associated with attenuated cardiac oxidative stress and inflammation. Given these results, it is likely that ectopic lipid accumulation in the heart and a subsequent increase in cardiac oxidative stress contribute to the development of cardiac remodeling and LV diastolic dysfunction associated with metabolic disorders.

Recent clinical studies also have demonstrated that CR, when accompanied by significant BW loss, has cardiac-specific effects that ameliorate LV diastolic function in healthy subjects [27], as well as in patients with T2DM [20]. Thus, the clinical application of CR and the development of CR mimetics that can replicate the effects of CR have considerable potential as novel therapeutic approaches for the treatment of patients with diastolic dysfunction. However, the exact mechanisms by which CR improves cardiac diastolic dysfunction in humans remain unknown. Recent investigations suggest that myocardial triglyceride content is an independent predictor of diastolic function in the elderly [76] and in patients with T2DM [20]. The decrease in myocardial triglyceride content produced by CR is associated with an improvement in LV diastolic function [20, 76]. It is plausible that enhanced autophagy contributes to the degradation of potentially toxic fatty acid intermediates. From the CALERIE study, Riordan et al. concluded that weight loss, whether induced by CR or exercise, has salutary effects on LV diastolic dysfunction associated with obesity in humans [21].

We focused on cardiac Sirt1 as a key molecule that regulates lipid metabolism and the capacity of cardiac triglyceride pool. Three-month-old male cardiomyocyte-specific Sirt1 KO mice and control littermates were fed a control diet (HFC) or a high fat diet (HFD) for 3 months [77]. The expression levels of Sirt1 mRNA did not change with the HFD in control mice. Echocardiographic evaluation revealed that there were no differences in LV systolic parameters, but interventricular septum wall thickness and corrected LV mass in cardiomyocyte-specific Sirt1 KO mice fed the HFD were higher than those in control mice fed the HFD. Liver weight increased with the HFD in both strains, and hematoxylin-eosin staining showed

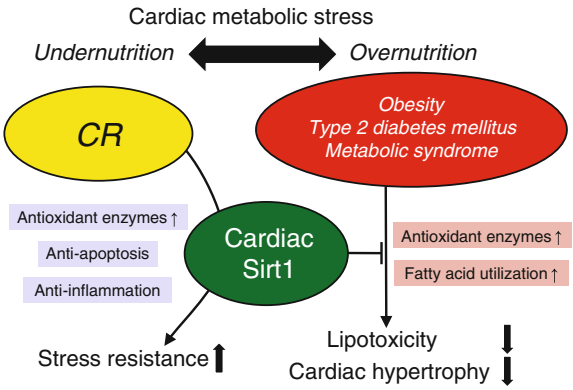

Fig. 9.5 The distinct role of cardiac Sirt1 during metabolic stress

similar degrees of steatohepatitis between both strains when fed the HFD. In contrast, oil red O staining exhibited massive fat deposition only in cardiomyocyte-specific Sirt1 KO mice fed the HFD and myocardial triglyceride content was significantly higher in these mice. These results demonstrate that a deficiency in cardiac Sirt1 exacerbates cardiac fat accumulation, which indicates that cardiac Sirt1 plays a protective role against HFD-induced cardiomyopathy. We speculate that cardiac Sirt1 is a guardian against metabolic stresses ranging from undernutrition to overnutrition (Fig. 9.5).

9.7 Potential for Caloric Restriction Mimetics as Cardiovascular Drugs

The use of CR mimetics would be much easier to incorporate into clinical practice than lifelong CR [78]. Promising CR mimetics with properties of cardiovascular protection include compounds that intersect with the critical signaling pathways identified above. These compounds include biguanides such as metformin, which targets the AMPK and insulin signaling pathways; resveratrol, which affects sirtuin activity; and rapamycin, which interacts with mTOR signaling. In particular, chemicals and compounds with autophagy-inducing properties would be promising as CR mimetics because enhanced autophagy seems to be one of the principle mechanisms by which CR exerts pleiotropic effects on CVDs. In contrast, the role of sirtuins in lifespan extension by CR remains controversial, but sirtuins regulate various aspects of the CR response. Thus, the development of specific sirtuin activators is potentially useful for managing age-associated CVDs. Table 9.3 provides possible candidates for CR mimetics as cardiovascular drugs.

Table 9.3 Possible candidates for CR mimetics as cardiovascular agents

Inhibitors of glycolytic pathway	2-deoxyglucose	Not recommended for long-term use. Reduces myocardial I/R injury
	Mannoheptulose	Hexokinase inhibitor. Might reduce myocardial I/R injury
	3-bromopyruvate	GAPDH inhibitor. Well-tolerated in humans, might reduce myocardial I/R injury
	Dichloroacetate	PDK inhibitor. Well-tolerated in humans, reduces myocardial I/R injury and might prevent cardiac hypertrophy
AMPK activators	AICAR	Reduces myocardial I/R injury
	Metformin	Reduces myocardial I/R injury. Not specific AMPK
Sirtuin-activating compounds (STACs)	Resveratrol	Activates AMPK and other many molecules. Prevents vascular aging, reduces myocardial I/R injury, and prevents cardiac hypertrophy
	SRT1720	Might reduce myocardial I/R injury
mTOR inhibitors	Rapamycin	Prevents cardiac hypertrophy
	mTORC1 selective inhibitor	Under development. Might prevent cardiac hypertrophy
Statin		Prevents vascular aging and reduces myocardial I/R injury
RAS inhibitors	ACE inhibitor and AT1R blocker	Prevents vascular aging, reduces myocardial I/R injury, and attenuates cardiac aging
Adiponectin and its agonist	Adiponectin	Reduces myocardial I/R injury and prevents cardiac hypertrophy
	AdipoRon	Agonist for both Adipo R1 and R2, and activates AMPK and PPAR-α. Might reduce myocardial I/R injury
GLP agonists and DPP-4 inhibitors		Reduces myocardial I/R injury

GAPDH glyceraldehyde 3-phosphate dehydrogenase, *PDK* pyruvate dehydrogenase kinase, *AICAR* aminoimidazole carboxamide ribonucleotide, *STACs* sirtuin-activating compounds, *mTORC1* mTOR complex 1, *RAS* renin-angiotensin-system, *ACE* angiotensin converting enzyme, *AT1R* angiotensin type 1 receptor, *GLP* glucagon-like peptide-1, *DPP-4* dipeptidyl peptidase-4, *PPAR-α* peroxisome proliferator activated receptor-α

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References

1. Masoro EJ (2005) Overview of caloric restriction and ageing. Mech Ageing Dev 126(9):913–922
2. McCay CM, Crowell MF, Maynard LA (1989) The effect of retarded growth upon the length of life span and upon the ultimate body size 1935. Nutrition 5(3):155–171 (discussion 172)
3. Shinmura K (2011) Cardiovascular protection afforded by caloric restriction: essential role of nitric oxide synthase. Geriatr Gerontol Int 11(2):143–156
4. Shinmura K (2013) Effects of caloric restriction on cardiac oxidative stress and mitochondrial bioenergetics: potential role of cardiac sirtuins. Oxid Med Cell Longev 2013:528935
5. Speakman JR, Mitchell SE (2011) Caloric restriction. Mol Aspects Med 32(3):159–221
6. Ungvari Z, Parrado-Fernandez C, Csiszar A, de Cabo R (2008) Mechanisms underlying caloric restriction and lifespan regulation: implications for vascular aging. Circ Res 102 (5):519–528
7. Schroeder JE, Richardson JC, Virley DJ (2010) Dietary manipulation and caloric restriction in the development of mouse models relevant to neurological diseases. Biochim Biophys Acta 1802(10):840–846
8. Shinmura K, Tamaki K, Sano M, Murata M, Yamakawa H, Ishida H, Fukuda K (2011) Impact of long-term caloric restriction on cardiac senescence: caloric restriction ameliorates cardiac diastolic dysfunction associated with aging. J Mol Cell Cardiol 50(1):117–127
9. Zainal TA, Oberley TD, Allison DB, Szweda LI, Weindruch R (2000) Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle. Faseb J 14(12):1825–1836
10. Heilbronn LK, de Jonge L, Frisard MI, DeLany JP, Larson-Meyer DE, Rood J, Nguyen T, Martin CK, Volaufova J, Most MM, Greenway FL, Smith SR, Deutsch WA, Williamson DA, Ravussin E (2006) Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA 295(13):1539–1548
11. Zanetti M, Gortan CG, Burekovic I, Barazzoni R, Stebel M, Guarnieri G (2010) Caloric restriction improves endothelial dysfunction during vascular aging: effects on nitric oxide synthase isoforms and oxidative stress in rat aorta. Exp Gerontol 45(11):848–855
12. North BJ, Sinclair DA (2012) The intersection between aging and cardiovascular disease. Circ Res 110(8):1097–1108
13. Mattson MP, Wan R (2005) Beneficial effects of intermittent fasting and caloric restriction on the cardiovascular and cerebrovascular systems. J Nutr Biochem 16(3):129–137
14. Ahmet I, Tae HJ, de Cabo R, Lakatta EG, Talan MI (2011) Effects of calorie restriction on cardioprotection and cardiovascular health. J Mol Cell Cardiol 51(2):263–271
15. Ahmet I, Wan R, Mattson MP, Lakatta EG, Talan MI (2010) Chronic alternate-day fasting results in reduced diastolic compliance and diminished systolic reserve in rats. J Card Fail 16 (10):843–853
16. Lakatta EG, Levy D (2003) Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: part II: the aging heart in health: links to heart disease. Circulation 107 (2):346–354
17. Shih H, Lee B, Lee RJ, Boyle AJ (2011) The aging heart and post-infarction left ventricular remodeling. J Am Coll Cardiol 57(1):9–17
18. Chatterjee K, Massie B (2007) Systolic and diastolic heart failure: differences and similarities. J Card Fail 13(7):569–576
19. Lakatta EG, Levy D (2003) Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: part I: aging arteries: a “set up” for vascular disease. Circulation 107 (1):139–146
20. Hammer S, Snel M, Lamb HJ, Jazet IM, van der Meer RW, Pijl H, Meinders EA, Romijn JA, de Roos A, Smit JW (2008) Prolonged caloric restriction in obese patients with type 2 diabetes mellitus decreases myocardial triglyceride content and improves myocardial function. J Am Coll Cardiol 52(12):1006–1012

21. Riordan MM, Weiss EP, Meyer TE, Ehsani AA, Racette SB, Villareal DT, Fontana L, Holloszy JO, Kovacs SJ (2008) The effects of caloric restriction- and exercise-induced weight loss on left ventricular diastolic function. Am J Physiol Heart Circ Physiol 294(3):H1174–H1182
22. Csiszar A, Labinskyy N, Jimenez R, Pinto JT, Ballabh P, Losonczy G, Pearson KJ, de Cabo R, Ungvari Z (2009) Anti-oxidative and anti-inflammatory vasoprotective effects of caloric restriction in aging: role of circulating factors and SIRT1. Mech Ageing Dev 130(8):518–527
23. Fontana L, Meyer TE, Klein S, Holloszy JO (2004) Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. Proc Natl Acad Sci USA 101 (17):6659–6663
24. Holloszy JO, Fontana L (2007) Caloric restriction in humans. Exp Gerontol 42(8):709–712
25. Pearson KJ, Lewis KN, Price NL, Chang JW, Perez E, Cascajo MV, Tamashiro KL, Poosala S, Csiszar A, Ungvari Z, Kensler TW, Yamamoto M, Egan JM, Longo DL, Ingram DK, Navas P, de Cabo R (2008) Nrf2 mediates cancer protection but not prolongevity induced by caloric restriction. Proc Natl Acad Sci USA 105(7):2325–2330
26. Stewart TM, Bhapkar M, Das S, Galan K, Martin CK, McAdams L, Pieper C, Redman L, Roberts S, Stein RI, Rochon J, Williamson DA (2013) Comprehensive assessment of long-term effects of reducing intake of energy phase 2 (CALERIE phase 2) screening and recruitment: methods and results. Contemp Clin Trials 34(1):10–20
27. Meyer TE, Kovacs SJ, Ehsani AA, Klein S, Holloszy JO, Fontana L (2006) Long-term caloric restriction ameliorates the decline in diastolic function in humans. J Am Coll Cardiol 47 (2):398–402
28. Varady KA, Bhutani S, Klempel MC, Kroeger CM, Trepanowski JF, Haus JM, Hoddy KK, Calvo Y (2013) Alternate day fasting for weight loss in normal weight and overweight subjects: a randomized controlled trial. Nutr J 12(1):146
29. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310(5746):314–317
30. Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE, Seals DR (2007) Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB. Circ Res 100 (11):1659–1666
31. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA (2004) Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 305(5682):390–392
32. Mattagajasingh I, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, DeRicco J, Kasuno K, Irani K (2007) SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci USA 104(37):14855–14860
33. Zhang QJ, Wang Z, Chen HZ, Zhou S, Zheng W, Liu G, Wei YS, Cai H, Liu DP, Liang CC (2008) Endothelium-specific overexpression of class III deacetylase SIRT1 decreases atherosclerosis in apolipoprotein E-deficient mice. Cardiovasc Res 80(2):191–199
34. Csiszar A, Sosnowska D, Tucsek Z, Gautam T, Toth P, Losonczy G, Colman RJ, Weindruch R, Anderson RM, Sonntag WE, Ungvari Z (2013) Circulating factors induced by caloric restriction in the nonhuman primate Macaca mulatta activate angiogenic processes in endothelial cells. J Gerontol A Biol Sci Med Sci 68(3):235–249
35. Mager DE, Wan R, Brown M, Cheng A, Wareski P, Abernethy DR, Mattson MP (2006) Caloric restriction and intermittent fasting alter spectral measures of heart rate and blood pressure variability in rats. Faseb J 20(6):631–637
36. Cain BS, Meldrum DR, Joo KS, Wang JF, Meng X, Cleveland JC Jr, Banerjee A, Harken AH (1998) Human SERCA2a levels correlate inversely with age in senescent human myocardium. J Am Coll Cardiol 32(2):458–467
37. Lakatta EG (2003) Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part III: cellular and molecular clues to heart and arterial aging. Circulation 107 (3):490–497

38. Lieber SC, Qiu H, Chen L, Shen YT, Hong C, Hunter WC, Aubry N, Vatner SF, Vatner DE (2008) Cardiac dysfunction in aging conscious rats: altered cardiac cytoskeletal proteins as a potential mechanism. Am J Physiol Heart Circ Physiol 295(2):H860–H866
39. Taffet GE, Pham TT, Hartley CJ (1997) The age-associated alterations in late diastolic function in mice are improved by caloric restriction. J Gerontol A Biol Sci Med Sci 52(6): B285–B290
40. Dhahbi JM, Tsuchiya T, Kim HJ, Mote PL, Spindler SR (2006) Gene expression and physiologic responses of the heart to the initiation and withdrawal of caloric restriction. J Gerontol A Biol Sci Med Sci 61(3):218–231
41. Seymour EM, Parikh RV, Singer AA, Bolling SF (2006) Moderate calorie restriction improves cardiac remodeling and diastolic dysfunction in the Dahl-SS rat. J Mol Cell Cardiol 41 (4):661–668
42. Yan L, Gao S, Ho D, Park M, Ge H, Wang C, Tian Y, Lai L, De Lorenzo MS, Vatner DE, Vatner SF (2013) Calorie restriction can reverse, as well as prevent, aging cardiomyopathy. Age 35(6):2177–2182
43. Inuzuka Y, Okuda J, Kawashima T, Kato T, Niizuma S, Tamaki Y, Iwanaga Y, Yoshida Y, Kosugi R, Watanabe-Maeda K, Machida Y, Tsuji S, Aburatani H, Izumi T, Kita T, Shioi T (2009) Suppression of phosphoinositide 3-kinase prevents cardiac aging in mice. Circulation 120(17):1695–1703
44. Taneike M, Yamaguchi O, Nakai A, Hikoso S, Takeda T, Mizote I, Oka T, Tamai T, Oyabu J, Murakawa T, Nishida K, Shimizu T, Hori M, Komuro I, Shirasawa T, Mizushima N, Otsu K (2010) Inhibition of autophagy in the heart induces age-related cardiomyopathy. Autophagy 6 (5):600–606
45. McMullen JR, Sherwood MC, Tarnavski O, Zhang L, Dorfman AL, Shioi T, Izumo S (2004) Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. Circulation 109(24):3050–3055
46. Dai DF, Karunadharma PP, Chiao YA, Basisty N, Crispin D, Hsieh EJ, Chen T, Gu H, Djukovic D, Raftery D, Beyer RP, MacCoss MJ, Rabinovitch PS (2014) Altered proteome turnover and remodeling by short-term caloric restriction or rapamycin rejuvenate the aging heart. Aging Cell 13(3):529–539
47. Chen K, Kobayashi S, Xu X, Viollet B, Liang Q (2013) AMP activated protein kinase is indispensable for myocardial adaptation to caloric restriction in mice. PLoS ONE 8(3):e59682
48. Zhang Y, Han X, Hu N, Huff AF, Gao F, Ren J (2014) Akt2 knockout alleviates prolonged caloric restriction-induced change in cardiac contractile function through regulation of autophagy. J Mol Cell Cardiol 71:81–91
49. Zheng Q, Zhao K, Han X, Huff AF, Cui Q, Babcock SA, Yu S, Zhang Y (2014) Inhibition of AMPK accentuates prolonged caloric restriction-induced change in cardiac contractile function through disruption of compensatory autophagy. Biochim Biophys Acta. doi:10.1016/j.bbadis.2014.1004.1023
50. Castello L, Froio T, Maina M, Cavallini G, Biasi F, Leonarduzzi G, Donati A, Bergamini E, Poli G, Chiarpotto E (2010) Alternate-day fasting protects the rat heart against age-induced inflammation and fibrosis by inhibiting oxidative damage and NF-kB activation. Free Radic Biol Med 48(1):47–54
51. Castello L, Maina M, Testa G, Cavallini G, Biasi F, Donati A, Leonarduzzi G, Bergamini E, Poli G, Chiarpotto E (2011) Alternate-day fasting reverses the age-associated hypertrophy phenotype in rat heart by influencing the ERK and PI3 K signaling pathways. Mech Ageing Dev 132(6–7):305–314
52. Tani M, Honma Y, Takayama M, Hasegawa H, Shinmura K, Ebihara Y, Tamaki K (1999) Loss of protection by hypoxic preconditioning in aging Fischer 344 rat hearts related to myocardial glycogen content and Na+ imbalance. Cardiovasc Res 41(3):594–602
53. Abete P, Cacciatore F, Testa G, Della-Morte D, Galizia G, de Santis D, Calabrese C, Cioppa A, Ferrara N, Rengo F (2010) Ischemic preconditioning in the aging heart: from bench to bedside. Ageing Res Rev 9(2):153–162

54. Abete P, Testa G, Ferrara N, De Santis D, Capaccio P, Viati L, Calabrese C, Cacciatore F, Longobardi G, Condorelli M, Napoli C, Rengo F (2002) Cardioprotective effect of ischemic preconditioning is preserved in food-restricted senescent rats. Am J Physiol Heart Circ Physiol 282(6):H1978–H1987
55. Broderick TL, Driedzic WR, Gillis M, Jacob J, Belke T (2001) Effects of chronic food restriction and exercise training on the recovery of cardiac function following ischemia. J Gerontol A Biol Sci Med Sci 56(1):B33–B37
56. Chandrasekar B, Nelson JF, Colston JT, Freeman GL (2001) Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 280(5):H2094–H2102
57. Edwards AG, Donato AJ, Lesniewski LA, Gioscia RA, Seals DR, Moore RL (2010) Life-long caloric restriction elicits pronounced protection of the aged myocardium: a role for AMPK. Mech Ageing Dev 131(11–12):739–742
58. Long P, Nguyen Q, Thurow C, Broderick TL (2002) Caloric restriction restores the cardioprotective effect of preconditioning in the rat heart. Mech Ageing Dev 123(10):1411–1413
59. Peart JN, See HL, Pepe S, Johnson P, Headrick JP (2012) Opposing effects of age and calorie restriction on molecular determinants of myocardial ischemic tolerance. Rejuvenation Res 15 (1):59–70
60. Shinmura K, Tamaki K, Bolli R (2005) Short-term caloric restriction improves ischemic tolerance independent of opening of ATP-sensitive K+ channels in both young and aged hearts. J Mol Cell Cardiol 39(2):285–296
61. Shinmura K, Tamaki K, Bolli R (2008) Impact of 6-mo caloric restriction on myocardial ischemic tolerance: possible involvement of nitric oxide-dependent increase in nuclear Sirt1. Am J Physiol Heart Circ Physiol 295(6):H2348–H2355
62. Shinmura K, Tamaki K, Saito K, Nakano Y, Tobe T, Bolli R (2007) Cardioprotective effects of short-term caloric restriction are mediated by adiponectin via activation of AMP-activated protein kinase. Circulation 116(24):2809–2817
63. Sung MM, Soltys CL, Masson G, Boisvenue JJ, Dyck JR (2011) Improved cardiac metabolism and activation of the RISK pathway contributes to improved post-ischemic recovery in calorie restricted mice. J Mol Med 89(3):291–302
64. Schneider CA, Taegtmeyer H (1991) Fasting in vivo delays myocardial cell damage after brief periods of ischemia in the isolated working rat heart. Circ Res 68(4):1045–1050
65. Ahmet I, Wan R, Mattson MP, Lakatta EG, Talan M (2005) Cardioprotection by intermittent fasting in rats. Circulation 112(20):3115–3121
66. Katare RG, Kakinuma Y, Arikawa M, Yamasaki F, Sato T (2009) Chronic intermittent fasting improves the survival following large myocardial ischemia by activation of BDNF/VEGF/PI3K signaling pathway. J Mol Cell Cardiol 46(3):405–412
67. Sciarretta S, Hariharan N, Monden Y, Zablocki D, Sadoshima J (2011) Is autophagy in response to ischemia and reperfusion protective or detrimental for the heart? Pediatr Cardiol 32 (3):275–281
68. Yamamoto T, Sadoshima J (2011) Protection of the heart against ischemia/reperfusion by silent information regulator 1. Trends Cardiovasc Med 21(1):27–32
69. Shinmura K, Yamamoto T, Tamaki K, Katsumata Y, Sano M, Fukuda K (2012) Caloric restriction ameliorates myocardial ischemia/reperfusion injury by suppressing complement activation in a Sirt1-dependent manner. Circulation 126:A12784
70. Shinmura K, Tamaki K, Sano M, Nakashima-Kamimura N, Wolf AM, Amo T, Ohta S, Katsumata Y, Fukuda K, Ishiwata K, Suematsu M, Adachi T (2011) Caloric restriction primes mitochondria for ischemic stress by deacetylating specific mitochondrial proteins of the electron transport chain. Circ Res 109(4):396–406
71. Shinmura K, Tamaki K (2011) Essential role of nitric oxide synthase in caloric restriction-induced cardioprotection. Circulation 124:A11579
72. Stein PK, Soare A, Meyer TE, Cangemi R, Holloszy JO, Fontana L (2012) Caloric restriction may reverse age-related autonomic decline in humans. Aging Cell 11(4):644–650

73. Sloan C, Tuinei J, Nemetz K, Frandsen J, Soto J, Wride N, Sempokuya T, Alegria L, Bugger H, Abel ED (2011) Central leptin signaling is required to normalize myocardial fatty acid oxidation rates in caloric-restricted ob/ob mice. Diabetes 60(5):1424–1434
74. AlGhatrif M, Watts VL, Niu X, Halushka M, Miller KL, Vandegaer K, Bedja D, Fox-Talbot K, Bielawska A, Gabrielson KL, Barouch LA (2013) Beneficial cardiac effects of caloric restriction are lost with age in a murine model of obesity. J Cardiovasc Transl Res 6(3):436–445
75. Takatsu M, Nakashima C, Takahashi K, Murase T, Hattori T, Ito H, Murohara T, Nagata K (2013) Calorie restriction attenuates cardiac remodeling and diastolic dysfunction in a rat model of metabolic syndrome. Hypertension 62(5):957–965
76. van der Meer RW, Rijzewijk LJ, Diamant M, Hammer S, Schar M, Bax JJ, Smit JW, Romijn JA, de Roos A, Lamb HJ (2008) The ageing male heart: myocardial triglyceride content as independent predictor of diastolic function. Eur Heart J 29(12):1516–1522
77. Yamamoto T, Shinmura K, Sano M, Fukuda K (2013) Protective role of cardiac Silent Information Regulator 1 against high fat diet-induced cardiac hypertrophy. Circulation 128: A14684
78. Ingram DK, Zhu M, Mamczarz J, Zou S, Lane MA, Roth GS, deCabo R (2006) Calorie restriction mimetics: an emerging research field. Aging Cell 5(2):97–108

Chapter 10
Calorie Restriction Mimetics: Progress and Potential

George S. Roth and Donald K. Ingram

Abstract Much has been written in recent years on the emerging field of calorie restriction mimetics (CRM), which appears to be the most efficient strategy for translating the huge body of research on the beneficial health and longevity promoting effects of actual calorie restriction (CR) into practice. While the latter has been shown to maintain, health, vitality, and function, as well as increase lifespan in many animal species, controversy still exists as to its relevance, particularly to primates/humans. While this review will assume enough consensus to at least warrant interest in obtaining the benefits suggested by CR, it will additionally weigh the evidence that CRMs can replicate some, if not most, of the same positive biological effects, and attempt to offer as objective an evaluation as possible of the current status of CRMs, and the best opportunities to extend this strategy to human applications.

10.1 Introduction

The concept of calorie restriction mimetics (CRM) was introduced by our group in 1998 with the first deliberate attempt to mimic the biological effects of CR (with the ultimate goal of replicating its anti-aging and health promoting benefits) without appreciably reducing food intake [1]. Male Fischer-344 rats were randomly divided into five groups, which included ad libitum-fed controls, three groups fed 2-deoxy-glucose (2DG) in the diet at 0.2, 0.4, and 0.6 % weight/weight, and a pair-fed group, whose intake was adjusted to match the group with the lowest intake. 2DG inhibits

G.S. Roth (✉)
GeroScience, Inc., Pylesville, MD 21132, USA
e-mail: geor@iximd.com

D.K. Ingram
Nutritional Neuroscience and Aging Laboratory, Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70809, USA
e-mail: donald.ingram@pbrc.edu

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the metabolism of glucose-6-phosphate, the second step in cellular glycolysis, thereby reducing overall glycolytic flux [2], which may represent a key strategy for the development of efficient CRM [3]. Those animals fed the medium 2DG dose weighed slightly less than controls and had reduced body temperatures and fasting insulin levels. Effects were apparent by 5 weeks and maintained until at least 24 weeks of treatment. Food intake was reduced very slightly early on as rats adjusted to the diet, but the reduction disappeared by about 16 weeks. Although 4 (of a total of 25) animals receiving the highest 2DG dose died of apparent cardiac toxicity, possibly related to myocardial vacuole formation (which also occurred in some of the other groups, but seemed to regress over the course of the study), no other degenerative changes were observed. In summary, it indeed appeared possible to duplicate key metabolic effects of CR without necessarily reducing food intake, although the range between effective and toxic 2DG doses appeared relatively narrow.

In that initial report [1], we suggested that, subsequent to additional confirmatory and mechanistic studies, CRMs might provide a useful compliment to CR itself. Indeed, the journal Editor provided a rather prescient note that concluded with, "Is it possible to develop a therapy which has the same cellular outcome that caloric restriction does, without restricting calories? In principle this approach is feasible, as shown by Lane and his colleagues." Demonstrating that the this field of research has expanded and matured, Table 10.1, which is derived from that found on the

Table 10.1 Current Wikipedia list of calorie restriction mimetics

Resveratrol
Metformin
Rimonabant (acomplia)
Lipoic acid (α-lipoic acid, alpha lipoic acid, or ALA)
2-deoxyglucose
Anti-glycators such as aminoguanidine and carnosine
Exanadin (exanatide), a GLP (glucagon-like peptide) modulator
Olbetam (acipimox)
PPAR gamma modulators, such as rosiglitazone and gugulipids
Leptin
Agents that modulate sirtuins (called STAC—sirtuin activating compounds), for example fisetin
4-phenylbutyrate (PBA)
Hydroxycitrate
Gymnemoside (modulates glucose metabolism)
Adiponectin, (together with leptin, it takes part in fat metabolism. It is activated by PPAR blockers such as rosiglitazone)
DPP-4 inhibitors (diapeptidyl peptidase 4)
Modulators of NPY, the neuropeptide Y
Iodoacetate
Rapamycin
Mannoheptulose (glycolytic inhibitor)

Wikipedia website, (www.en.wikipedia.org/wiki/Caloric_restriction_mimetic), provides a long list of purported candidate CRMs. Indeed, so many research groups, commercial enterprises, and other interested parties have now joined the quest for what might be the "Holy Grail of Biogerontology" that this is now a multimillion dollar industry, which at last count had generated nearly 80 peer-reviewed papers, review articles/chapters [4], and books [5, 6]. Consequently, it has become a challenge to provide any fresh perspective on CRMs in the face of an expanding literature, especially with so many experts entering the discussion, and interfacing it with the parent paradigm of actual CR. Nevertheless, herein we have attempted to provide a selective review of the current state of the CRM research, with emphasis primarily on what might be our best expectations for both individual CRM candidates and the field in general.

Given the current state of disagreement as to whether CR itself is a "real" paradigm that can benefit "normal" animals, let alone have its health and longevity benefits extrapolated to primates/humans, a few words of background are appropriate. Long considered the most robust paradigm in experimental gerontology, CR, also known as diet restriction (DR), involves restricting intake of a nutritious diet by 20–60 % from ad libitum levels [7, 8]. This intervention, used in many rodent studies, increases lifespan (both median and maximum) as well as healthspan by retarding the onset and delaying the incidence of age-related disease and maintaining physiological and behavioral function later into life [8, 9]. In other vertebrate species, including dogs and monkeys, studies have indicated the beneficial effects of CR on indices of aging to suggest relevance to human aging [7]. Related, epidemiological studies of long-term CR practitioners and short-term experimental studies of gradually imposed CR demonstrated that reductions in risk factors for major age-related diseases as well as improvements in putative biomarkers of aging are possible as well as feasible for human applications [7, 10]. Nevertheless, even if evidence existed that life-long CR could produce beneficial effects for humans paralleling those observed in rodents, implementation of this intervention might be extremely difficult. Compliance issues, as well as other quality of life issues impacted by CR, including thermoregulation, satiety, libido, and bone health could conceivably arise [11].

Moreover, gerontologists have challenged the relevance of the CR paradigm itself and whether it applies to long-lived species [12, 13]. Selman [14] recently reviewed the controversy for both rodents and nonhuman primates. Briefly, in the case of wild caught mouse populations, moderate CR was actually detrimental to most of the animals in terms of longevity, although 20–30 % did seem to exhibit increased lifespans [15], while a number of laboratories have utilized both inbred and recombinant strains to achieve both positive and negative longevity effects depending on animals, the degree of restriction, and other experimental variables including gender [16].

The situation regarding CR benefits to nonhuman primates is less complicated, as there are only two major ongoing studies, at the National Institute on Aging (NIA) and the University of Wisconsin (UW), and both report better health and function under 30 % CR conditions [17–19]. However, the UW group has observed increased

survival with respect to both age-related and non age-related causes of death [17, 18], while the NIA group, using cohorts of both substantially older and younger rhesus monkeys at the onset of CR, has reported no statistically significant longevity effects after over 20 years on CR [19]. When subjected to power analyses, the current results project this situation will not to change in the future [19]. It is, however, interesting to note that the older cohort of NIA monkeys yielded 5 animals who survived beyond 40 years of age, and 4 of these were on CR [19]. Possible reasons for apparent discrepancies between the results of these two studies have been, and are being (the authors were the initiators of the NIA study in 1987, and continue with very limited involvement to monitor both projects) reviewed separately, although the major factor appears to be the relatively high sucrose and fat diet at the UW, which has produced heavier and fatter "control" animals, with a greater chance of exhibiting CR benefits than NIA counterparts, which are fed slightly less than ad libitum to minimize the possibility of complications of obesity and over-nutrition. Both criticisms are often leveled at the CR paradigm to suggest it is simply a "laboratory artifact" that benefits primarily sedentary, overweight controls.

The above complications and possible confusion in interpretation notwithstanding, it is important to note that both studies should be viewed together as a continuum of caloric intake. Moreover, the UW control monkeys more resemble the majority of people in developed countries, who are indeed overweight, and therefore have true relevance to the practical human situation. Thus, we would argue that CR is indeed relevant and may be beneficial for primates/humans, but may be difficult for most individuals to implement for the reasons outlined above. Thus, we come full circle to the realm of CRMs as the best possible strategy for maintenance of healthy years.

10.2 Categories of CRMs

The CRM concept continues to evolve and has generated widespread discussion. In a broad context, considerations of CRMs have pertained to virtually any intervention that produces benefits on aging, healthspan, and lifespan similar to those of CR. These have included antioxidants, hormones, metal chelators, and appetite suppressants. One paper suggested broadening the definition to include exercise [20]. In our earlier discussions [21], we proposed a more narrowly defined concept entailing the following criteria: (1) mimics the metabolic, hormonal, and physiological effects of CR; (2) activates stress response pathways observed in CR and enhances stress protection; (3) produces CR-like effects on longevity, reduces age-related disease, and maintains more youthful function; and (4) does not significantly reduce food intake, at least over the short-term. The last is intended more as an experimental control rather than an absolute criterion. Various interventions that reduce food intake are by that fact inducing CR; thus, it becomes difficult to ascertain that they are in fact "mimicking" CR unless careful pair-feeding studies are conducted. Nonetheless, with further regard to this criterion, we must recognize that if a candidate CRM alters body composition as expected, it is highly likely, but

not certain, that such an intervention might also result in reduced food intake over the long-term. Consequently, the criterion of no significant effects on food intake should apply only to initial introduction of the intervention. Nevertheless, the paradigm remains one of "Having one's cake and eating it, too."

Another consideration is whether an effective CRM must demonstrate the ability to increase lifespan. CR has garnered extensive research attention because of this unique quality of extending median and maximum lifespan over a broad range of species. More recently, increased attention has been given to the benefits of candidate anti-aging interventions for extending healthspan [22]. In this regard, the Intervention Testing Program (ITP) sponsored by the National Institute on Aging (NIA) has brought the needed standardization and rigor to the complex challenge of evaluating anti-aging interventions [23, 24]. After investigating over a dozen compounds to date, only one, rapamycin, has shown consistent effects on lifespan extension in both male and female mice [25, 26]. Various other candidates, such as aspirin, nordihydroguaiaretic acid, and acarbose have demonstrated sex-specific benefits on lifespan [24, 27]. Previously, many of these interventions had shown beneficial effects in delaying age-related disease or functional declines, but they did not significantly increase lifespan. A particularly pertinent example is resveratrol, which benefited a wide range of healthspan measures, but did not significantly increase lifespan in mice on a normal diet [23, 28].

The ultimate goal for developing CRM is translation to human interventions. In this regard, while we can acknowledge that CR can induce physiological and molecular changes in humans that parallel findings in rodent CR studies and portend reduced risk of age-related disease and increased healthspan, we do not know, and might not ever know, if long-term CR in humans would extend lifespan. Moreover, addressing this question in closely related primate species has now yielded mixed results, as discussed in the Introduction. Since our focus will emphasize best expectations (function/vitality, lifespan, and/or healthspan), it would be efficient at this point to provide a list of CRM candidate categories, and attempt to determine how each may exert potential benefits. For purposes of this review then, we will utilize the following operational classification:

1. Alternative dietary and mechanical strategies, which include appetite suppressants, nutrient substitutes (such as Olestra), manipulation of specific nutrients (e.g. methionine restriction), intermittent feeding regimens, and bariatric surgery
2. Inhibition of nutrient absorption/digestion.
3. Glycolytic inhibition and related sequellae
4. Endocrine regulation
5. Regulation of specific gene expression (e.g. sirtuins, mTOR, and PEPCK)
6. Miscellaneous other CRM strategies.

It should be recognized that many ways exist to group the above CRM candidates. The arbitrary categories defined here are best suited to our current discussion of common health, vitality, and longevity benefits. Moreover, due to editorial constraints, references are limited to the most recent (for a more comprehensive list, see Ingram and Roth [4]).

10.2.1 Alternative Dietary and Mechanical Strategies

Although the CRM candidates in this category appear to have quite different biological mechanisms of action, they will be considered together since their mode of administration, delivery vehicle/process, etc. fall somewhat outside the more stringent definition(s) of CRMs described above. For conciseness, we will not include exercise or other more esoteric/ascetic lifestyle modifications [29], which have been briefly reviewed elsewhere, although in the broadest sense, these may also mimic the beneficial effects of CR to some extent.

Table 10.2 lists the current Wikipedia compilation of anorectics or appetite suppressants (www.en.wikipedia.org/wiki/Appetite_suppresant). It is immediately obvious that this field is even broader than that of CRMs, and impossible to cover in any depth here, except to restate that such agents, by definition, reduce food intake and therefore are, in effect, actually CR. Since, with the exception of a few traditional folk medicinals and other herbal derivatives, most current commercial appetite suppressant products have only been marketed/utilized for the last few decades, few individuals have had the opportunity to fully experience either the beneficial or negative effects, and there appear to be few controlled long-term studies. Thus, expectations (assuming minimal side effects particular to each agent) might be similar to extrapolations from the few short-term human CR studies, and less-well controlled longer term practice, mentioned in the Introduction. Specifically, delayed and reduced incidence of cardiovascular disease, cancer, and diabetes, as well as inflammatory diseases, and, of course, better weight management have been either directly observed or inferred through beneficial changes in biomarkers such as LDL-cholesterol, triglycerides, C-reactive protein, fat mass, insulin levels and sensitivity [7, 10, 30]. As stated above, no predictions regarding lifespan extension can be made, especially in light of current questions regarding CR effects in primates and extrapolation to humans. Nevertheless, amelioration of potential diseases, and even predisposition to diseases, would seem to bode well for increased healthspan, if not actual lifespan.

Moreover, since the quality of life is equally, if not more important than the quantity of life, it should be pointed out that appetite suppressants reduce the former somewhat by decreasing enjoyment of eating; hence supporting the time-worn adage that "CR doesn't actually lengthen life, it only SEEMS longer!" Consequently, the stricter definition of CRMs again offers a better alternative for the "Having one's cake and eating it, too" model suggested above. In this regard, specific dietary nutrient substitutes that allow for the pleasure of eating without the negative effects of calories or fat, such as Olestra, provide another alternative CRM strategy.

Olestra. Olestra, $C_{n+}12H_{2n+22}O_{13}$ (where fatty acids are saturated), is a fat substitute first developed by Procter and Gamble. It was synthesized from sucrose and can bond 6–8 fatty acids. The resulting radial arrangement results in a compound that is too large and irregular to be digested and absorbed through the intestine, and thus allows consumption without appreciable calories. Thus, in some respects, it is

Table 10.2 Current Wikipedia list of anorectics/appetite suppressants

The following drugs listed as "centrally-acting antiobesity preparations" in the anatomical therapeutic chemical classification system:
• Phentermine (adipex, duromine, fastin, ionamin, metermine, etc.)
• Diethylpropion (tenuate)
• Rimonabant (acomplia; cannabinoid antagonist selective for CB1. Withdrawn amidst concerns about psychiatric consequences of drug treatment)
• Sibutramine (meridia, reductil; withdrawn from the market due to concerns regarding its cardiovascular effects)
• Oxymetazoline (over the counter afrin nasal decongestant)
The following are listed as appetite depressants by medical subject headings (MeSH):
• Benfluorex
• Butenolide
• Cathine
• Diethylpropion
• FG-7142
• Phenmetrazine
• Phentermine
• Phenylpropanolamine
• Pyroglutamyl-histidyl-glycine
• Sibutramine
• Other compounds with known appetite suppressant activity include:
• Amfetamine (also known as amphetamine, US brand name of mixed amfetamine salts is Adderall)
• Benzphetamine (didrex)
• Bupropion (formerly known as amfebutamone; brand names: Prexaton, Wellbutrin, Zyban) - An atypical antidepressant
• Dexamfetamine (also known by its USAN and brand names: dexamphetamine, dexedrine, dextrostat)
• Dexfenfluramine† (adifax; the d-enantiomer of fenfluramine; withdrawn for the same reason as its racemate)
• Dexmethylphenidate (focalin)
• Fenfluramine [ponderal, ponderax, pondimin; one of the two components (the other being phentermine) of fen-phen. Since discontinued to its potential for causing valvulopathies and pulmonary hypertension]
• Glucagon (GlucaGen)
• Methylenedioxypyrovalerone (MDPV)
• Lorcaserin (belviq)
• Lisdexamfetamine (vyvanse)
• Metamfetamine (also known as methamphetamine, desoxyn)

(continued)

Table 10.2 (continued)

• Methylphenidate (concerta, ritalin)
• Phendimetrazine (bontril)
• Phenethylamine
• Topiramate (topamax, an anticonvulsant medication. Sometimes combined with phentermine under the brand name qsymia as a treatment for obesity)

similar to the inhibitors of nutrient absorption and digestion that will be discussed subsequently, but unlike the latter does not itself directly inhibit absorption and digestion of other nutrients. End results of long-term consumption of all of these agents may still be similar to actual CR, especially if secondary to reduced caloric consumption, weight loss, and reduction in body fat.

Amino Acid Restriction. Manipulation of specific nutrients may be another means to achieve some of the benefits of CR without actually dieting. Both tyrosine, and more recently methionine, restriction has exhibited some promise [31, 32], although the mechanism(s) of action are unclear, and potential for negative side effects remains substantial. Nevertheless, extension of lifespan and healthspan in rodents by such interventions necessitates the need for further research. Apparently, diets low in specific nutrients may not be particularly palatable or easy to formulate, and identification of mimetics to substitute for such diets may be even more difficult.

Intermittent Feeding. Fasting regimens may offer another alternative to CR, although a compromise at best, since periods of refraining from eating is necessary to achieve benefits. A tradition of fasting exists in many religions and cultures, and the resulting physiological benefits suggest a selective advantage for practitioners over the years, although again, no well controlled long-term studies appear to have been conducted [29, 33]. In animal studies, both lifespan and healthspan extension have been observed, with specific protection against cancer, glucose intolerance, obesity, and oxygen radical damage [14]. Thus, many of the benefits of full CR may accrue for intermittent feeding practitioners, if they are willing to undergo periods of abstinence from food.

Bariatric Surgery. Finally, bariatric surgery offers another alternative in this particular category. The nearest approximation to actual CR would be blocking absorption of food products that are converted to calories. Bariatric surgery offers a means of reducing energy production by reducing the energy ingested from reaching circulation. The many health benefits of this surgical intervention to treat obesity are now well documented with a rapidly expanding literature, which has been reviewed extensively elsewhere [4]. However, those that would accrue following bariatric surgery in normal weight individuals, are largely unknown., Nevertheless, possibilities for benefit exist, but with the relatively high risks associated with the surgery, this intervention may be necessarily confined to obese, diabetic, or other “at risk” individuals in need therapy.

10.2.2 Inhibition of Nutrient Absorption/Digestion

Moving to strategies that fit the more stringent definition(s) of CRM (i.e. NOT reducing food intake, requiring anatomical manipulations, etc.), we will briefly deal with specific inhibitors of nutrient adsorption and/or digestion. Three main types of inhibitors of fat absorption and digestion have been investigated, including chitosan, tetrahydrolipstatin, and mannanoligosaccharides. Several of these have already undergone extensive clinical evaluation and results have been reviewed elsewhere [4]. In summary, fat blockers as a strategy for developing CRM appear to have merit, although the results on body weight and composition as well as other blood parameters relevant to CR are modest at best. Additionally, there appear to be undesirable side effects to some of the products. Most important regarding this review is the fact that long-term animal studies have yet to be conducted to ascertain beneficial effects of these compounds on healthspan and lifespan. Thus, more work is required to determine practical feasibility as viable CRMs.

A final strategy of gut intervention to affect energy availability is the targeting oft carbohydrate digestion to affect glucose availability. Acarbose (ACA), composed of an acarviosin moiety with a maltose at the reducing terminus, is a leading candidate for this approach. It inhibits glycoside hydrolases, the enzymes required to digest carbohydrates, specifically, alpha-glucosidase enzymes, within the brush border of the small intestines and also inhibits pancreatic alpha-amylase [34]. The latter hydrolyzes complex starches to oligosaccharides in the lumen of the small intestine, and the membrane-bound alpha-glucosidases hydrolyze oligosaccharides, trisaccharides, and disaccharides to glucose and other monosaccharides in the small intestine. By inhibiting these enzymes, there is a reduction in the digestion rate of complex carbohydrates resulting in less glucose being absorbed, since carbohydrates are not broken down into glucose molecules. Like the fat blockers above, ACA has been reviewed by us elsewhere [4]. Similar to metformin, to be discussed in a later section, ACA has widespread use for the treatment of diabetes; thus, its effects on healthspan and lifespan in human populations will be subject to many subsequent analyses. It is sold generically in Europe and China as Glucobay (Bayer) and in North America as Precose and in Canada as Prandase. The popularity of ACA in China is much greater, likely due to two reasons. First, effects of ACA appear to be higher in persons on high carbohydrate diets, such as in Eastern cultures. Second, some side effects, including diarrhea and increased flatulence, are not uncommon among Western users, so these can outweigh the benefits. Nevertheless, large well-controlled studies of Eastern populations have found ACA to be safe and effective for diabetes and even prediabetes with apparent equal effectiveness as metformin [35]. Consequently, inhibition of carbohydrate digestion by agents such as acarbose offers yet another possible strategy by which to mimic at least some beneficial CR effects. Whether or not long-term administration to normal, nondiabetic individuals will support the promise of such CRM candidates (gender specific differences in rodent studies notwithstanding) will require additional documentation.

10.2.3 Glycolytic Inhibition and Related Sequellae

The next step "downstream" from blocking energy availability and absorption at the gut level is blocking energy utilization at the cellular level. As a potentially fertile target, we previously proposed the glycolytic pathway and have subsequently supported this proposal in other publications [3, 4]. This metabolic pathway offers several points for intervention, specifically inhibiting the enzymes–hexokinase, phosphoglucose isomerase, and phosphofructokinase–involved in the intracellular conversion of glucose and glucose products to ATP.

2-deoxy-D-glucose. As described in the Introduction, as an initial approach, we proposed in 1998 to target phosphoglucose isomerase, the second step in this pathway [1]. The candidate molecule was 2-deoxy-D-glucose (2DG) as it offered a well-established history as a glycolytic inhibitor. Moreover, the literature on 2DG offered several insights suggesting the potential of this compound as a CRM. Injections of 2DG had been shown to inhibit tumor growth, induce torpor, and increase circulating levels of glucocorticoids, all of which are hallmarks of CR [3]. Also as mentioned previously, our first study of 2DG as a candidate CRM [1], fed young male Fischer-344 (F344) rats diets supplemented (by weight) with 0.2, 0.4, or 0.6 % 2DG to approximate doses of 100–150, 250–300, or 400–450 mg kg, respectively. The high dose turned out to be toxic, which would be consistent with the U-shaped effects of CR on mortality. Nonetheless, at the lower doses the 2DG diets affected two important biomarkers of CR, but without significant effects on food intake. Specifically, plasma insulin and body temperature were reduced at the 0.4 % dose. A persistent concern regarding glycolytic inhibition was the possibility of hyperglycemia; however, we saw no significant effects on plasma glucose levels.

Since that initial study of 2DG, evidence from other studies strengthened its prospect as a CRM. Several investigations demonstrated 2DG protection against various in vivo and in vitro stressors similar to CR which have been reviewed [4].

Cancer research has experienced a rebirth of interest in glycolytic inhibition. Based on the classic Warburg effect [36], cancer cells up-regulate glucose metabolism to support their rapid growth. Depending upon cancer cell type, glycolytic enzymes are up-regulated while mitochondria production is down-regulated [37]. Cancerous cells utilize this metabolic shift to gain an energetic advantage over normal cells because they do not need to depend on oxygen. Thus, similar to the abundant reports of robust effects of CR on tumor induction and growth, glycolytic inhibition may be an effective anti-cancer intervention [37].

Several studies had reported beneficial effects of 2DG injections on inhibiting tumor growth [38] prior to our proposal of 2DG as a CRM, and the effectiveness of 2DG as an anti-tumor agent has been confirmed in several other studies. Notably, Zhu et al. [39] demonstrated that 2DG markedly reduced mammary tumor growth in female Sprague-Dawley rats induced by injection of 1-methyl-1-nitrosourea. It is important to note that these investigators used dietary concentrations of 0.02 and 0.03 % 2DG after observing that the concentrations used in our first study [1] significantly inhibited body weight growth in this rat strain, while these lower

concentrations did not. Nevertheless, these lower concentrations reduced serum insulin and raised serum corticosterone levels consistent with CR effects, but there were no significant effects on glucose, leptin, or IGF-1 values. Utilizing cultures of cancer cells (MCF-7) treated with 2DG, these researchers additionally noted upregulation in important CR-related signaling pathways, specifically, increased levels of phosphorylated AMPK and SIRT1.

The ability of a compound to increase both median and maximum lifespan stands as the highest standard of proof for a CRM. In regard to 2DG, this proof has been provided in the nematode model of aging in the laboratory of Michael Ristow. Using various concentrations of 2DG in worm cultures, Schulz et al. [40] reported significant increases in lifespan, which were dependent upon AMPK signaling [40]. Additionally, these investigators proposed a major hypothesis of how glycolytic inhibition could produce the health benefits of CR, described as the concept of "mitohormesis." This phenomenon was supported by results demonstrating short-term evidence of oxidative stress in response to 2DG that in turn induced adaptive responses to increase stress resistance. In support of this hypothesis, they showed that the effects of 2DG on longevity could be eliminated when the worm cultures were treated with antioxidants, including N-acetyl-cysteine, vitamin C, or vitamin E. In essence, it was proposed that hormesis is the induction of a mild stress that results in improved responses to greater stressors. Interest in the concept of hormesis as a major mechanism of CR [41] is expanding. Thus, we should consider that this concept could apply to the actions CRM as well.

In summary, an impressive literature has emerged which shows marked parallels between CR and 2DG treatment to strongly support its candidacy as a CRM [42]. Unfortunately, some major caveats have emerged to attenuate this positive profile. For example, we conducted toxicity studies and discovered the concentrations we had used (0.2–0.4 %) produced cardiotoxicity in both F344 and Brown-Norway rats [43]. Several aspects of the expected phenotype of a CRM were again reproduced in 2DG-treated groups, including reduced blood levels of glucose and insulin as well as lower body temperature. Cardiotoxicity was observed in the form of vacuolarization of cardiac myocytes leading to heart failure in many rats in the long-term study. The cause of this pathology has not been established, but it appeared to be dose-dependent. However, these negative results can be contrasted to positive results reported in other studies examining 2DG effects on cancer [42] and Alzheimer's disease [44] at lower doses than we employed. Thus, while discouraging to some degree, we must consider that 2DG could remain a viable candidate if the dose dependency of the toxicity can be fully established.

D-glucosamine. Considerable attention has recently been drawn to a compound related to 2DG that represents a commonly used dietary supplement, D-glucosamine (GlcN), which is used to treat symptoms of osteoarthritis [45]. GlcN is a component of chitosan described in the previous section. This monosaccharide (2-amino-2-deoxy-D-glucose) acts through the hexosamine biosynthesis pathway to provide a precursor for the synthesis of glycosylated proteins and lipids, and is part of the structure of chitosan and chitin. Its application for treating osteoarthritis

derives from it being a precursor for glycosaminoglycans, which are major components of joint cartilage. Beyond this application, however, GlcN in its phosphorylated form, GlcN-6-phosphate, acts as an inhibitor of hexokinase, with high activity directed toward glucokinase, the liver specific isoform. As described above, the Ristow laboratory first reported lifespan extension in nematodes using 2DG, which seemed to act via mitohormesis [40]. In their most recent paper, Weimer et al. [45] demonstrated significant lifespan extension in nematodes treated with GlcN that appeared to be independent of the hexosamine pathway. Duplicating their earlier 2DG study, they observed evidence of mitohormesis, demonstrated by transient increases in mitochondrial ROS production in GlcN-treated worm cultures. In the newer study [45], they expanded their studies to a mouse model and observed that aged mice (100 weeks old) had significantly increased lifespan, with no treatment effects on food intake or body composition, or energy expenditure. One notable difference was reduced blood glucose under random fed, but not fasted, conditions. Gene array analysis in both species pointed to decreased glycolysis compensated for by increased amino acid metabolism that was linked to SKN-1/NRF-2-dependent transcription. Another recent study has provided evidence that GlcN activates autophagy [46], an essential mechanism of cellular homeostasis that will be discussed below. Given the impressive safety profile of this dietary supplement along with findings from a formal toxicity study in rats [47], this recent report on prolongevity effects of GlcN will likely generate new studies investigating its effects on healthspan.

Mannoheptulose. Mannoheptulose (MH) represents another potentially promising glycolytic inhibitor. In a recent review of the CRM literature, we have proposed further research on this seven carbon sugar as a candidate CRM [3]. Described in reports dating to the 1970s, MH was investigated as a treatment for hypoglycemia, since when delivered IV at high doses, it would produce huge spikes in glucose with a marked decrease in insulin. This action was hypothesized to result from inhibition of glycolysis at the level of hexokinase II (HKII) in pancreatic β-cells. High doses of MH have also demonstrated efficacy in rodent tumor models [48, 49]. A logical follow-up to previous work with 2DG was to identify a compound that reduces insulin levels without producing hyperglycemia and without toxicity and/or other negative side effects when given chronically. Thus, we initiated experiments to support that proposal using in vitro and in vivo cell models [50, 51]. A major advantage for translational purposes is the ability to utilize an extract of unripened avocados found to contain high concentrations of this rare sugar. Preliminary studies support the safety and efficacy of an avocado extract enriched in MH when given to dogs. Of particular interest was the dose-dependent reduction in fasting insulin levels without increased glucose [51].

3-bromopyruvate. As mentioned previously, the cancer field is also actively involved in identifying glycolytic inhibitors. 3-bromopyruvate (3BP) is one compound that has attracted considerable attention. A simple lactic acid analog, a brominated derivative of pyruvic acid, 3BP acts, like MH, to inhibit HKII, the first step in the glycolytic process [37]. While no studies have focused on this compound as a CRM for attenuating aging, many studies have examined its efficacy against a

variety of tumor models. As stated, the Warburg effect accounts for the increased glycolytic activities of many tumors and has thus generated efforts to identify HKII inhibitors for cancer treatment, since many tumor lines greatly upregulate HKII activity and increase its binding to mitochondria [37]. Ko et al. [52] used a rabbit liver cancer model to demonstrate that a high level of HKII activity could be lowered that was accompanied by a marked reduction in tumor growth following IP 3BP treatment over the course of several days. When 3BP was delivered to rabbits IV, again these investigators reported even more impressive tumor size reductions with no evidence of pathology in other tissues. Using AS-30D hepatoma cells in a rat tumor model, Ko et al. [53] noted similar efficacy to the point that most animals showed no residual signs of cancer. Additional studies have noted the effectiveness of 3BP against leukemia cells [54]. 3BP purportedly enters tumor cells via lactic acid transporters and inhibits HKII bound to mitochondria [37].

Based on the results demonstrating its anti-tumor effects, 3BP could qualify as a candidate CRM, but further evaluation is clearly needed regarding long-term toxicity. While many previous studies have reported little or no toxicity from 3BP treatment [37], a few have noted issues. For example, one report observed dose-related toxicity to the liver and gastrointestinal tract in rabbits treated with 3BP via intra-arterial delivery in doses similar to those applied in previous studies [55]. Moreover, ICV delivery of 3BP in rats has been reported to reduce brain metabolism, neurotransmitter function, particularly in the cholinergic system, and behavioral impairment [56]. Finally, 3BP has been noted to negatively impact spermatozoal metabolism [57]. All these effects, whether anti-tumor effects or negative effects on brain metabolism, appear to be dose dependent. Of course, inhibition of energy production, or major suppression of ATP production, which could be an effective anti-tumor treatment, could be lethal for the cell and the animal, so careful dose studies of 3BP are still required. A patent for 3BP for cancer treatment has been approved and a company organized to promote its development (www.presciencelabs.com). Following marginal success in a single case clinical trial targeted to fibrolamellar hepatocellular carcinoma in a young man [58], the company has implemented a Phase I clinical trial utilizing this compound.

Iodoacetate. There are several possible points of intervention for inhibiting glycolysis, so numerous candidates could be proposed as CRMs. Targets might include glucose transporters as well as other enzymatic steps in glycolysis. For example, iodoacetate is known to inhibit glyceraldehyde-3-phosphate dehydrogenase. In a preliminary in vitro analysis, pretreatment of fetal rat hippocampal neurons with iodoacetate provided protection against several stresses, including excessive glutamate, iron, and trophic factor withdrawal, while up-regulating heat shock proteins, HSP70 and HSP90 [59].

Other Candidate Glycolytic Inhibitors. Many other candidate HK inhibitors can be explored to evaluate their efficacy as CRMs. Moreover, additional efforts to develop anti-cancer drugs have investigated other steps in the glycolytic pathway. For example, several inhibitors of the catalytic subunit of glucose-6-phosphatase have been suggested including Ilicicolinic acid (B), oxodiperoxo(1,10-

phenanthrolin)vanadate, and tetrahydrothienylpyridine [60]. Additionally, dichloroacetic acid has been proposed as another anti-cancer drug acting via glycolytic inhibition [61]. Its direct action is to inhibit pyruvate dehydrogenase kinase which increases the flux of pyruvate into mitochondria to promote glucose oxidation over glycolysis. A potentially powerful strategy would be to evaluate the individual efficacy of these candidate compounds, but also in combination with other glycolytic inhibitors on important endpoints beyond tumor inhibition.

Additionally, sequellae of glycolytic inhibition, such as upregulation of enzymes in the pathway as a consequence of reduced substrate availability at preceding steps might also represent targets/candidates for CRM. A prime example of this phenomenology is the upregulation of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis, by fasting and/or CR itself [62]. The CR-like beneficial effects of such up-regulation are quite apparent in the PEPCK-C (mus) mouse [63], which will be discussed in a later section. In summary, interference with glucose flux at the cellular level, by manipulation of enzymes involved in the glycolytic pathway, and elsewhere in carbohydrate metabolism presents an intriguing opportunity to mimic both a principle biochemical mechanism of CR and many of its physiological benefits.

10.2.4 Endocrine Regulation

When considering endocrine targets for developing CRM, insulin and IGF1 production, the insulin receptor, and its interaction with insulin signaling pathways should appear at the top of the list [21, 64]. These targets began to emerge in studies using invertebrate models in which genetic manipulation of the daf-2 pathway, a putative primitive insulin signaling pathway, elicited increased lifespan [64]. A key biomarker of CR is reduced plasma levels of insulin, which has also been reported to predict longevity in healthy humans [65].

Metformin. Biguanides are a class of compounds that include phenformin, buformin, and metformin. The latter has emerged as a particularly strong candidate CRM [66, 67]. Since the 1950s, biguanides have served as major anti-diabetic treatments due to their robust ability to reduce hyperglycemia, insulin, gluconeogenesis, intestinal glucose absorption, serum lipids and somatomedin. Phenformin had early success in many preclinical studies related to aging. In several rodent investigations, this compound was reported to increase lifespan and reduce cancer in mouse strains susceptible to tumors [68]. Clinical use of phenformin and buformin, however, had to be halted because of problems with lactic acidosis noted in many patients [68]. As a result, metformin became the principal drug for treating type 2 diabetes [69]. Apparently acting via several mechanisms, metformin has been demonstrated in many long-term studies to improve the metabolic profile of diabetes. Its actions include suppressed hepatic gluconeogenesis; enhanced peripheral glucose uptake; decreased absorption of glucose from the gastrointestinal

tract; and increased fatty acid oxidation [70]. Many of these are driven by the activation of adenosine monophosphate-activated protein kinase (AMPK), which increases expression of small heterodimer protein (SHP), acting to inhibit expression of hepatic gluconeogenic genes, including PEPCK and glucose 6-phosphatase [70]. This effect would appear counterintuitive in light of the activation of PEPCK and gluconeogenesis observed during fasting and CR mentioned above, and to be discussed later with respect to the PECK-C (mus) transgenic mouse. However, many candidate CRMs must be considered "segmental" in that few, if any, will replicate 100 % of the biological effects of full CR. In this case, the antidiabetic effects of metformin may derive in part from mimicking satiety, rather than CR or fasting. Additionally, activation of AMPK also stimulates GLUT4 translocation to the plasma membrane to improve insulin independent glucose uptake. Increased peripheral glucose utilization results from increased insulin binding to insulin receptors [70]. Thus, metformin may be a two-edged sword in regard to antidiabetic and CRM actions.

Because of the large scale use of metformin, many epidemiological studies have been able to analyze its long-term use and have noted increased survival from all-cause mortality in diabetic and cardiovascular disease patients [71]. There are also many studies of reduced incidence of age-related diseases, including cancer [72], chronic kidney disease [73], and cardiovascular disease [74]. These findings describing robust anti-disease effects of metformin treatment strongly support its candidacy as a CRM. However, it should be acknowledged that a recent meta-analysis of metformin studies suggested no significant overall mortality benefit [72].

Further supporting its candidacy as a CRM, Dhahbi et al. [75] reported that the transcriptional profile of mice treated with metformin for 8 weeks closely resembled that produced by CR. Again, with regard to PEPCK, effects on transcription and activation may be dissociated under some conditions. In a series of studies using cancer-prone mouse strains, Anismov and colleagues reported positive effects of metformin treatment on lifespan [76–78]. However, results in invertebrate models of aging have been mixed regarding beneficial effects of metformin. Using the nematode model and a variety of doses, Onken and Driscoll [66] observed increased lifespan in metformin treated worm cultures, which was shown to require AMPK expression. Cabreiro et al. [79] also demonstrated increased lifespan in nematode cultures treated with metformin, but these investigators focused on the microbial folate and methionine metabolism as important mechanisms mediating its prolongevity effects. In the *Drosophila* model, Slack et al. [80] observed AMPK activation following metformin treatment, but no significant effects on lifespan were observed and at higher doses toxicity was noted.

In other rodent studies examining effects on longevity, we observed no significant effects of metformin (300 mg/kg) on lifespan of F344 rats; however, this study used only the one dose [43]. In a more recent study in C57BL/6 mice using two doses of metformin (1 and 0.1 % in the diets), a clear and contrasting dose response was observed [81]. Survival was significantly reduced compared to controls at the higher dose; whereas, at the lower dose, survival was significantly increased, even though the treated mice were consuming more food. Additionally, the lower dose

increased healthspan evidenced by improvements in a number of parameters measured at older ages, including improved glucose tolerance, increased treadmill endurance, better rotarod performance, higher levels of locomotor activity, and reduced incidence of cataracts. In summary, metformin remains at the top of the list of candidate CRMs; however, additional investigation is needed to clarify the dose dependency of the effects on lifespan and healthspan, and possibly paradoxical effects, such as on PEPCK. Given the drug's widespread clinical use, research will no doubt expand over the next decade to examine effects on a wide range of age-related conditions and diseases.

Growth Hormone/Insulin-Like Growth Hormone. Although reductions in growth hormone (GH) and insulin-like growth factor-1 (IGF-1) in various animals have been reported in CR animals, there is less consistency in the literature regarding effects of CR on this axis in humans [82]. One recent study of long-lived individuals found that lifespan was associated with low serum levels of IGF-1 in females, but not in males; however, cancer survival was increased in both females and males with low IGF-1 levels [83]. As demonstrated in dwarf mutant mice as well as in genetically engineered mice, however, manipulation of GH/IGF-1 axis can exert profound effects on lifespan [84]. Excessive GH as produced in transgenic mice overexpressing GH as well as in the human condition, acromegaly, reportedly increases incidence of age-related diseases and mortality [84]. A small group with mutations in the GH receptor gene who live in Ecuador have a syndrome known as Laron dwarfism, with severe GHR and circulating IGF-1 deficiencies. These individuals exhibit reduced risk for T2 diabetes, presumably due to the absence of the anti-insulinemic action of GH [85]. Although these individuals also exhibit lower cancer incidence, they apparently do not live any longer than control subjects [85].

Pegvisomant. Although no strong candidate CRM has yet to be identified from this line of research, one drug has been used very effectively to treat acromegaly. Pegvisomant (trade name Somavert) is an antagonist of the GH receptor, which reduces production of **IGF-1** [84]. It appears to be highly efficacious and safe [84], and can normalize IGF-1 levels in patients receiving chronic injections. Recently, there has been increased interest in its use for cancer therapy [84]. The major deterrent to further investigation of pegvisomant as a candidate CRM is its prohibitively high costs even for preclinical investigations, such as in the ITP. Moreover, timing treatment during the life course will be a difficult issue to address as well. Other, less costly antagonists of the GH receptor may emerge to increase the number of candidates directed toward this target.

All in all, current findings suggest that optimizing the IGF-1 axis to promote healthy aging in humans may be a complex proposition. Increased understanding of an array of interactions and tissue specificity will be required to advance the field and generate practical candidate CRMs directed toward specific GH/IGF1 targets [83].

10.2.5 Regulation of Specific Gene Expression

Another strategy for developing CRMs is the direct targeting of specific genes in order to regulate their level of expression. This can be achieved by recombinant DNA technology as well as with agents that act at the genomic level.

PEPCK-C. A good example of the former, and also in keeping the concept of glycolytic control as a robust strategy for CRMs, is the PEPCK-C (mus) transgenic mouse [63]. Briefly, transgenically manipulated and selectively bred mouse lines with 9.0 units PEPCK/g skeletal muscle, as compared to 0.080 units/g in muscle from wild type control animals, were developed and characterized with respect to phenotype and behavior. As mentioned above, PEPCK is a key gene in the control of gluconeogenesis, by which carbohydrates are produced from non-carbohydrate sources, and is upregulated under conditions of fasting and CR [62]. Remarkably, these animals actually eat more than twice as much as wild type, but apparently live almost two years longer, although detailed longevity studies have not been published. The mice can also have litters at up to 35 months of age, which is really an advanced age for reproduction. Other phenotypic similarities to CR animals include almost 50 % smaller size, reduced adiposity, increased numbers of skeletal muscle mitochondria, lower levels of blood insulin and leptin, and apparently increased sensitivity of skeletal muscle to insulin. Interestingly, the PEPCK transgenics are quite hyperactive, running 7–10 times more on homecage runwheels than controls, and for long distances (up to 5 km at 20 m/min).

Mechanistically, the increased activity likely accounts for the smaller size of the mice, despite increased food consumption. Increase in PEPCK-C activity in skeletal muscle may dramatically alter citric acid cycle dynamics, allowing a greater flux of intermediates. This may be reflected in an elevated flow of carbon to glyceride-glycerol via glyceroneogenesis or an increased rate of recycling of the carbon skeletons of amino acids through the citric acid cycle. One of the citric acid cycle intermediates is oxaloacetic acid (OAA), which will be discussed below in the final section. Regarding our theme of best expectations for various CRM candidates, it is interesting to note that both PEPCK transgenic nematodes analogous to PEPCK-C (mus) mice and nematodes fed OAA exhibit longer lifespans as well [86, 87]. Unfortunately, since OAA supplementation increases the NAD+/NADH ratio analogous to actual CR, a process that also activates the sirtuin genes that will be discussed next, it seems somewhat paradoxical that activation of the SIRT1 gene suppresses the activity of PEPCK, while knockdown has the opposite effect [88]. A possible explanation may be differences in tissue specificity, since the above results were obtained in hepatic-derived cell lines, while overexpression muscle and adipose PEPCK can have quite opposite effects on overall metabolism and body composition. Other studies suggest the relationship between sirtuins and PEPCK may not be so simple [88]. In summary, however, some type of activation of muscle PEPCK, most likely via a selective CRM type agent, may provide an interesting opportunity for increased function and lifespan.

Sirtuins. Probably most active example of gene regulation as a strategy for developing CRMs pertains to the activation of a class of genes known as sirtuins [89, 90]. These genes code for histone deacetylases that regulate DNA structure and specific transcriptional capability. Led by David Sinclair, Leonard Guarente, and others, this search has generated a massive amount of research on several fronts. Sirtris Pharmaceuticals was formed in 2004 to take the lead in developing this strategy. In 2008 the company was purchased by Galaxo Smith Kline (GSK), with a strategy described as follows: "Our drug candidates are designed to mimic certain beneficial health effects of calorie restriction, without requiring a change in eating habits by activation of sirtuins, a recently discovered class of enzymes that control the aging process."

Identified through a compound screen as an activator of SIRT1 in mammals and its invertebrate homolog, SIRT2 [91], the plant polyphenol, resveratrol, emerged as the lead compound for the initial Sirtris research efforts. The potential of resveratrol to increase healthspan and lifespan has been examined in a wide variety of studies [89]. Early reports showed that adding resveratrol to the diet significantly increased median and maximal lifespan in invertebrate studies including yeast [91], nematodes [92], and *Drosophila* [92]. Given that knock-out of SIRT2 signaling was shown to block effects of CR on lifespan in invertebrate models [92, 93], these findings provided strong evidence of the importance of signaling in this pathway for mediating effects of CR [93].

Support for the postulated role of SIRT1 was more limited when these intervention studies were expanded to mammalian models. Resveratrol increased lifespan when fed to short-lived fish [94], but when fed to middle-aged mice (12 months) on a normal diet, however, a resveratrol supplemented diet did not increase mean or maximum lifespan [28]. Nonetheless, findings from these studies revealed beneficial effects of the compound on healthspan. Specifically, mice fed resveratrol exhibited less cardiac pathology, greater bone health, reduced cataract incidence, and improved motor function compared to mice on a control diet [28]. Moreover, there were clear-cut benefits on survival in mice fed a high fat diet supplemented with resveratrol compared to mice on the same diet minus resveratrol [28]. Healthspan was also increased in the resveratrol-fed mice, as measured by several functional indices in addition to a gene transcriptional profile that more closely resembled that of CR mice in this experiment than control fed mice [28]. Complimentary research, examining the beneficial effects of resveratrol, has been rapidly expanding. In rodent models resveratrol treatment has exhibited protection against a great variety of insults, including ischemic stroke [95]; heart failure [96], seizures [97, 98], Parkinson's disease [99], and Alzheimer's disease [100].

In moving beyond the research on resveratrol, the major objective of Sirtris was to synthesize and characterize unique compounds that were direct activators of SIRT1, or STACs. Several candidates were developed and subjected to preclinical studies, with some moving forward to clinical studies primarily focused on diabetes [101]. The rationale for this focus is that since aging is not a diagnostic entity recognized by the US FDA, pharmaceutical development for CRM must identify

other appropriate targets. Given the robust effects of CR on the glucoregulation, diabetes was a very logical target for these efforts.

In contrast to the early robust findings investigating resveratrol as a CRM, many negative reports also emerged that failed to replicate the prolongevity effects of resveratrol in invertebrate models [102] or that demonstrated increased lifespan independent of effects on SIRT1/2 [103, 104]. Moreover, further studies feeding resveratrol to rodents failed to replicate the pattern of gene expression stimulated by CR in mice [105]. A major criticism also arose regarding the validity of the original assay used to identify resveratrol as a SIRT2/1 activator [106]. The argument was that a technical artifact was present involving the flurophore in the assay [106]. These investigators contended that resveratrol did not directly activate SIRT1. Moreover, they claimed that the synthetic compounds developed by Sirtris did not even activate the targeted gene [107]. One compound, SIRT1720, also did not activate SIRT1 in vitro, even after considering possible assay confounds. Additionally, this compound did not have any beneficial effects on health in vivo when delivered to an *ob/ob* mouse model [106]. Other studies provided evidence that the metabolic benefits of resveratrol were generated through inhibition of phosphodiesterase 4 (PDE4), thus leading to elevated levels of cAMP, which increased signaling through the CamKK-β-AMPK pathway [108]. These contradictory findings have generated intense arguments by many investigators from many sides [107].

Regarding the criticism of the original assay, recent subsequent work by the Sinclair laboratory provided evidence supporting their original conclusions. They reported that SIRT1 was directly activated by resveratrol and other STACs via binding in the N-terminus of SIRT1 [109]. Specifically, a mutation in this site (SIRT1-E230K) appeared to block activation by resveratrol and by STACs. It remains uncertain whether the controversy has now been settled, since the initial objections are often still raised. Recent studies conducted in the de Cabo laboratory at NIA demonstrated beneficial effects on lifespan and healthspan of the Sirtris compound, SRT1720, in mice on both standard and high fat diets [110]. Nevertheless, it should be remembered that resveratrol itself was originally studied as an antioxidant, and that its role in the beneficial effects of red wine was long believed to derive from protection against oxyradical damage, an area of gerontology that has both dominated as well as divided the field in recent years. Thus, there is still much to be learned before definitive conclusions can be drawn regarding mechanisms of action and potential as a CRM.

Beyond these arguments based on preclinical studies, the true value of the STACs strategy has yet to be fully realized in clinical trials. Sirtris went forward with several randomized, placebo controlled, double-blind trials involving the compound, SRT2104, In an initial trial examining tolerability and pharmacokinetics, the compound was provided orally to healthy volunteers for 7 days which yielded evidence for its safety and bioavailability [111]. In another preliminary study involving healthy elderly subjects, oral doses of this compound were provided for 28 days [112], and results supported the safety of the treatment over this time period, with significant reductions in serum cholesterol, LDL and triglycerides, but no significant treatment effects on glucose responses. A similar 28-day trial was

conducted in cigarette smokers again with reported safety and efficacy as well as significant treatment-related reductions in serum cholesterol, LDL, and triglycerides and improved measures of blood flow [113]. Finally, a recent 28-day study in adult diabetics again replicated the beneficial treatment effects of SRT2104 on blood lipids, but this investigation noted no significant effects on glucose and insulin parameters [114]. Therefore, while some beneficial results have emerged from these clinical trials regarding blood lipids, there has been no progress regarding the original target for development of these STACs, specifically improved insulin sensitivity and glucose control. GSK has now begun new clinical trials of SRT2104 and other compounds focused on ulcerative colitis and psoriasis in an effort to identify other biological targets. Given the failure to make substantial progress in initial clinical targets, the Cambridge headquarters of Sirtris was closed in 2013, although active research in sirtuin biology will continue.

As evidence of further progress in this gene target, a major refocus has shifted emphasis on activation of SIRT1 to activation of SIRT3. The latter sirtuin is a protein located within the mitochondrial matrix and has been implicated in regulating metabolic processes, particularly oxidative stress by inhibiting components of the mitochondrial permeability transition pore [115]. A major driver of this interest was the report of the relationship between longevity in an older Italian cohort and alleles of SIRT3 [116]. Others have suggested a role of SIRT3 as a tumor suppressor protein, since mitochondria damaged by oxidative stress may trigger tumor development [117]. As supporting evidence, mice with Sirt3 deleted develop breast mammary tumors [118].

Studies on the actions of sirtuins have also redirected attention to nicotinamide adenine dinucleotide (NAD), also known as vitamin B3 [119]. Because of its role in redox balance, NAD is a central metabolic cofactor involved in numerous metabolic transformations. Sirtuins utilize NAD+ to deacetylate proteins, thus making its concentrations rate-dependent. Increasing the levels of NAD+ represents a parallel strategy for activating sirtuins; however, there is a clear dose-dependency since high levels can inhibit sirtuins [119]. Reasonable progress applying this strategy has been made in preclinical studies using mouse models of Alzheimer's disease. When nicotinamide is fed to mice for several months, significant reductions in amyloid and tau protein pathology as well as improvements in cognition were reported [120–122]. Hypothesized mechanisms for the above phenomena include increased activation of PGC1α to stimulate mitochondrial biogenesis [121] and increased autophagy to improve cellular health and function [122]. A recent study in nematodes demonstrated lifespan extension in worms treated with nicotinamide. However, surprisingly this effect was also observed in worms in which Sir-2 had been deleted. These investigators noted that nicotinamide underwent methylation and generated hydrogen peroxide via an aldehyde oxidase in mitochondria, which likely served as a hormetic signal to activate protective pathways promoting longevity in this model [123]. In light of these findings, it is highly likely that many new investigations will elevate the candidacy of nicotinamide as a CRM. To our knowledge there is currently a clinical trial to evaluate its effects in patients with Alzheimer's disease (www.clinicaltrials.gov/ct2/show/NCT00580931).

mTOR. Mammalian target of rapamycin (mTOR) represents another genetic signaling pathway that has become a major focus of interest for developing CRM [124]. Interestingly, the debate over the centrality of SIRT1 in mediating the anti-aging effects of CR generated this interest. Additionally, interest was rapidly growing in autophagy as a key cellular process involved in aging [124]. mTOR is a serine/threonine protein kinase involved in regulating cell survival, cell growth, cell proliferation, cell motility, cell protein synthesis and transcription [125, 126] and autophagy [127]. The pathway is positioned to sense cellular nutrient and energy levels and redox status that can mediate effects of CR [126]. mTOR can function to integrate input from pathways further upstream, including insulin, IGF1, and mitogens [125]. Additional research has characterized what is now known to be the mTOR complex; mTOR Complex 1 (mTORC1) consists of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (MLST8) and the non-core components PRAS40 and DEPTOR [128]. This complex operates as a nutrient/energy/redox sensor and controller for protein synthesis [125], and mTORC1 activity can be stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress [129, 130]. mTOR Complex 2 (mTORC2) is composed of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), MLST8, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) [131]. The primary role of mTORC2 is regulation of the cytoskeleton through stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKCα) [132]. mTORC2 also phosphorylates the serine/threonine protein kinase to affect metabolism and survival [133].

Many CR studies in animals have reported reduced mTOR [134]. Genetic downregulation has been demonstrated to increase lifespan in several model systems, including yeast, worms, and flies [134]. A major boost in interest in rapamycin as a CRM came from the ITP study reporting in 2009 that pharmacological inhibition of mTOR signaling induced via dietary supplementation with rapamycin increased mean and maximal lifespan in heterogeneous mice placed on treatment at middle age [135]. However, a number of questions remain in light of gender differences in survival effects, as well as differences between the different laboratories conducting the studies. Rapamycin, also known as sirolimus, is a FDA-approved drug used as an immunosuppressant for prevention of organ transplantation rejection. The primary mechanism for this action is the targeting of T-cells and B-cells to inhibit their response to IL-2. Regarding application as an anti-aging intervention, the principle hypothesized mechanism of rapamycin is the inhibition of mTOR to upregulate pathways involved in autophagy, thereby removing damaged or misfolded proteins and preventing their aggregation [136]. It should be noted that another of the CRM candidates listed in Table 10.1, Olbetam/acipimox, apparently also works via increase in autophagy, and although not discussed here, removal of damaged macromolecules that accumulate during aging may indeed be a key mechanism of other CRMs and CR itself.

Other mouse studies, including new ones from the ITP, replicated the positive effects of rapamycin on lifespan when added to the diet [26, 137–140] including

mice on a high fat diet [141]. Some studies of effects on healthspan have come to different conclusions. In a recent one, Zhang et al. [137] noted that rapamycin introduced late in life (19 months) to B6 mice could increase lifespan and function as indicated by measures of gait and balance as well as several markers of pathology. Neff et al. [142] were able to replicate the lifespan extension in rapamycin-treated mice as well as a few pathological markers; however, they noted only a few effects on functional measures could be observed in aged mice exclusively. Therefore, these investigators argued that rapamycin treatment did not retard aging. Studies focused more on functional measures of aging have reported that rapamycin treatment can attenuate age-related declines in tests of cognitive performance in normal mice [143] as well as in mouse models of Alzheimer's disease [144]. Flynn et al. [145] noted that rapamycin treatment initiated late in life could improve also cardiac function. Other studies have explored the beneficial effects of rapamycin on survival in cancer-prone mouse strains [146], as it has a long history in treatment of cancers [147]. Many clinical trials have made progress, applying an analog of rapamycin, temsirolimus, in treating a variety of different tumors [147]. In fact, several groups argue that the appearance of anti-aging effects of rapamycin emerging from longevity studies is due primarily to the effects of the compound on inhibiting cancer [140, 142].

Another controversy challenging the development of rapamycin as candidate CRM pertains to the reported toxic effects observed in many mouse studies. Specifically, chronic rapamycin treatment can produce insulin resistance, hyperlipidemia and glucose intolerance, increased incidence of cataracts, as well as testicular degeneration [139, 142, 148–150]. Importantly, however, a recent study by Fang et al. [151] noted that the negative metabolic responses were dependent upon time and duration of dosing. When rapamycin treatment was continued beyond 20 weeks, the negative effects on glucose/insulin metabolism were absent.

A final concern about long-term rapamycin treatment at high doses is suppression of the immune system, rendering individuals more susceptible to dangerous infections. When administered at low doses, rapamycin treatment has been reported to enhance immune response to tuberculosis [152]. Most likely the solution to avoiding the negative effects of rapamycin treatment will emerge from efforts to develop compounds that are more specific to mTORC1. Although rapamycin has strong affinity for mTORC1, detrimental effects on glucose/insulin metabolism have been linked to its inhibition of mTORC2 [153]. Nevertheless, despite these safety issues and complexity of responses observed with rapamycin treatment, inhibition of mTOR has been clearly established as a leading target for development of CRM at a specific gene site.

10.2.6 Miscellaneous Other CRM Strategies

Oxaloacetate. As mentioned earlier in relationship to PEPCK regulation, another strategy directed towards increasing levels of NAD+ to activate sirtuins is supplementation with oxaloacetic acid (OA). OA is a Kreb's cycle intermediate that increases levels of NAD+ and restores redox balance, NAD+/NADH, and has been reviewed more thoroughly elsewhere [4]. Effects of OA on healthspan and lifespan have not been very extensive. In a nematode study, OA was shown to significantly increase lifespan acting by through an AMPK pathway, but not involving sirt2 [87]. Effects of OA supplementation on lifespan in mice were investigated by the ITP, which reported no significant effects. Unfortunately, however, there were questions about whether effective blood levels of the compound were achieved [23]. Having been developed as a nutraceutical product, OA is currently being marketed as a CRM (www.benegene.com).

Spermidine. Finally, we can discuss the polyamine, spermidine, as the newest candidate CRM [154]. Polyamines comprise a ubiquitous group of polycationic aliphatic amines that serve multifunctional roles in the cell, many of which involve survival. In particular, spermidine is involved with numerous cellular processes (Ca^{2+}, Na^{+}, K^{+}—ATPase), which maintain membrane potential and control intracellular pH and volume. Spermidine is the source of many other polyamines, such as spermidine and thermospermine, which are involved in osmolality in many organisms. Spermidine is also found in a wide variety of foods, including mushrooms, soy products, legumes, corn, whole grains, aged cheese, and wheat germ [155].

The recent interest in spermidine as a candidate CRM was largely generated by a 2009 paper in which lifespan was increased in a variety of invertebrate models (yeast, worms, flies) by spermidine administration [156]. Although there was evidence of reduced oxidative stress in these models, as well as in mice treated with spermidine, the primary mechanism suggested was enhanced autophagy acting through inhibition of mTOR. Other studies confirmed this effect, and showed that these results were independent of SIRT1 [157]. Nishimura et al. [158] determined polyamine levels in different tissues from female mice at 3, 10, and 26 weeks of age, and noted marked age-related declines, particularly in spermidine detected in skin, thymus, spleen, ovary, liver, stomach, lung, kidney, heart and muscle. Age-related decline in serum spermidine concentrations have also been noted in humans [156], and interestingly, a recent paper observed higher blood levels of spermidine in centenarians [159]. To our knowledge, no reports of lifespan effects have emerged in mammalian models associated with spermidine treatment, although several are likely in progress. These have also been covered in our recent review [4], but in summary, the safety profile of this polyamine seems acceptable, and the results thus far are encouraging regarding beneficial effects on a wide-range of age-related function and diseases.

10.3 Discussion and Conclusions

Clearly, many CRM candidate agents and strategies are currently under investigation. At this junction, it would require an entire volume, rather than a single chapter, to comprehensively and critically review the entire field. We have attempted to describe some of the more promising candidates with particular emphasis on the best possible expectations for human application, based on data largely obtained from animal studies. Nevertheless, both potential efficacy vs side effects as well as mechanism of action offer two key criteria by which to evaluate feasibility. Regarding the former criteria, agents such as 2DG and rapamycin present obvious problems in need of resolution (e.g. narrow efficacy to toxicity window and various possible negative biological effects), while we have recently suggested that the latter criteria should, among other things, reflect maximal CR efficacy by acting as far "upstream" in the metabolic process as possible [3]. Inhibition of nutrient absorption/digestion and glycolytic inhibition would seem to be the most efficient strategies to achieve this end, despite the greater amount of data available for "downstream" agents such as sirtuin activators and related compounds. Additionally, targeting of some "midstream" or peripheral processes, such as autophagy, may play very important roles in assuring the efficacy of CRM candidates.

Despite growing interest and research in the concept, no proven CRM has yet to be identified, but several candidates appear highly promising. In arguing that CR induces beneficial effects through multiple signaling pathways, previously we have proposed the development of "cocktails" of CRMs to affect multiple systems [3]. Many potential targets can be identified, and many new candidates, including cocktails of candidates, will likely be identified in the near future. Such advances will be greatly facilitated by better resolution of apparent discrepancies in the mechanistic investigations discussed above, by utilization of more sophisticated methodology, and equally important, better agreement on the applicability of CR and CRMs to humans.

Finally, we would offer the following assessment, predictions, and advice. Many academic, government, and industrial laboratories are now involved in CRM research, and it has become a major scientific and commercial undertaking, which will only increase in scope as the population continues to "gray" and become ever more interested in successful aging and maintenance of healthspan and vitality. The CRM literature, both technical and lay, will expand exponentially to the point that only very careful evaluation will be able to discern the facts from the hype. It is our challenge as both investigators and interpreters to assure that only the highest quality science is accepted, and that the biogerontological field in general, which only in the last half century or so began transitioning out of the "wild west," is transformed as seamlessly and transparently as possible into the new civilization and culture that it promises.

References

1. Lane MA, Ingram DK, Roth GS (1998) 2-deoxy-D-glucose feeding in rats mimics physiological effects of calorie restriction. J Anti-Aging Med 1:327–337
2. Wick AN, Drury DR, Nakada HI (1957) Localization of the primary metabolic block produced by 2-deoxyglucose. J Biol Chem 224:963–969
3. Ingram DK, Roth GS (2011) Glycolytic inhibition as a strategy for developing calorie restriction mimetics. Exp Gerontol 46:148–155
4. Ingram DK, Roth GS (In press) Calorie restriction mimetics: can you have your cake and eat it, too? Ageing Res Rev, Dec 19. pii: S1568-1637(14)00127-5. doi: 10.1016/j.arr.2014.11.005. [Epub ahead of print]
5. Roth GS (2005) The truth about aging, can we really live longer and healthier?. Windstorm Creative, Port Orchard
6. Stipp D (2010) The youth pill. Penguin Books, London
7. Fontana L, Partridge L, Longo VD (2010) Extending healthy life span—from yeast to humans. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys Science 328:321–326
8. Chung KW, Kim DH, Park MH, Choi YJ, Kim ND, Lee J, Yu BP, Chung HY (2013) Recent advances in calorie restriction research on aging. Exp Gerontol 48:1049–1053
9. Masoro EJ (2005) Overview of caloric restriction and ageing. Mech Ageing Dev 126:913–922
10. Heilbronn LK, de Jonge L, Frisard MI, DeLany JP, Larson-Meyer DE, Rood J, Nguyen T, Martin CK, Volaufova J, Most MM, Greenway FL, Smith SR, Deutsch WA, Williamson DA, Ravussin E, Pennington CALERIE Team (2006) Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA 295:1539–1548
11. Dirks AJ, Leeuwenburgh C (2006) Caloric restriction in humans: potential pitfalls and health concerns. Mech Ageing Dev 127:1–7
12. Phelan JP, Austad SN (1989) Natural selection, dietary restriction, and extended longevity. Growth Dev Aging 53:4–6
13. Shanley DP, Kirkwood TB (2006) Caloric restriction does not enhance longevity in all species and is unlikely to do so in humans. Biogerontology 7(3):165–168
14. Selman C (2014) Dietary restriction and the pursuit of effective mimetics. Proc Nutr Soc 10:1–11
15. Harper JM, Leathers CW, Austad SN (2006) Does caloric restriction extend life in wild mice? Aging Cell 5:441–449
16. Liao CY, Johnson TE, Nelson JF (2013) Genetic variation in responses to dietary restriction—an unbiased tool for hypothesis testing. Exp Gerontol 48:1025–1029
17. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R (2009) Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Science 325:201–204
18. Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R, Anderson RM (2014) Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat Commun 1(5):3557
19. Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, Longo DL, Allison DB, Young JE, Bryant M, Barnard D, Ward WF, Qi W, Ingram DK, de Cabo R (2012) Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature 489:318–321
20. Huffman DM (2010) Exercise as a calorie restriction mimetic: implications for improving healthy aging and longevity. Interdiscip Top Gerontol 37:157–174
21. Ingram DK, Zhu M, Mamczarz J, Zou S, Lane MA, Roth GS, de Cabo R (2006) Calorie restriction mimetics: an emerging research field. Aging Cell 5:97–108

22. Mercken EM, Carboneau BA, Krzysik-Walker SM, de Cabo R (2012) Of mice and men: the benefits of caloric restriction, exercise, and mimetics. Ageing Res Rev 11:390–398
23. Strong R, Miller RA, Astle CM, Baur JA, de Cabo R, Fernandez E, Guo W, Javors M, Kirkland JL, Nelson JF, Sinclair DA, Teter B, Williams D, Zaveri N, Nadon NL, Harrison DE (2013) Evaluation of resveratrol, green tea extract, curcumin, oxaloacetic acid, and medium-chain triglyceride oil on life span of genetically heterogeneous mice. J Gerontol A Biol Sci Med Sci 68:6–16
24. Harrison DE, Strong R, Allison DB, Ames BN, Astle CM, Atamna H, Fernandez E, Flurkey K, Javors MA, Nadon NL, Nelson JF, Pletcher S, Simpkins JW, Smith D, Wilkinson JE, Miller RA (2014) Acarbose, 17-α-estradiol, and nordihydroguaiaretic acid extend mouse lifespan preferentially in males. Aging Cell 13:273–282
25. Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, Fernandez E, Flurkey K, Javors MA, Nelson JF, Orihuela CJ, Pletcher S, Sharp ZD, Sinclair D, Starnes JW, Wilkinson JE, Nadon NL, Strong R (2011) Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. J Gerontol A Biol Sci Med Sci 66:191–201
26. Miller RA, Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, Javors MA, Li X, Nadon NL, Nelson JF, Pletcher S, Salmon AB, Sharp ZD, Van Roekel S, Winkleman L, Strong R (2013) Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. Aging Cell 17. doi:10.1111/acel.12194
27. Strong R, Miller RA, Astle CM, Floyd RA, Flurkey K, Hensley KL, Javors MA, Leeuwenburgh C, Nelson JF, Ongini E, Nadon NL, Warner HR, Harrison DE (2008) Nordihydroguaiaretic acid and aspirin increase lifespan of genetically heterogeneous male mice. Aging Cell 7:641–650
28. Pearson KJ, Baur JA, Lewis KN, Peshkin L, Price NL, Labinskyy N, Swindell WR, Kamara D, Minor RK, Perez E, Jamieson HA, Zhang Y, Dunn SR, Sharma K, Pleshko N, Woollett LA, Csiszar A, Ikeno Y, Le Couteur D, Elliott PJ, Becker KG, Navas P, Ingram DK, Wolf NS, Ungvari Z, Sinclair DA, de Cabo R (2008) Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. Cell Metab 8:157–168
29. Mehta LH, Roth GS (2009) Caloric restriction and longevity: the science and the ascetic experience. Ann NY Acad Sci 1172:28–33
30. Weiss EP, Racette SB, Villareal DT, Fontana L, Steger-May K, Schechtman KB, Klein S, Holloszy JO, Washington University School of Medicine CALERIE Group (2006) Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. Am J Clin Nutr 84:1033–1042
31. Orentreich N, Matias JR, DeFelice A (1993) Low methionine ingestion by rats extends life span. J Nutr 123:269–274
32. Sanchez-Roman I, Barja G (2013) Regulation of longevity and oxidative stress by nutritional interventions: role of methionine restriction. Exp Gerontol 48:1030–1042
33. Roth GS, Lane MA, Ingram DK (2005) Caloric restriction mimetics: the next phase. Ann NY Acad Sci 1057:365–371
34. Derosa G, Maffioli P (2012) Efficacy and safety profile evaluation of acarbose alone and in association with other antidiabetic drugs: a systematic review. Clin Ther 34:1221–1236
35. Zhang W, Kim D, Philip E, Miyan Z, Barykina I, Schmidt B, Stein H (2013) The Gluco VIP study. A multinational, observational study to investigate the efficacy, safety and tolerability of acarbose as add-on or monotherapy in a range of patients: the Gluco VIP study. Clin Drug Investig 33:263–274
36. Warburg O, Posener K, Negelein E (1930) Ueber den Stoffwechsel der Tumoren. Biochemische Zeitschrift 152:319–344

37. Pedersen PL (2007) Warburg, me and hexokinase 2: multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg Effect", i.e., elevated glycolysis in the presence of oxygen. J Bioenerg Biomembr 39:211
38. Gridley DS, Nutter RL, Kettering JD, Mantik DW, Slater JM (1985) Mouse neoplasia and immunity: effects of radiation, hyperthermia, 2-deoxy-D-glucose, and *Corynebacterium parvum*. Oncology 42:391–398
39. Zhu Z, Jiang W, McGinley JN, Thompson HJ (2005) 2-deoxyglucose as an energy restriction mimetic agent: effects on mammary carcinogenesis and on mammary tumor cell growth in vitro. Cancer Res 65:7023–7030
40. Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M (2007) Glucose restriction extends *Caenorhabditis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab 6:280–293
41. Rattan SIS (2008) Hormesis in aging. Ageing Res Rev 7:63–78
42. Kang HT, Hwang ES (2006) 2-Deoxyglucose: an anticancer and antiviral therapeutic, but not any more a low glucose mimetic. Life Sci 78:1392–1399
43. Minor RK, Smith DL Jr, Sossong AM, Kaushik S, Poosala S, Spangler EL, Roth GS, Lane M, Allison DB, de Cabo R, Ingram DK, Mattison JA (2010) Chronic ingestion of 2-deoxy-D-glucose induces cardiac vacuolization and increases mortality in rats. Toxicol Appl Pharmacol 243:332–339
44. Yao J, Chen S, Mao Z, Cadenas E, Brinton RD (2011) 2-Deoxy-D-glucose treatment induces ketogenesis, sustains mitochondrial function, and reduces pathology in female mouse model of Alzheimer's disease. PLoS ONE 6(7):e21788. doi: 10.1371/journal.pone.0021788
45. Weimer S, Priebs J, Kuhlow D, Groth M, Priebe S, Mansfeld J, Merry TL, Dubuis S, Laube B, Pfeiffer AF, Schulz TJ, Guthke R, Platzer M, Zamboni N, Zarse K, Ristow M (2014) D-Glucosamine supplementation extends life span of nematodes and of ageing mice. Nature Commun. doi:10.1038/ncomms4563
46. Caramés B, Kiosses WB, Akasaki Y, Brinson DC, Eap W, Koziol J, Lotz MK (2013) Glucosamine activates autophagy in vitro and in vivo. Arthritis Rheum 65:1843–1852
47. Takahashi M, Inoue K, Yoshida M, Morikawa T, Shibutani M, Nishikawa A (2009) Lack of chronic toxicity or carcinogenicity of dietary N-acetylglucosamine in F344 rats. Food Chem Toxicol 47:462–471
48. Rasschaert J, Kadiata MM, Malaisse WJ (2001) Effects of D-mannoheptulose upon D-glucose metabolism in tumoral pancreatic islet cells. Mol Cell Biochem 226:77–81
49. Ramirez R, Rasschaert J, Laghmich A, Louchami K, Nadi AB, Jijakli H, Kadiata MM, Sener A, Malaisse WJ (2001) Uptake of D-mannoheptulose by normal and tumoral pancreatic islet cells. Int J Mol Med 7:631–638
50. Roth G, Hayek M, Massimino S, Davenport G, Arking R, Bartke A, Bonkowski M, Ingram D (2009) Mannoheptulose: glycolytic inhibitor and novel caloric restriction mimetic. Exp Biol Abstract 553:1
51. Davenport G, Massimino S, Hayek M, Burr J, Michael Ceddia M, Yeh C-H, Roth G, Ingram D (2010) Biological activity of avocado-derived mannoheptulose in dogs. Exp Biol Abstract 725:4
52. Ko YH, Pedersen PL, Geschwind JF (2001) Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase. Cancer Lett 173:83–91
53. Ko YH, Smith BL, Wang Y, Pomper MG, Rini DA, Torbenson MS, Hullihen J, Pedersen PL (2004) Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. Biochem Biophys Res Commun 324:269–275
54. Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, Keating MJ, Huang P (2005) Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res 65:13–621
55. Chang JM, Chung JW, Jae HJ, Eh H, Son KR, Lee KC, Park JH (2007) Local toxicity of hepatic arterial infusion of hexokinase II inhibitor, 3-bromopyruvate: in vivo investigation in normal rabbit model. Acad Radiol 14:85–92

56. Froelich L, Ding A, Hoyer S (1995) Holeboard maze-learning deficits and brain monoaminergic neurotransmitter concentrations in rats after intracerebroventricular injection of 3-bromopyruvate. Pharmacol Biochem Behav 51:917–922
57. Jones AR, Porter KE, Dobbie MS (1996) Renal and spermatozoal toxicity of alpha-bromohydrin, 3-bromolactate and 3-bromopyruvate. J Appl Toxicol 16:57–63
58. Ko YH, Verhoeven HA, Lee MJ, Corbin DJ, Vogl TJ, Pedersen PL (2012) A translational study "case report" on the small molecule "energy blocker" 3-bromopyruvate (3BP) as a potent anticancer agent: from bench side to bedside. J Bioenerg Biomembr 44:163–170
59. Guo Z, Mattson MP (2000) In vivo 2-deoxyglucose administration preserves glucose and glutamate transport and mitochondrial function in cortical synaptic terminals after exposure to amyloid β-peptide and iron: evidence for a stress response. Exp Neurol 166:173–179
60. Parker JC (2001) Glucose-6-phosphate translocase as a target for the design of antidiabetic agents. Drugs Fut 26:687
61. Michelakis ED, Webster L, Mackey JR (2008) Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. Br J Cancer 99(7):989–994
62. Hanson RW, Reshef L (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 66:581–611
63. Hakimi P1, Yang J, Casadesus G, Massillon D, Tolentino-Silva F, Nye CK, Cabrera ME, Hagen DR, Utter CB, Baghdy Y, Johnson DH, Wilson DL, Kirwan JP, Kalhan SC, Hanson RW (2007) Overexpression of the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) in skeletal muscle repatterns energy metabolism in the mouse. J Biol Chem 282:32844–32855
64. Bartke A (2008) Insulin and aging. Cell Cycle 7:3338–3344
65. Roth GS, Lane MA, Ingram DK, Mattison J, Elahi D, Tobin J, Muller D, Metter EJ (2002) Biomarkers of caloric restriction may predict longevity in humans. Science 297:881
66. Onken B, Driscoll M (2010) Metformin induces a dietary restriction-like state and the oxidative stress response to extend *C. elegans* healthspan via AMPK, LKB1, and SKN-1. PLoS ONE 5:e8758
67. Anisimov VN (2013) Metformin: do we finally have an anti-aging drug? Cell Cycle 15:3483–3489
68. Anisimov VN (2003) Insulin/IGF-1 signaling pathway driving aging and cancer as a target for pharmacological intervention. Exp Gerontol 38:1041–1049
69. Kirpichnikov D, McFarlane SI, Sowers JR (2002) Metformin: an update. Ann Intern Med 137:25–33
70. Kim YD, Park KG, Lee YS, Kim DK, Nedumaran B, Jang WG, Cho WJ, Ha J, Lee IK, Lee CH, Choi HS (2008) Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. Diabetes 57:306–314
71. Eurich DT, Majumdar SR, McAlister FA, Tsuyuki RT, Johnson JA (2005) Improved clinical outcomes associated with metformin in patients with diabetes and heart failure. Diabetes Care 28:2345–2351
72. Boussageon R1, Supper I, Bejan-Angoulvant T, Kellou N, Cucherat M, Boissel JP, Kassai B, Moreau A, Gueyffier F, Cornu C (2012) Reappraisal of metformin efficacy in the treatment of type 2 diabetes: a meta-analysis of randomised controlled trials. PLoS Med 9(4):e1001204. doi:10.1371/journal.pmed.1001204
73. Pilmore HL (2010) Metformin: potential benefits and use in chronic kidney disease. Nephrology 15:412–418
74. Papanas N, Maltezos E (2009) Oral antidiabetic agents: anti-atherosclerotic properties beyond glucose lowering? Curr Pharm Des 15:3179–3192
75. Dhahbi JM, Mote PL, Fahy GM, Spindler SR (2005) Identification of potential caloric restriction mimetics by microarray profiling. Physiol Genomics 23:343–350
76. Anisimov VN, Berstein LM, Egormin PA, Piskunova TS, Popovich IG, Zabezhinski MA, Kovalenko IG, Poroshina TE, Semenchenko AV, Provinciali M, Re F, Franceschi C (2005) Effect of metformin on life span and on the development of spontaneous mammary tumors in HER-2/neu transgenic mice. Exp Gerontol 40:685–693

77. Anisimov VN, Berstein LM, Egormin PA, Piskunova TS, Popovich IG, Zabezhinski MA, Tyndyk ML, Yurova MV, Kovalenko IG, Poroshina TE, Semenchenko AV (2008) Metformin slows down aging and extends life span of female SHR mice. Cell Cycle 7:2769–2773
78. Anisimov VN, Egormin PA, Piskunova TS, Popovich IG, Tyndyk ML, Yurova MN, Zabezhinski MA, Anikin IV, Karkach AS, Romanyukha AA (2010) Metformin extends life span of HER-2/neu transgenic mice and in combination with melatonin inhibits growth of transplantable tumors in vivo. Cell Cycle 9:188–197
79. Cabreiro F1, Au C, Leung KY, Vergara-Irigaray N, Cochemé HM, Noori T, Weinkove D, Schuster E, Greene ND, Gems D (2013) Metformin retards aging in *C. elegans* by altering microbial folate and methionine metabolism. Cell 28:228–239
80. Slack C1, Foley A, Partridge L (2012) Activation of AMPK by the putative dietary restriction mimetic metformin is insufficient to extend lifespan in *Drosophila*. PLoS ONE 7(10):e47699. doi:10.1371/journal.pone.0047699
81. Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, Gomes AP, Ward TM, Minor RK, Blouin MJ, Schwab M, Pollak M, Zhang Y, Yu Y, Becker KG, Bohr VA, Ingram DK, Sinclair DA, Wolf NS, Spindler SR, Bernier M, de Cabo R (2013) Metformin improves healthspan and lifespan in mice. Nat Commun 4:2192
82. Longo VD, Fontana L (2010) Calorie restriction and cancer prevention: metabolic and molecular mechanisms. Trends Pharmacol Sci 31:89–98
83. Milman S, Atzmon G, Huffman DM, Wan J, Crandall JP, Cohen P, Barzilai N (2014) Low insulin-like growth factor-1 level predicts survival in humans with exceptional longevity. Aging Cell. doi: 10.1111/acel.12213 (Epub ahead of print)
84. Kopchick JJ, Kelder B, Gosney ES, Berryman DE (2014) Evaluation of growth hormone (GH) action in mice: discovery of GH receptor antagonists and clinical indications. Mol Cell Endocrinol 386:34–45
85. Guevara-Aguirre J, Balasubramanian P, Guevara-Aguirre M, Wei M, Madia F, Cheng CW, Hwang D, Martin-Montalvo A, Saavedra J, Ingles S, de Cabo R, Cohen P, Longo VD (2011) Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. Sci Transl Med 3:70ra13. doi:10.1126/scitranslmed.3001845
86. Yuan Y, Kadiyala CS, Ching TT, Hakimi P, Saha S, Xu H, Yuan C, Mullangi V, Wang L, Fivenson E, Hanson RW, Ewing R, Hsu AL, Miyagi M, Feng Z (2012) Enhanced energy metabolism contributes to the extended life span of calorie-restricted *Caenorhabditis elegans*. J Biol Chem 287:31414–31426
87. Williams DS, Cash A, Hamadani L, Diemer T (2009) Oxaloacetate supplementation increases lifespan in *Caenorhabditis elegans* through an AMPK/FOXO-dependent pathway. Aging Cell 8:765–768
88. Yang J, Kong X, Martins-Santos ME, Aleman G, Chaco E, Liu GE, Wu S, Samols D, Hakimi P, Chiang C, Hanson RW (2009) 3 Activation of SIRT1 by resveratrol represses transcription of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) by deacetylating hepatic nuclear factor 4α. J Biol Chem 284(40):27042–27053
89. Baur JA (2010) Resveratrol, sirtuins, and the promise of a DR mimetic. Mech Ageing Dev 131:261–269
90. Kume S, Uzu T, Kashiwagi A, Koya D (2010) SIRT1, a calorie restriction mimetic, in a new therapeutic approach for type 2 diabetes mellitus and diabetic vascular complications. Endocr Metab Immune Disord Drug Targets 10:16–24
91. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA (2003) Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 425:191–196
92. Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 430:686–689
93. Chen D, Guarente L (2007) SIR2: a potential target for calorie restriction mimetics. Trends Mol Med 13:64–71

94. Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A (2006) Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr Biol 16:296–300
95. Sakata Y, Zhuang H, Kwansa H, Koehler RC, Doré S (2010) Resveratrol protects against experimental stroke: putative neuroprotective role of heme oxygenase 1. Exp Neurol 224:325–329
96. Yang DL, Zhang HG, Xu YL, Gao YH, Yang XJ, Hao XQ, Li XH (2010) Resveratrol inhibits right ventricular hypertrophy induced by monocrotaline in rats. Clin Exp Pharmacol Physiol 37:150–155
97. Gupta YK, Briyal S, Chaudhary G (2002) Protective effect of trans-resveratrol against kainic acid-induced seizures and oxidative stress in rats. Pharmacol Biochem Behav 71:245–249
98. Wu Z, Xu Q, Zhang L, Kong D, Ma R, Wang L (2009) Protective effect of resveratrol against kainate-induced temporal lobe epilepsy in rats. Neurochem Res 34:1393–1400
99. Khan MM, Ahmad A, Ishrat T, Khan MB, Hoda MN, Khuwaja G, Raza SS, Khan A, Javed H, Vaibhav K, Islam F (2010) Resveratrol attenuates 6-hydroxydopamine-induced oxidative damage and dopamine depletion in rat model of Parkinson's disease. Brain Res 1328:139–151
100. Karuppagounder SS, Pinto JT, Xu H, Chen HL, Beal MF, Gibson GE (2009) Dietary supplementation with resveratrol reduces plaque pathology in a transgenic model of Alzheimer's disease. Neurochem Int 54:111–118
101. Smith JJ, Kenney RD, Gagne DJ, Frushour BP, Ladd W, Galonek HL, Israelian K, Song J, Razvadauskaite G, Lynch AV, Carney DP, Johnson RJ, Lavu S, Iffland A, Elliott PJ, Lambert PD, Elliston KO, Jirousek MR, Milne JC, Boss O (2009) Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo. BMC Syst Biol 10:31
102. Zou S, Carey JR, Liedo P, Ingram DK, Müller HG, Wang JL, Yao F, Yu B, Zhou A (2009) The prolongevity effect of resveratrol depends on dietary composition and calorie intake in a tephritid fruit fly. Exp Gerontol 44:472–476
103. Aljada A, Dong L, Mousa SA (2010) Sirtuin-targeting drugs: Mechanisms of action and potential therapeutic applications. Curr Opin Investig Drugs 11:1158–1168
104. Das DK, Mukherjee S, Ray D (2010) Resveratrol and red wine, healthy heart and longevity. Heart Fail Rev 15:467–477
105. Barger JL, Kayo T, Vann JM, Arias EB, Wang J, Hacker TA, Wang Y, Raederstorff D, Morrow JD, Leeuwenburgh C, Allison DB, Saupe KW, Cartee GD, Weindruch R, Prolla TA (2008) A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS ONE 3:e2264
106. Pacholec M, Bleasdale JE, Chrunyk B, Cunningham D, Flynn D, Garofalo RS, Griffith D, Griffor M, Loulakis P, Pabst B, Qiu X, Stockman B, Thanabal V, Varghese A, Ward J, Withka J, Ahn K (2010) SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J Biol Chem 285:8340–8351
107. Schmidt C (2010) GSK/Sirtris compounds dogged by assay artifacts. Nat Biotechnol 28:185–186
108. Park SJ, Ahmad F, Philp A, Baar K, Williams T, Luo H, Ke H, Rehmann H, Taussig R, Brown AL, Kim MK, Beaven MA, Burgin AB, Manganiello V, Chung JH (2012) Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. Cell 148:421–433
109. Hubbard BP, Gomes AP, Dai H, Li J, Case AW, Considine T, Riera TV, Lee JE, E SY, Lamming DW, Pentelute BL, Schuman ER, Stevens LA, Ling AJ, Armour SM, Michan S, Zhao H, Jiang Y, Sweitzer SM, Blum CA, Disch JS, Ng PY, Howitz KT, Rolo AP, Hamuro Y, Moss J, Perni RB, Ellis JL, Vlasuk GP, Sinclair DA (2013) Evidence for a common mechanism of SIRT1 regulation by allosteric activators. Science 339:1216–1219
110. Mitchell SJ, Martin-Montalvo A, Mercken EM, Palacios HH, Ward TM, Abulwerdi G, Minor RK, Vlasuk GP, Ellis JL, Sinclair DA, Dawson J, Allison DB, Zhang Y, Becker KG, Bernier M, de Cabo R (2014) The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet. Cell Rep 6:836–843

111. Hoffmann E, Wald J, Lavu S, Roberts J, Beaumont C, Haddad J, Elliott P, Westphal C, Jacobson E (2013) Pharmacokinetics and tolerability of SRT2104, a first-in-class small molecule activator of SIRT1, after single and repeated oral administration in man. Br J Clin Pharmacol 75:186–196
112. Libri V, Brown AP, Gambarota G, Haddad J, Shields GS, Dawes H, Pinato DJ, Hoffman E, Elliot PJ, Vlasuk GP, Jacobson E, Wilkins MR, Matthews PM (2012) A pilot randomized, placebo controlled, double blind phase I trial of the novel SIRT1 activator SRT2104 in elderly volunteers. PLoS ONE 7(12):e51395. doi:10.1371/journal.pone.0051395 (Epub 20 Dec 2012)
113. Venkatasubramanian S, Noh RM, Daga S, Langrish JP, Joshi NV, Mills NL, Hoffmann E, Jacobson EW, Vlasuk GP, Waterhouse BR, Lang NN, Newby DE (2013) Cardiovascular effects of a novel SIRT1 activator, SRT2104, in otherwise healthy cigarette smokers. J Am Heart Assoc e000042. doi:10.1161/JAHA.113.000042
114. Baksi A, Kraydashenko O, Zalevkaya A, Stets R, Elliott P, Haddad J, Hoffmann E, Vlasuk GP, Jacobson EW (2014) A phase II, randomized, placebo-controlled, double-blind, multi-dose study of SRT2104, a SIRT1 activator, in subjects with type 2 diabetes. J Clin Pharmacol. doi:10.1111/bcp.12327
115. Kincaid B1, Bossy-Wetzel E (2013) Forever young: SIRT3 a shield against mitochondrial meltdown, aging, and neurodegeneration. Front Aging Neurosci 5:48
116. Bellizzi D, Rose G, Cavalcante P, Covello G, Dato S, De Rango F, Greco V, Maggiolini M, Feraco E, Mari V, Franceschi C, Passarino G, De Benedictis G (2005) A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages. Genomics 85:258–263
117. Park SH, Ozden O, Jiang H, Cha YI, Pennington JD, Aykin-Burns N, Spitz DR, Gius D, Kim HS (2013) Sirt3, mitochondrial ROS, ageing, and carcinogenesis. Int J Mol Sci 12:6226–6239
118. Kim HS, Patel K, Muldoon-Jacobs K, Bisht KS, Aykin-Burns N, Pennington JD, van der Meer R, Nguyen P, Savage J, Owens KM, Vassilopoulos A, Ozden O, Park SH, Singh KK, Abdulkadir SA, Spitz DR, Deng CX, Gius D (2010) SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. Cancer Cell 17:41–52
119. Mouchiroud L, Houtkooper RH, Auwerx J (2013) NAD^+ metabolism: a therapeutic target for age-related metabolic disease. Crit Rev Biochem Mol Biol 48:397–408
120. Green KN, Steffan JS, Martinez-Coria H, Sun X, Schreiber SS, Thompson LM, LaFerla FM (2008) Nicotinamide restores cognition in Alzheimer's disease transgenic mice via a mechanism involving sirtuin inhibition and selective reduction of Thr231-phosphotau. J Neurosci 28:11500–11510
121. Gong B, Pan Y, Vempati P, Zhao W, Knable L, Ho L, Wang J, Sastre M, Ono K, Sauve AA, Pasinetti GM (2013) Nicotinamide riboside restores cognition through an upregulation of proliferator-activated receptor-γ coactivator 1α regulated β-secretase 1 degradation and mitochondrial gene expression in Alzheimer's mouse models. Neurobiol Aging 34:1581–1588
122. Liu D, Pitta M, Jiang H, Lee JH, Zhang G, Chen X, Kawamoto EM, Mattson MP (2013) Nicotinamide forestalls pathology and cognitive decline in Alzheimer mice: evidence for improved neuronal bioenergetics and autophagy procession. Neurobiol Aging 34:1564–1580
123. Schmeisser K, Mansfeld J, Kuhlow D, Weimer S, Priebe S, Heiland I, Birringer M, Groth M, Segref A, Kanfi Y, Price NL, Schmeisser S, Schuster S, Pfeiffer AF, Guthke R, Platzer M, Hoppe T, Cohen HY, Zarse K, Sinclair DA, Ristow M (2013) Role of sirtuins in lifespan regulation is linked to methylation of nicotinamide. Nat Chem Biol 9:693–700
124. Lamming DW, Ye L, Sabatini DM, Baur JA (2013) Rapalogs and mTOR inhibitors as anti-aging therapeutics. J Clin Invest 123:980–989
125. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18:1926–1945
126. Tokunaga C, Yoshino K, Yonezawa K (2004) mTOR integrates amino acid- and energy-sensing pathways. Biochem Biophys Res Commun 313:443–446

127. Salminen A, Kaarniranta K (2009) Regulation of the aging process by autophagy. Trends Mol Med 15:217–224
128. Kim D, Sarbassov D, Ali S, Latek R, Guntur K, Erdjument-Bromage H, Tempst P, Sabatini D (2003) GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell 11:895–904
129. Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science 294:1942–1945
130. Kim D, Sarbassov D, Ali S, King J, Latek R, Erdjument-Bromage H, Tempst P, Sabatini D (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110:163–175
131. Frias M, Thoreen C, Jaffe J, Schroder W, Sculley T, Carr S, Sabatini D (2006) mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. Curr Biol 16:1865–1870
132. Sarbassov D, Ali S, Kim D, Guertin D, Latek R, Erdjument-Bromage H, Tempst P, Sabatini D (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14:1296–1302
133. Betz C, Stracka D, Prescianotto-Baschong C, Frieden M, Demaurex N, Hall MN (2013) mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology. Proc Natl Acad Sci USA 110:12526–12534
134. Blagosklonny MV (2010) Calorie restriction: decelerating mTOR-driven aging from cells to organisms (including humans). Cell Cycle 15:683–688
135. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 460:392–395
136. Zemke D, Azhar S, Majid A (2007) The mTOR pathway as a potential target for the development of therapies against neurological disease. Drug News Perspect 20:495–499
137. Zhang Y, Bokov A, Gelfond J, Soto V, Ikeno Y, Hubbard G, Diaz V, Sloane L, Maslin K, Treaster S, Réndon S, van Remmen H, Ward W, Javors M, Richardson A, Austad SN, Fischer K (2014) Rapamycin extends life and health in C57BL/6 mice. J Gerontol A Biol Sci Med Sci 69:119–130
138. Fok WC, Chen Y, Bokov A, Zhang Y, Salmon AB, Diaz V, Javors M, Wood WH 3rd, Zhang Y, Becker KG, Pérez VI, Richardson A (2014) Mice fed rapamycin have an increase in lifespan associated with major changes in the liver transcriptome. PLoS ONE 9(1):e83988
139. Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, Woodward MA, Miller RA (2012) Rapamycin slows aging in mice. Aging Cell 11:675–682
140. Richardson A (2013) Rapamycin, anti-aging, and avoiding the fate of Tithonus. J Clin Invest 123:3204–3206
141. Leontieva OV, Paszkiewicz GM, Blagosklonny MV (2014) Weekly administration of rapamycin improves survival and biomarkers in obese male mice on high-fat diet. Aging Cell. doi:10.1111/acel.12211 (Epub ahead of print)
142. Neff F, Flores-Dominguez D, Ryan DP, Horsch M, Schröder S, Adler T, Afonso LC, Aguilar-Pimentel JA, Becker L, Garrett L, Hans W, Hettich MM, Holtmeier R, Hölter SM, Moreth K, Prehn C, Puk O, Rácz I, Rathkolb B, Rozman J, Naton B, Ordemann R, Adamski J, Beckers J, Bekeredjian R, Busch DH, Ehninger G, Graw J, Höfler H, Klingenspor M, Klopstock T, Ollert M, Stypmann J, Wolf E, Wurst W, Zimmer A, Fuchs H, Gailus-Durner V, Hrabe de Angelis M, Ehninger D (2013) Rapamycin extends murine lifespan but has limited effects on aging. J Clin Invest 123:3272–3291
143. Majumder S, Caccamo A, Medina DX, Benavides AD, Javors MA, Kraig E, Strong R, Richardson A, Oddo S (2012) Lifelong rapamycin administration ameliorates age-dependent cognitive deficits by reducing IL-1β and enhancing NMDA signaling. Aging Cell 11:326–335

144. Ozcelik S, Fraser G, Castets P, Schaeffer V, Skachokova Z, Breu K, Clavaguera F, Sinnreich M, Kappos L, Goedert M, Tolnay M, Winkler DT (2013) Rapamycin attenuates the progression of tau pathology in P301S tau transgenic mice. PLoS ONE 8(5):e62459
145. Flynn JM, O'Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, Zykovich A, Mooney SD, Strong R, Rosen CJ, Kapahi P, Nelson MD, Kennedy BK, Melov S (2013) Late-life rapamycin treatment reverses age-related heart dysfunction. Aging Cell 12:851–862
146. Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Rosenfeld SV, Blagosklonny MV (2011) Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. Cell Cycle 10:4230–4236
147. Sparks CA, Guertin DA (2010) Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. Oncogene 29:3733–3744
148. Fraenkel M, Ketzinel-Gilad M, Ariav Y, Pappo O, Karaca M, Castel J, Berthault MF, Magnan C, Cerasi E, Kaiser N, Leibowitz G (2008) mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. Diabetes 57:945–957
149. Chang GR, Wu YY, Chiu YS, Chen WY, Liao JW, Hsu HM, Chao TH, Hung SW, Mao FC (2009) Long-term administration of rapamycin reduces adiposity, but impairs glucose tolerance in high-fat diet-fed KK/HlJ mice. Basic Clin Pharmacol Toxicol 105:188–198
150. Houde VP, Brûlé S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, Marette A (2010) Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. Diabetes 59:1338–1348
151. Fang Y, Westbrook R, Hill C, Boparai RK, Arum O, Spong A, Wang F, Javors MA, Chen J, Sun LY, Bartke A (2013) Duration of rapamycin treatment has differential effects on metabolism in mice. Cell Metab 217:456–462
152. Jagannath C, Bakhru P (2012) Rapamycin-induced enhancement of vaccine efficacy in mice. Methods Mol Biol 821:295–303
153. Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin DA, Sabatini DM, Baur JA (2012) Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science 335:1638–1643
154. Minois N (2014) Molecular basis of the 'anti-aging' effect of spermidine and other natural polyamines—a mini-review. Gerontology (Epub ahead of print)
155. Ali AA, Poortvliet E, Strömberg R, Yngve A (2011) Polyamines in foods: development of a food database. Food Nutr Res 55:5572
156. Eisenberg T, Knauer H, Schauer A, Büttner S, Ruckenstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L, Fussi H, Deszcz L, Hartl R, Schraml E, Criollo A, Megalou E, Weiskopf D, Laun P, Heeren G, Breitenbach M, Grubeck-Loebenstein B, Herker E, Fahrenkrog B, Fröhlich KU, Sinner F, Tavernarakis N, Minois N, Kroemer G, Madeo F (2009) Induction of autophagy by spermidine promotes longevity. Nat Cell Biol 11:305–314
157. Morselli E, Mariño G, Bennetzen MV, Eisenberg T, Megalou E, Schroeder S, Cabrera S, Bénit P, Rustin P, Criollo A, Kepp O, Galluzzi L, Shen S, Malik SA, Maiuri MC, Horio Y, López-Otín C, Andersen JS, Tavernarakis N, Madeo F, Kroemer G (2011) Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. J Cell Biol 192:615–629
158. Nishimura K, Shiina R, Kashiwagi K, Igarashi K (2006) Decrease in polyamines with aging and their ingestion from food and drink. J Biochem 139:81–90
159. Pucciarelli S, Moreschini B, Micozzi D, De Fronzo GS, Carpi FM, Polzonetti V, Vincenzetti S, Mignini F, Napolioni V (2012) Spermidine and spermine are enriched in whole blood of nona/centenarians. Rejuvenation Res 15:590–595

Chapter 11
History of the Study of Calorie Restriction in Nonhuman Primates Conducted by the National Institute on Aging: The First Decade

Donald K. Ingram, Julie A. Mattison, Rafael de Cabo and George S. Roth

Abstract Beginning in 1987 a long-term study was initiated by the National Institute on Aging to evaluate the effects on health, disease, and aging of a regimen of calorie restriction (CR) in nonhuman primates. Specifically, different age groups of rhesus monkeys (*Macaca mulatta*) were gradually introduced to a nutritionally balanced diet that represented about 30 % less of the calories provided to comparably aged cohorts. A small group of squirrel monkeys (*Saimiri sciureus*) was also initiated at the beginning of the study but was not expanded due to several issues with husbandry. The study had several objectives including determining the safety and feasibility of such interventions for humans, development of a nonhuman primate model of aging, and identification of possible biomarkers of aging to evaluate this and other aging interventions. Over the years, many reports have been published describing measures of aging in numerous organ systems and the effects of CR thereon. The objective of this chapter is to document important events in the early history of this landmark study.

D.K. Ingram (✉)
Nutritional Neuroscience and Aging Laboratory, Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70809, USA
e-mail: Donald.ingram@pbrc.edu

J.A. Mattison · R. de Cabo
Translational Gerontology Branch, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA
e-mail: mattisonj@mail.nih.gov

R. de Cabo
e-mail: deCaboRa@grc.nia.nih.gov

G.S. Roth
GeroScience, Inc., Pylesville, MD 21132, USA
e-mail: geor@iximd.com

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11.1 Introduction and Background

By the 1980s the anti-aging and health and longevity promoting effects of dietary calorie restriction (CR) were fairly well characterized, although mechanism(s) of action were only slightly more controversial than they are today [1, 2]. Following the pioneering studies in the laboratories of Osborne [3] and McKay [4–6] early in the twentieth century, the beneficial effects of CR had been established for a variety of species from invertebrates to mammals, although most studies had been conducted in rodents [1, 2]. Consequently, the other major question was whether CR might promote health and longevity in humans, and perhaps even more basic, would it elicit the same biological effects in primates or longer-lived species demonstrated in rodents and lower species? This was an essential question for two primary reasons. One, from the scientific perspective, it can be argued that the anti-aging effects of CR would be far less robust in long-lived species than in short-lived species because the former had evolved the prolongevity mechanisms evoked by CR [7–9]. Second, from a public health perspective, the emerging obesity epidemic in industrialized societies put greater emphasis on the research on nutrition and aging.

At that time, two of the authors (Donald K. Ingram (DKI) and George S. Roth (GSR)) were working within the Intramural Research Program (IRP) of the National Institute on Aging (NIA), National Institutes of Health (NIH), which was located at the Gerontology Research Center (GRC) in Baltimore, Maryland. Much historical CR work had already been conducted there in the laboratories of Barrows [10, 11] and Goodrick [12, 13]. Following postdoctoral training at the Fels Institute at Temple University under the mentorship of Richard Adelman, GSR arrived at the GRC in 1972 as a Staff Fellow in the laboratory of Takashi Makinoden. Progressing through various assignments, he was appointed Chief of the Molecular Physiology and Genetics Section within the Laboratory of Cellular and Molecular Biology in 1986 at the GRC. Following postdoctoral training at the Jackson Laboratory under the mentorship of Richard Sprott, DKI began his appointment at the GRC in 1980 as a Staff Fellow in the Laboratory of Behavioral Sciences working under the supervision of Charles Goodrick, who died in 1981 of lymphoma. Charles Barrows retired shortly thereafter.

After Goodrick's death, DKI undertook the task of salvaging many ongoing Goodrick studies, resurrecting unpublished data, and otherwise maintaining an active CR program at the NIA. At that time, there were relatively few scientists at the GRC who were focused primarily on basic biological mechanisms of aging. Since those so engaged were well aware that the CR paradigm was the most reproducible and robust means of "slowing aging," several collaborations were generated to examine the effects of CR on specific biological systems already under study in the various NIA laboratories. In this regard, GSR was already working with James Joseph on age changes in neurotransmitter signal transduction as they related to impaired control of motor function in the dopaminergic system. He approached DKI about the possibilities of examining the effects of CR on rat motor control mechanisms. A three-way effort was established along with Joseph. To this end,

GRC Wistar rats that were already on CR in the DKI laboratory were assessed to demonstrate for the first time a functionally significant CR effect on brain aging. Specifically, the team reported that the concentration of dopamine receptors in the striatal region of the brain were maintained much later in life than observed in ad libitum controls [14] and similarly to have maintained responsiveness to dopaminergic agonists for motor control [15].

These studies provided a highly useful biochemical complement to other ongoing studies on behavior by both Joseph and DKI. When Joseph moved in 1982 to a new position at Lederle Laboratories (Pearl River, NYC), Richard G. Cutler (RGC), another GRC scientist who had also focused on basic aging mechanisms at the molecular level, expressed an interest in exploring additional possibilities for interfacing the CR intervention with key biogerontological questions. The remaining three continued to discuss potential CR experiments as well as basic aging questions, mostly at seminars, internal "journal club" type groups at the GRC, national and international meetings, and informally over an occasional beer or two. Organizational issues, NIA priorities, and funding uncertainties precluded the planning of any meaningful work until 1986. That year, following the approval of the Scientific Director of NIA, Richard Greulich, GSR was appointed as Chief of the newly formed Molecular Physiology and Genetics Section, and was joined there by DKI and RGC, a move which consolidated a keen interest in the CR paradigm.

During the summer of that year, the three spent a leisurely Sunday exploring the Eastern Shore of the Chesapeake Bay on Cutler's sailboat, the Vector. Late in the afternoon, after the usual mix of shop talk and less substantive conversation, a mutual idea emerged. Specifically, it was agreed that a coordinated effort was needed to expand the rodent CR work, particularly as related to lifespan, health, and function, into a system in which it would be possible to determine whether all the considerable effort up to that time would have relevance to humans, or at least longer-lived species. Essentially all of the previous studies had utilized experimental models with maximal lifespans less than 3–4 years, allowing for the much leveled criticism that CR-induced lifespan extension (generally not more than about 1 year in the models under study) might simply be an evolutionary adaptation to the under-nutrition of a famine, and might only serve to prolong survival until the next year of available food (reviewed in [16]). It should be noted that all three were aware of anecdotal historical human CR attempts as well as informal groups of practitioners, essentially all uncontrolled and mostly relatively short-term (reviewed in [17, 18]). It was also known that several short-term studies of dietary manipulation had been or were currently underway in nonhuman primates [19, 20], but were not structured in a way that would shed much light on the critical question(s), let alone answer them convincingly. Consequently, the team wondered whether it might be possible to persuade the NIA that a proper "long-term, high risk" project examining CR in a longer-lived species (such as dogs, nonhuman primates, or another suitable model) was appropriate to the mission of the IRP.

That sailboat trip was eventually to spawn one of the longest term projects in NIA history (second only to the Baltimore Longitudinal Study on Aging). With the idea germinating for such a study, there was a tremendous amount of background

and preparatory work to be done. The next several months were spent reviewing the CR literature, talking with various experts in the field, and evaluating the potential of various longer-lived aging models for study. Initially, there was some discussion with our colleagues at the GRC within the Laboratory of Cardiovascular Science (Harold Spurgeon and Edward Lakatta, in particular) about the possibility of establishing a colony of dogs on the roof of the GRC building. Several collaborative studies were conducted with Lakatta and his colleagues, but on rodents. Eventually monkeys emerged as the choice of animal model, since the dog colony never materialized. Ironically, a study conducted by Nestle Purina, which began shortly after, did indeed eventually report health and longevity benefits of CR in Labrador retrievers [21], and only required about a decade to complete due to the shorter lifespan of dogs compared to the best characterized monkey species.

The team started to investigate the literature on primate/monkey aging, visited several primate facilities, and talked to a number of investigators with experience in the area on nonhuman primate aging. At first consideration, an ideal model would be a primate with a lifespan of less than 10 years. There were candidates with such short lifespans reported in the literature at that time; however, after careful consideration and deliberation with experts, we soon realized that these reported estimates were not likely reliable as they were based on small samples and more importantly were obtained with less than ideal husbandry for these species. After crafting a shortlist of possible candidates that included rhesus monkeys (*Macaca mulatta*), squirrel monkeys (*Saimiri sciureus*), galagos (*Galago*), and marmosets (*Callithrix jacchus*), and discussing it with NIA staff as well as outsiders, it became obvious that rhesus were truly the "*E. coli*" of monkeys in terms of characterization and research use for essentially every physiological, behavioral, and pathological question, including aging. Unfortunately, they lived longer than just about every other primate we had seriously considered. The team had considered the use of great apes given their close genetic association to humans, but these presented their own sets of problems, welfare and cost issues as well as lifespans over 40 years. However, on balance, much shorter-lived primates such as dwarf lemurs and pygmy marmosets seemed to require exceptional care, were not readily available in the required quantities, and had major issues with their husbandry and diet, which rendered rhesus the best choice at that time. Nevertheless, some of the other candidates did offer other advantages in addition to shorter lifespan, such as "twinning" in marmosets (which could have been used to place siblings on control and CR diets, respectively, to minimize genetic variability), although this was offset by susceptibility to specific viruses. We did, however, decide to "hedge our bet" a bit, by proposing a secondary group of squirrel monkeys that were believed at that time to live only about half as long as rhesus. In retrospect, this choice turned out to be problematic for a number of reasons. Essentially all had a "benign" microfilaria infestation, which will be discussed in a later section, and some confusion existed over subspecies, and, in our own particular case, a critical misclassification of the ages of the monkeys, prior to admission to the study.

11.2 Crafting the Proposal and Administrative Strategy

Once we had decided on the best models for testing the hypothesis that CR could elicit the same health, function, and longevity benefits in primates/longer-lived species as it did in rodents and lower animals, it became necessary to craft a proposal that could be presented to the NIA administration in an effort to obtain both organizational and financial support. Although not quite a biogerontological "moonshot," the scope and cost of such a project closely balanced its importance as a critical question in the ever-expanding field. Consequently, the team needed to rally both outside scientific/political, as well as internal NIA administrative support, in a way that had probably never been attempted in the IRP. The three of us had already obtained somewhat of a reputation for "out of the box" thinking as well as always looking for a way to get a "yes" answer from administrators whose job seemed to be "finding ways to say no," so we expected to be eyed rather suspiciously whenever we presented new ideas that required money.

Because of the size of initial budget required for the project, we decided that the best means would be to utilize a funding source known as an "Inter-Agency Agreement" available through the "NIA contract pool." Under the direct control of the NIA Director and his appointed committee, called the Planning and Contracts Committee, comprised of staff within the extramural program of the NIA, this money was used by NIA to fund various large-scale projects, for example, epidemiological studies of Alzheimer's disease. We were greatly aided in our efforts by the Associate Director for Extramural Affairs, Miriam Kelty, who chaired this committee and patiently assisted us in learning how to make a proposal through this channel. The principals we had to convince of the value of our proposal were the NIA Director, T. Franklin Williams, and the Deputy Director, Edward Schneider. Before coming to the NIA, Dr. Williams had been an eminent geriatrician at the University of Rochester, who was not entirely familiar with basic research in the biology of aging. On the other hand, before being appointed Deputy Director, Dr. Schneider had served as Head of the Molecular Genetics Section at the GRC; thus, he was familiar with the CR paradigm, the controversies surrounding it, and the need to conduct studies in human relevant models. One major issue that Dr. Schneider had was protecting the study from any undue scrutiny by animal rights groups. To this point, he advised against referral in the official title of the project to the terms "calorie restriction" or "diet restriction." Therefore, we settled on the official title of the project as "Assessment of Primate Aging: Effects of Caloric Modification."

11.3 The Pilot Study

After securing the initial $225,000 from the Planning and Contracts Committee in 1985 for conducting a feasibility study to establish a primate CR and aging project, the team had to find ways to obtain the animals and a venue in which to conduct it,

in addition to supporting it logistically and scientifically for the approximately 1 year initial funding period. Since the financial support came from an Intra-Agency Agreement, the most expeditious way to do this was to find another government facility, preferably within NIH, where such a study could be operated. Therefore, we put the equally important scientific aspects (the details of which still needed to be fleshed out at many levels) temporarily on hold to seek out a suitable location. Ideally, this would include proper housing, logistics, husbandry, veterinary care and experience, ability to help procure animals, and a host of other related qualifications. Several NIH veteran colleagues were aware of the NIH Large Animal Facility, operated by the Division of Research Resources (DRR) of NIH just outside of the rural town of Poolesville, Maryland, in northwestern Montgomery County. It was about 20 miles from the main Bethesda campus and approximately 70 miles from the GRC in Baltimore. Nobody seemed to know much about it, except that it was apparently underutilized. The facility maintained "large" animals there, which included farm livestock and primates. There were other facilities for dogs, cats, and swine. We learned from various sources that the NCRR veterinarians there were supposedly looking for projects to house, so as to support the operation. It was recommended that we make an appointment with the Poolesville staff to tour the facilities and discuss the proposed project with them.

Thus, towards the end of 1986, we scheduled a meeting with David Renquist and Milton April, the veterinarians who were running the Poolesville Unit which housed primates for several NIH investigators. The primary mission there was to procure monkeys, hold them, screen and treat them for any health problems, prior to shipping them to the NIH main campus in Bethesda. Drs. Renquist and April seemed very positive about the proposed project, accommodating, and interested in coming to an agreement that would benefit both sides–them filling empty animal rooms and us starting a long-term monkey CR study. An attractive part of the deal would be for them to procure the animals we needed from sources with which they were familiar. In addition, they would supply veterinary care and husbandry, and we would also have access to DRR nutritionists with extensive nonhuman primate experience (Joseph Knapka and Dennis Barnard).

After considering the budget available to us along with current prices for procuring monkeys and their per diem costs, our initial plan was to obtain approximately 30 male rhesus; 12 juveniles, 12 young adults, and 6 old, and a same number of male squirrel monkeys distributed along similar age lines, the two youngest groups of each species to be divided into control and CR subgroups. As we had not made a decision to induce CR in older monkeys, the inclusion of this age group was to provide an anchor for determining the degree of age effects on any parameter selected to assess in the initial study. Additionally, we did not include any females at the outset, since most rodent CR studies had conducted using males (this later raised administrative issues that will be discussed below).

After decisions about the species to be investigated and the sample size, the next most important decision was what diet to incorporate. For these discussions, we had

the expert advice of the renowned lab animal nutritionist at NIH, Joseph Knapka, and his assistant, Dennis Bernard. In early discussions, Dr. Knapka insisted that we should plan for a totally synthetic diet for the study. There were several advantages of this approach, most importantly the reliability and stability of ingredients over the life of the study. We argued strongly that since the study was expected to span 5–10 years, we could be risking the exclusion of an essential micronutrient, perhaps unidentified at that time, if we chose a purified diet. After further reflection, Dr. Knapka agreed that we could develop a highly standardized natural ingredient diet for the study. The details of this diet are discussed in later section.

After ample consultation with various colleagues and other experts, the next discussion concerned the degree of restriction to be imposed. We considered ranges from 20–40 % restriction, which was the range that had proven most effective in rodent studies. We finally decided on a target of 30 % less than controls. We considered that a higher degree of restriction, e.g. 40 % which is often applied in rodent CR studies, might be too severe, particularly for younger monkeys. We also decided that the CR regimen should be gradually introduced. All animals were to receive control levels of food for the first month; then the monkeys on CR would receive 10 % less each month until full 30 % restriction was gradually achieved.

Once we reached an agreement on the budget, the real work began as we realized how much more there was to do, both to successfully obtain and maintain the monkeys under the proper conditions as well as to implement the science. Little did we realize at that point how "fluid" the project was to become in terms of constant adjustment to changing biological, pathological, and environmental conditions, as well as scientific opportunities that would arise as the biogerontological field (and CR paradigm itself) rapidly advanced.

Given the expected length of the study planned and the attendant administrative and financial challenges, we can say that our original intent was not specifically directed toward assessing the effects of CR on survival. Rather the "short-term", objective which we estimated might take at least a decade, was to assess biomarkers of aging. During the inception of the study, biogerontology had begun to recognize the need to develop a battery of age-sensitive tests that might be applied to assess the success of any intervention aimed to slow the rate of aging [22–24]. Several workshops had been held and proposals made without any clear consensus emerging. Nonetheless, great optimism for making progress in this area had been generated by research identifying candidate cellular and molecular biomarkers of aging, e.g. DNA damage [25], advanced glycation endproducts [26], oxidative stress [27]. Thus, it was our intention to team up with noted experts in these areas to develop and utilize such biomarkers of aging to assess the hypothesis that CR attenuated aging in monkeys. Beyond the emphasis on cellular and molecular biomarkers of aging, we also recognized the need to assess age-related changes in physiology and behavior; therefore, we began making plans to implement such assays into our study, e.g. cardiac function, sensory abilities, and locomotor activity.

11.3.1 Monkeys, Housing, and Health

As first steps for the project, we had to make decisions on three key issues: (1) husbandry issues, including how to procure the monkeys; (2) securing their housing; and (3) establishing procedures to protect their health.

Our DRR colleagues were familiar with monkey procurement procedures, but not long-term maintenance. The latter issue went on to cause us considerable consternation over the years, as we sought to maintain the animals and their health for the longest possible time, regardless of experimental group. The veterinarians formulated a plan for acquiring the 30 rhesus in the approximate age ranges sought. Male monkeys were obtained between December, 1986 and February, 1987 [28]. Twelve juveniles (aged 0.6–1 year, mean = 0.9 years) were born and raised at a NIH facility in Perrine, Florida, and briefly resided at the Primate Research Institute of New Mexico State University at Holloman Air Force Base. Twelve young adults (aged 3–5 years, mean = 4.2 years) were obtained from a research colony in The People's Republic of China via the Texas Primate Center, and 6 old animals (aged approximately 18–25 years, mean approximately = 19.5 years) were acquired from breeding colonies at the Perrine facility, where they had been housed in social, mixed aged and sex breeding groups. Unfortunately, several of these older monkeys were originally wild-caught in India, so age could only be estimated from dentition records. As all of the monkeys arrived with fairly comprehensive health reports, we were assured that none of them had been used in invasive experiments.

The supply of available squirrel monkeys was not so liberal. Nevertheless, after some creative negotiating, the animals arrived in Poolesville in February, 1987 and consisted of 12 juveniles (aged 1–4 years), 13 adult (aged 5–10 years), and 4 old (aged >10 years). Most were wild-caught and purchased from World Wide Primates in Dallas, Texas. Virtually all were identified by morphometric characteristics and selected cytogenetic typing to be of the *sciureus* subspecies, except for 6 (2 adult and 4 old obtained from the primate facility in Iquitos, Peru) that were typed as the *boliviensis* subspecies. One additional monkey (of the *sciureus* subspecies) was obtained from the Naval Medical Research Center to replace an adult monkey that died shortly after arrival. Like the rhesus monkeys, none of the squirrel monkeys had been used in invasive experimentation.

An early indication that the squirrel monkeys were to be more problematic than the rhesus came when a visiting expert from Goucher College, Bernadette Marriott, informed us upon close examination that the ages of some animals had been misestimated by the suppliers. She graciously offered to reclassify them, and the above numbers represent her more reliable estimates.

All the monkeys were housed under the care of the DRR veterinarians and staff, and were maintained using standard DRR Primate Unit procedures with some slight modifications [28]. With the exception of the old rhesus, which were housed individually because of their larger size as well as to avoid injuries from fighting, all animals were originally housed in pairs with cage mates of similar age, diet, and size at the outset of the study. Pair housing was used to balance social needs with

feeding constraints, by interconnecting two or three stainless steel cages (88.9 cm × 61 cm × 68.5 cm each). Triple cages were used for juvenile and adult rhesus pairs, while double cages were employed for the largest old single animals. All squirrel monkey pairs were housed in double cages. Interconnected cages possessed sliding partitions to isolate cagemates during feeding and allow for the precise allotment required by the study. Wire screens below the cages allowed monkeys to retrieve dropped food and enabled measurement of actual food intake by collecting and weighing the residue retrieved from the screens.

Animal rooms were 2.9 × 8.2 m, without windows, but with temperature automatically controlled at 22–28 °C, humidity at 50–60 %, and light cycle at 12 h on/12 h off [28]. Light was fluorescent at 30 ft candles of illumination per room. Ventilation was fresh air, with 10–15 changes per hour through standard filtration. Water was provided ad libitum, via an automatic, filtered, and chlorinated (2–3 ppm) system connected to each cage. Cages were washed twice daily in situ and removed regularly for steam cleaning. Initially, environmental enrichment was provided via white Teflon balls. Further discussion of socialization, welfare, and environmental enrichment will be discussed in later sections. All animals were observed daily, and physical examinations were conducted every 3 months. Complete blood chemistry and hematology were included, and will be discussed further in regard to scientific issues. Fecal analysis for parasites was also conducted quarterly, and routine tuberculin testing and dental cleaning were performed every 6 months. In sum, the monkeys were subject to continuous health monitoring over the course of the study.

11.3.2 The Diet and Implementation of CR

The diet formulation for rhesus monkeys is presented in Table 11.1. It represents a modification of the high fiber diet that was routinely fed to monkeys at NIH at that time [28]. Crude fiber content was reduced from 7 to 5 % in an effort to more closely match the levels of most commercial primate diets. It was manufactured by Agway (Ithaca, NY) by extruding it into biscuits (0.71–0.95 × 1.59–2.54 cm). The squirrel monkey diet (Table 11.2) was a modification of that used for marmosets and tamarins [29], with the crude fat content reduced from 10 to 8 % [28]. It was manufactured at Ziegler Brothers, Gardners, PA as cold pellets, 1.27 × 1.91–3.18 cm. The nutrient concentrations of the respective diets for rhesus and squirrel monkeys are shown in Table 11.3. To accommodate the need for adequate "micronutrient" intake in CR animals, the diet was supplemented with extra vitamins, minerals, and trace elements. Specifically, the vitamin and mineral mix was increased by 40 % above the amount considered adequate for ad libitum fed monkeys. To assure reliability of ingredients, safety, and levels of macro and micronutrients, both diets were routinely assayed for quality control as previously described [28].

Table 11.1 Formulation for the rhesus monkey diet

Ingredient	Amount (% by weight)
Basal mix	
Ground wheat	32.50
Ground corn	22.10
Soybean hulls	12.00
Soybean meal (48 % protein)	8.50
Fish meal (60 % protein)	5.47
Sugar	4.00
Alfalfa meal	3.00
Dried whey	3.00
Brewer's yeast	2.00
Limestone	1.30
Dicalcium phosphate	1.00
Iodized salt	0.60
Mineral mix	0.40
dl-methionine	0.13
Vitamin mix	1.00
	100.00

Table 11.2 Formulation for the squirrel monkey diet

Ingredient	Amount (% by weight)
Basal mix	
Fish meal (60 % protein)	10.00
Soybean meal (48 % protein)	12.00
High fat milk solids	6.50
Corn flour	25.50
Casein	6.40
Glucose	17.90
Apple pomace	10.00
Beet pulp	5.00
Soybean oil	1.90
Soybean lecithin	0.60
Dicalcium phosphate	1.00
Salt	0.60
Mineral mix	0.60
Vitamin mix	1.00
	100.00

Decisions regarding the degree of CR to implement were discussed above. Now a major challenge beyond that decision was what the control group should be provided. A conventional CR paradigm for rodents would be to continuously measure the intake of the control group and provide 30 % less of the estimated

Table 11.3 Calculated nutrient concentration of the diets

Nutrient	Units	Rhesus monkey diet	Squirrel monkey diet
Crude protein	%	15.37	20.34
Crude fat	%	5.01	7.97
Crude fiber	%	5.00	4.99
Gross energy	kcal/g	3.77	4.03

group average to the experimental group. This approach would not take into account the great variance in body weight and composition that existed within our groups, particularly the adult groups (range 4–6 kg for rhesus and 0.5–0.9 kg for squirrels). Another approach would be to monitor the food intake of each monkey over the course of several weeks, and then use this estimate as a baseline from which to reduce the food provided by 30 %. This procedure could not be implemented in our study either, specifically because there were groups of young, growing juvenile monkeys. In consultation with Dr. Knapka and other experts, we agreed that the nutritional objective for our study should be to provide sufficient food for control groups to meet their energy requirements based on species, age, and body weight. Therefore, to this end, we developed the feeding protocol presented in Table 11.4 designed to provide a precise amount of food to each monkey based on their age at that time and their mean body weight measured over the previous 3 months.

Food was provided on stainless steel trays mounted outside the cages. Each animal was moved into an individual cage (except larger rhesus that were already singly housed) at approximately 700 and fed 50 % of his daily allotment, with the remainder of the ration offered at 1,300. All unconsumed food was removed at 1,500, and the uneaten amount determined. Monkeys were then returned to their designated cages for pairing where necessary until the next morning's feeding. Initially each monkey was also provided with a small amount of fresh fruit on a weekly schedule, primarily as another source of environmental enrichment. Later in the study, non-caloric alternative treats were employed.

From the description provided above, it should be noted that we implemented meal feeding and thus intentionally avoided implementing ad libitum feeding, which is a conventional control paradigm used in rodent CR studies. To this point, in early discussions Barbara Hansen from the University of Maryland School of Medicine, who was a leading expert in rhesus monkey nutrition and physiology, had warned us that most of our monkeys were likely to become obese and diabetic if fed ad libitum. Thus, we designed our protocol with the objective of avoiding "overfeeding" our controls. This criticism had been frequently leveled against rodent CR work, to the effect that the CR paradigm was simply a "laboratory artifact" that would only benefit sedentary overweight animals. In retrospect, we believe the choice of the natural ingredient diet and controlled feeding regimen was prudent and sound.

Table 11.4 Daily food allotments according to diet, body size, and monkey species

Body weight (kg)	Control (g)	Degree of restriction		
		−10 % (g)	−20 % (g)	−30 % (g)
Rhesus monkeys				
1.5	130	117	104	91
2.0	137	124	111	98
2.5	137	124	111	98
3.0	150	137	124	104
3.5	156	143	130	111
4.0	176	163	143	124
4.5	189	176	156	130
5.0	195	176	156	137
5.5	215	195	176	150
6.0	221	202	182	156
6.5	234	215	189	163
7.0	241	221	195	169
7.5	247	221	202	176
8.0	254	234	208	176
8.5	260	234	208	176
9.0	260	234	208	182
12.0	280	254	228	195
15.0	299	273	241	215
18.0	319	292	260	228
Squirrel monkeys				
0.20	13	12	11	10
0.25	16	15	13	12
0.30	23	21	19	16
0.35	26	24	21	18
0.40	31	28	25	22
0.45	36	33	29	25
0.50	41	37	33	29
0.55	44	40	36	31
0.60	46	42	37	33
0.65	47	43	38	34
0.70	48	44	39	34
0.75	48	44	39	34
0.80	48	44	39	34
0.85	50	45	40	35
0.90	51	46	41	36
0.95	54	49	44	38
1.00	57	52	46	40
1.05	60	54	48	42

(continued)

Table 11.4 (continued)

Body weight (kg)	Control (g)	Degree of restriction		
		−10 % (g)	−20 % (g)	−30 % (g)
1.10	63	57	51	44
1.15	66	60	53	46
1.20	69	63	56	49

11.3.3 The Science and Candidate Biomarkers of Aging

At the time of the "pilot" study, it had not yet been determined what candidate biomarkers of aging would be suitable to assess rates of aging in the respective monkey age and diet groups. Since panels of blood chemistry and hematology were an essential part of their routine health screening, we attempted to determine whether these might serve a dual purpose. Monkeys were, therefore, anesthetized monthly with ketamine, prior to the morning feeding. Standard hematology and blood chemistries were determined initially by Maryland Medical Laboratories (Baltimore, Maryland) and subsequently by their successors. Some of these measures exhibited age, but not diet group, differences essentially from the outset [28], and were later to become incorporated into a biomarker battery (also more on this later).

11.3.4 Early Results and Modifications: Reality Sets In

Despite the complexities of getting the project launched, those were somewhat "heady" times for the team. The opportunity to conduct a study to address a major question in biogerontology at a prestigious institution, not to mention successfully jumping through so many administrative/bureaucratic hoops to obtain the initial funding, inspired us to work ever harder. There were no shortages of issues that required quick responses, even from day one. Most of the issues had to be handled by phone, as we maintained our other extensive research program at the GRC in Baltimore. We made treks dutifully to Poolesville on the last Wednesday of every month to meet with the veterinarians and staff to basically check on all the activities.

As the first problem mentioned above, within a month of arrival, it was determined that the age estimates of some squirrel monkeys were grossly inaccurate (e.g. one juvenile had been estimated to be >10 years old!) and had to be reclassified. Shortly after, we established collaboration with William Erschler and Joseph Kemnitz at the University of Wisconsin (UW) to assess immune function (already established as a potential biomarker of aging). Upon microscopic examination of freshly shipped blood samples, it was determined that essentially all the squirrel monkeys were infested with the blood borne parasitic nematode, microfilaria. For the first time we learned that this infection was quite common, for this species, even in laboratories, and supposedly "benign," but our naiveté with respect to the

peculiarities of primate models was just beginning to surface. The long-term utility of the squirrel monkeys was now certainly in question.

The next problem involved a few of the juvenile rhesus on CR that began to lose some hair during the first couple of months after reaching the 30 % level on protocol. Since hair loss is observed with protein malnutrition, the veterinarians advised us to make adjustments to our diet table for younger monkeys; thus, we increased the control levels of food up by about 10 % with corresponding changes in the amounts provided to CR monkeys. This change eliminated any further observations of hair loss and the juvenile monkeys exhibited steady growth.

Then over the course of that year, we experienced what we began to refer euphemistically to as the "Friday Phone Call" (FPC). These were usually either from animal technicians, who had primary "hands on" responsibility for our animals, and began something like, "Doc, I'm sorry to tell you this, but…." Of course, our biggest fear all along was that some monkey malady or epidemic, unknown to basic bench scientists, would strike the colony and wipe out the entire project in one fell swoop. This fear never actually subsided, and was later the basis for suggesting a "two site" venue when expansion of the project was recommended.

One of the earliest reasons for the FPC was "bloat," a common problem in livestock when they consumed too much food and/or water, and usually occurred in our CR monkeys after a bleeding procedure, since, being deprived of food for longer than usual, they gorged themselves after coming out of anesthesia, resulting in this gaseous syndrome. Alleviation is easily possible by aspiration when detected early, and it was consequently necessary to institute "bloat patrols" to monitor susceptible animals. Bloat was just one of many "mechanical" issues that seemed to be more frequent in CR monkeys than controls, and became particularly problematic when evaluating longevity/survival data.

Finally, there were many more such issues, but a not totally unexpected one was fighting among rhesus males as they reached puberty. For this reason, despite our best hopes for continued "socialization" of bonded pairs, these animals had to be separated and relegated to single housing for their own safety.

As another challenge to the science of the project, we had to address the issue of monkey injuries from fighting and other trauma, which happened on a few occasions early in the study. We soon learned that the propensity of the veterinarians was to recommend euthanasia in such situations; whereas, we felt that a unique long-term study of this nature necessitated every effort to continue the animals as long as they were not subject to unrelieved pain and/or suffering. One salient example was a squirrel monkey that caught his arm in the cage and severely injured the limb. In one of our early "victories" in this regard, we convinced the veterinarians to amputate the injured arm, rather than sacrifice the animal. Following surgery, the monkey continued in an apparent state of compensation for the amputated limb and good health for many years thereafter. This episode was characteristic of the challenges we faced in convincing the numerous veterinarians who filtered through the study over the years to take a long-term "research interest" in the project, rather than simply providing good health care and husbandry (see next section).

11.4 Progress and Expansion

Despite the many challenges and the few major hiccups after the first "pilot" feasibility year, we were able to convince the leadership of NIA to renew the project and obtain additional funding to double the size of the study from 30 to 60 monkeys. This action involved procurement of an additional 4 monkeys for each of juvenile and adult diet groups an and additional 14 monkeys for the aged group, which included 4 for the control group and 10 for a CR group that was initiated. The objective of instituting the latter group was to evaluate whether CR imposed late in life could still have significant beneficial health effects. An outline of the origins of these and other monkeys added to the study is presented in Table 11.5.

Within 3 years we had finally shaped the project into a cohesive operation and preliminary, believable results were beginning to emerge; thus, we began to assemble a report on the study, the first of what would become more than 100 reports to the present date. Titled as "Dietary Restriction and Aging: The Initiation of a Primate Study," the paper was submitted to the *Journal of Gerontology* in 1989 and published in 1990 [28]. It was not in the traditional vein of that publication, in that the only "gerontology" was a description of the older monkeys in our study and preliminary comparisons with the younger ones. Nevertheless, the scope and hypothesis were certainly a major question in the field. Our close colleague and Editor at that time, the late Vincent Cristofalo, was instrumental in having the paper published, despite reviews suggesting that we should wait for some actual CR results before publishing. For co-authors we included just about anybody who had anything to do with the establishment and physical operation of the study. Thus, beyond the three principle investigators–GSR, DKI, and RGC, the authors included the veterinarians, David Reinquist and Milton April, three of the main laboratory

Table 11.5 Origins of the NIA study rhesus monkeys

Origin	Source	Sex	Sample size	Age at study onset (years)	Study onset date		
			CON	CR	Total		
Indian	Perrine, FL	Male	6	6	12	0.5–1.0	1986–1987
Chinese	Alice, TX	Male	6	6	12	3–5	1986–1987
Indian	Perrine, FL	Male	3	3	6	18–25	1986–1987
Chinese	Sino-Tech	Male	4	4	8	1–4	1988
Chinese	Sino-Tech	Male	4	4	8	5–9	1988
Chinese	Sino-Tech	Male	7	7	14	>20	1988
Indian	Morgan Island, SC	Female	10	10	20	1–3	1992
Chinese	Alice, TX or Aberdeen Proving Ground, MD	Female	10	10	20	6–14	1992
Indian	Morgan Island, SC	Female	10	10	20	16–21	1992

animal technicians—Claude T. Belcher, Margaret Clark, and Charles Hatcherson—the nutritionist, Joseph Knapka, and two scientific colleagues, Bernadette Marriott and Richard Weindruch. As mentioned earlier, Dr. Marriott, who had now taken a position within the Department of Comparative Medicine at the Johns Hopkins University, was instrumental in correcting mistakes in the age estimations for the squirrel monkeys and also provided valuable advice on other matters of monkey husbandry and physiology. As one of the leading experts in the CR paradigm, Dr. Weindruch had been a consultant on the project during our exploratory phases who provided valuable advice for many issues. Between 1987 and 1990, Rick was working as a Health Scientist Administrator in the extramural program of NIA. Within this position, he was given time off weekly for research, so he had already put in many hours on-site in Poolesville doing various measurements that would be included in this and later publications. Additionally, in 1990 he departed NIA to accept a position as Associate Director of the Institute on Aging at UW, where he became involved in the CR study in rhesus monkeys that had started up in 1989, and for which we maintained a close relationship over the years.

With Rick's departure along with the demands of the growing number of assays that we had initiated, it became clear that we needed to hire a scientist to station full-time in Poolesville to supervise the daily operations and minimize the "Friday phone calls." To this end, we advertised for a post-doctoral position, which was the only type position that would suit our budget at that time. To be sure, we would be asking much from a person hired into this training position. We received applications from many suitable candidates; however, we were highly fortunate to hire an especially qualified and talented one, Mark Lane, who joined the project in 1991. Following a stint as an Emergency Medical Technician, Mark had been broadly trained in gerontology at the excellent Pennsylvania State University program. We met with him at the annual meeting of the Gerontological Society of America, where he expressed a strong interest in joining the project. We promised him a "unique" post-doctoral experience to say the least. In return for his willingness to relocate to a somewhat remote outpost of NIH, he would receive not only cutting edge scientific training, but also obtain valuable managerial and administrative experience dealing with the Primate Unit staff. Dr. Lane proved to be highly capable, and the study flourished during his 11-year stint.

Beyond the scientific advances, several major administrative accomplishments were achieved during Mark Lane's stay in Poolesville. One was his recruitment and training of an outstanding and dedicated group of technicians. Two of the most distinguished in this group with the longest tenure were Edward Tilmont and April Handy. Both were highly skilled in animal physiology and became experts in many techniques and assays that allowed numerous projects from collaborators from around the world to be accomplished. Moreover, their dedication to the project and attention to detail assured the highest degree of health and care for the monkeys. As a second accomplishment, Mark was able to build close working relationships with the veterinarians at Poolesville that engendered a better understanding and appreciation of the science, which also paid huge dividends over time in terms of the health and welfare of the monkeys. Mark steadily advanced from his post-doctoral

position to higher positions within NIA, and he had entered the tenure-track program at NIA before departing in 2002 to pursue a career in the pharmaceutical field. Mark maintained contact with the study for the first years after his departure, but that involvement dissipated due to his heavy work obligations.

Mark Lane arrived at the time of a great expansion of the study. Given the progress that was being made in the study and the need for greater sample size to boost statistical power, a second expansion of the study occurred in 1992 under very interesting and fortuitous circumstances. Returning to the NIA Planning and Contracts Committee with a proposal to double the size of the study, again requesting only the use of male monkeys, we learned in 1991 that our proposal had been approved. This news was brought to us by George R. Martin who had been appointed Scientific Director for the NIA in 1988. Bernadine Healy had just been appointed as the first female Director of NIH and was mandating equal inclusion of female subjects in all relevant NIH-sponsored clinical studies. To our astonishment, we were told that our CR monkey project would be under this new mandate, and thus we were instructed to procure only female rhesus monkeys for the project. To this end, we made excursions to a NIH-sponsored facility on Morgan Island, South Carolina, headed by David Taub and to the Aberdeen Proving Ground in Maryland, headed by Michael Flynn. As a result, we were able procure 60 female rhesus monkeys comprising the age groups presented in Table 11.5.

Another smaller expansion of the study was made a couple of years later. This involved the procurement of small groups of rhesus monkeys for which more intensive studies could be made during the transition to CR. In 1994, we began studies on the first group of 12 male 1–2 year old rhesus monkeys with 6 assigned to both the control and CR groups. We would later obtain other small batches of monkeys of different genders and ages for short-term studies and referred to as the "acute monkeys". Eventually most of these monkeys were shipped to the Oregon National Primate Research Center (ONPRC) to take advantage of an important technology that they had perfected for rhesus monkey there, specifically the ability to obtain continuous blood sampling through indwelling catheters. This technology would permit us to more accurately measure hormones and other proteins that manifested circadian rhythms in their production/secretion.

Around 1994, one of the founding members of our research team, Richard Cutler, resigned from NIA to head a start-up company, Genox. Located in Baltimore, this company was focused on development of new assays to measure oxidative stress in various tissues. A few years later, Richard was recruited to head up the clinical laboratory at the newly founded Kronos Longevity Research Institute in Phoenix, Arizona. His involvement with the study dissipated greatly after leaving the NIA.

11.5 Expanding the Collaborator Base and Rolling Out the Reports

As stated earlier, one of primary objectives for the study was to identify possible cellular and molecular biomarkers of aging that might enable the team to evaluate whether CR could attenuate the rate of aging. To this end, we sought to enlist the collaboration of scientists who were involved in such research. Although we will not provide an exhaustive list of studies and collaborations generated, we can describe a few of the early ones directed toward this objective.

As mentioned previously, we were examining immunological markers in collaboration with William Ershler at University of Wisconsin, who was now joined by Rick Weindruch. We also recruited the help of Peter Rabinovitch and Norm Wolf at the University of Washington for examining immune function. Beyond simple assays for assessing proliferative capacity of lymphocytes in response to specific mitogens, many new assays were being developed in the Rabinovitch laboratory and elsewhere, particularly as related to specific subsets of lymphocytes, e.g. CD4+ cells. Norm Wolf and William Pendergrass from the University of Washington also helped us with assays to examine proliferative capacity of fibroblasts using new techniques developed in their laboratory. We sought collaboration with Vince Monnier and David Sell at Case Western Reserve University to examine markers of glycation, such as pentosidine, which was a hot new area of research that they were developing. Judson Aiken at University of Wisconsin also began a collaboration to examine mitochondrial DNA damage in muscle tissue obtained from the monkeys. We also recruited the help of Cal Harley, who had recently moved to Geron Corporation. Cal had helped develop new assays for examining telomere length in cells, which was another exciting new area in biogerontology. Additionally Hiroshi Kondo from the Tokyo Metropolitan Institute of Gerontology came on sabbatical to NIA to examine the migration of fibroblasts in vitro in response to treatment with sera from different age and diet groups.

As a general summary of these early studies on cellular and molecular biomarkers of aging, we can say that most of these assays proved to have significant, yet modest, age sensitivity; however, none of them could distinguish clearly the effects of CR. We concluded at that time that the monkeys had not been on the CR regimen sufficiently long enough to significantly retard markers of aging. Additionally because we observed significant trends in some of the assays denoting possible CR effects, we were concerned that the study was not sufficiently powered for many of these assays in which substantial variability was observed, making many of the age effects not so remarkable. Indeed, some notable exceptions to observing significant age effects were studies on telomere length in lymphocytes and measures of oxidative stress, specifically 8-Oxo-2′-deoxyguanosine and oxidized proteins. Instead, what we did observe in early studies were age and diet effects in several simpler assays. A few of these findings can be described as follows:

Skeletal Growth and Health. In collaboration with Abraham Reznick from the Technion-Israel Institute of Technology and Sheldon Ball from the University of Mississippi, we were able to show that CR slowed the rate of skeletal growth in monkeys without affecting measures of bone health [30]. This study was made possible with the new bone scanning technology dual-beam X-ray absorptiometry (DEXA) that was previously acquired with funds secured by George Martin. However, the most dramatic effect was observed in serum measures of alkaline phosphatase, which is a marker of active bone growth. The marked decline in alkaline phosphatase during early years was attenuated about a year in the juvenile monkeys on CR. Rather than emerging as a measure of aging, however, this difference more likely reflected a slower rate of skeletal development in the CR group.

IGF-1. Collaborating with Daniela Cocchi from the University of Bari, Italy, we analyzed IGF-1 levels in serum and found lower levels in the young groups of CR monkeys consistent with findings in rodents [31]. This study had only one sample timed to best ensure we hit the highest peak given the circadian rhythmicity of this hormone. This issue was later resolved in the studies conducted at the ONPRC that employed continuous blood sampling.

DHEA. One of the most highly age-sensitive parameters that could be measured in a simple blood test was dehydroepiandrosterone (DHEA). This adrenal steroid, particularly its sulfated form (DHEAs), exhibits marked decline in monkeys and humans past puberty. Thus, we were very excited to report that the age-related decline in DHEAs appeared to be significantly attenuated in monkeys on CR [32]. Unfortunately we would have to refine that finding in later reports published in collaboration with Henryk Urbanski at the ONPRC [33]. Henryk had cautioned us that DHEA had a circadian rhythm in its secretion, so that our one-time sample might not represent the true picture of diet effects. Thus, in later studies conducted in Oregon using small groups of monkeys with indwelling catheters to permit continuous blood sample monitoring, we were able to replicate the age-related decline in DHEA levels but not the significant attenuation of this decline in CR monkeys. In fact, old-onset CR significantly reduced DHEAs compared to age-matched controls [34].

Lipids. Among the first of several high visibility papers to emerge from the study was the report published in the *American Journal of Physiology* in collaboration with Roy Verdery at the University of Arizona [35]. Roy's lab had been developing assays to look at specific fractions of high density lipoproteins (HDL). The main findings reported were that CR reduced plasma levels of triglycerides in the younger monkeys and elevated levels of HDL 2b, which was a fraction that had been associated with reduced risk for cardiovascular disease.

Body Temperature and Energy Expenditure. Another paper reporting on a straightforward physiological parameter published in a prestigious journal (*Proceedings of the National Academy of Science*) was conducted in collaboration with William Rumpler and David Baer of the Department of Agriculture, Beltsville, Maryland [36]. Bill and Dave were experts in large animal metabolism and assisted us with measures of body temperature and whole body calorimetry. For monkeys involved in the long-term study, temperature data were recorded from rectal probes

during the quarterly examinations. However, this recording was obtained while the monkeys were lightly anesthetized, a situation which could affect the measurements. Fortunately, new technology became available permitting continuous collection of body temperature data from subcutaneous sensors. The young acute males were fitted with the implanted sensors. We considered this too invasive for the long-term monkeys. Based on the rodent literature, we expected to observe generally lower body temperature in monkeys on CR to indicate a diet-induced shift in metabolism. In both the long-term (rectal temperatures) and acute monkeys (implanted sensors), this prediction was confirmed, and thus, another important biomarker of the CR phenotype was established in the nonhuman primate model. This observation gained additional significance after we analyzed several key biomarkers of CR in the large human study at the NIA, known as the Baltimore Longitudinal Study of Aging (BLSA), in collaboration with our colleagues at GRC, Jeffrey Metter, Dennis Muller, and Jordan Tobin. In a paper published in Science 2002, we reported that healthy men who maintained lower body temperature had a significantly lower risk of mortality [37].

Using whole body calorimeters built with the assistance of Rumpler and Baer, we were also able to measure metabolism during the phase-in of CR in the acute monkeys. Over the course of 3 months of decreasing diet allotments by 10 % every 4 weeks, we observed a steady decline in energy expenditure (kcal/day/kg). Indeed, a significant reduction of about 24 % was recorded after implementation of 30 % CR for 2 months, confirming another key biomarker of CR that was predicted from the rodent literature. Moreover, again using data from the BLSA, Luigi Ferrucci who is the current Scientific Director of NIA, and his colleagues would confirm that healthy individuals with lower basal metabolic rates had a lower probability of dying [38] and a better functional health status [39].

Employing the indwelling sensors which recorded body temperature, we could also obtain telemetered data on locomotor activity and heart rate in this small group of monkeys. Examining the data after 4 months of study, we saw clear trends towards increased locomotor activity and decreased heart rates in the CR monkeys, but these did not reach a level of statistical significance, given the small samples and the variability obtained in these measures. Thus, we did not substantiate the predictions from the rodent literature for these two key biomarkers of CR.

Locomotor Activity. Shortly after these efforts were initiated, we also began to examine locomotor activity in the first cohorts of monkeys on long-term CR for about 6 years. To meet this need, we employed the expertise of the GRC fabrication shop, specifically, Guenter Baartz and Ray Banner, who helped to design a motion detection device based on an infrared sensor that was used as a commercial burglar alarm. When mounted onto the cage, the system could reliably detect gross movements of the monkeys. Additionally, we were fortunate to bring on board as a post-doctoral fellow, James Weed, who had extensive experience in primate behavior. While helping with the locomotor project, Jim also led the effort to train monkeys to present their arms for venipuncture, thus reducing the need for anesthesia and the stress associated with the procedure.

The home-made apparatus proved to be very useful, as we could detect circadian rhythms in the monkey's activity [40]. There was a clear age-related decline in activity, as well as enhanced activity in the CR monkeys as compared to controls and predicted from the rodent literature.

Thyroid Hormones. Another key biomarker of CR is the response of the thyroid axis. We had made several attempts to get reliable measures from plasma over the years; however, specific antibodies for several rhesus thyroid hormones took a while to develop. The clinical core at the Yerkes Regional Primate Center was one of the first places to perfect such assays, so we recruited its help. To this end, we reported in 2002 that plasma levels of thyroxin (T4), and thyroid-stimulating hormone (TSH), but not triiodothyronine (T3), declined significantly with age [41]. However, regarding the effects of CR on the thyroid axis, T3 was significantly reduced in both long-term and acute CR monkeys. The latter group of male monkeys was 4 years old and 20 years old, respectively, at introduction of CR. The CR-induced reduction in T3, but not T4 or TSH, was clearly observed during the phase-in of CR from 0 to 30 % over the course of 3 months in the younger monkeys, but the diet effect did not achieve statistical significance in the older group. Thus, again we had confirmed another key metabolic marker in monkeys in response CR. This observation was later confirmed in healthy humans undergoing 25 % CR [42]. Additionally, Ferrucci and colleagues confirmed that subclinical hyperthyroidism was associated with reduced physical function in a longitudinal analysis [43]. Baranowska et al. [44] also reported lower levels of T3 in their sample of centenarians.

Glucose and Insulin. Other notable simple biomarkers of CR in rodents are decreased fasting blood levels of glucose and insulin. Our analysis of these markers did not get off to a good start as it turned out that their measurement was not as simple as first considered. Specifically, in a 1992 report collaborating with Barbara Davis from the University of Rochester, we noted no significant age or diet effects in plasma concentrations of glucose or percent glycosylated hemoglobin in rhesus or squirrel monkeys [45]. Also, when measuring insulin in rhesus monkeys (no antibody for squirrel monkey insulin was available at that time), we observed no significant age or diet effects. The major reason for these negative effects was due to the huge variability in the data. We soon realized that major changes were needed in the procedures for obtaining the blood samples. To this end, under the supervision of Mark Lane, new standard operating procedures (SOPs) were established to assure uniformity in how the monkeys were treated on the day of the blood draws, procedures of anesthesia and standardizing times between anesthesia and blood draws. Thus, in a subsequent analysis published in 1995 with the SOPs in place, we reported significant reductions in glucose and insulin in monkeys on CR begun at young ages; however, no diet effects in glycosylated hemoglobin were noted [46]. Additionally, in a carefully conducted glucose tolerance test, responses of glucose and insulin were significantly improved in the CR monkeys. On the issue of how reliable fasting plasma glucose levels are as a biomarker of CR, we should note that a recent analysis of the NIA dataset reported in *Nature* that involved 1,260 observations for 81 monkeys over their lifetime, no significant diet effect was

observed [47]. Over the years we have evolved a clear appreciation that fasting insulin levels or the response of glucose to insulin (insulin tolerance test) are much better biomarkers of CR than are static glucose measures. To this point, we noted that like body temperature, lower serum insulin measures were predictive of lower mortality in healthy men from the BLSA [37].

Index of Biological Age. Beyond the evaluation of individual biomarkers of CR and aging, we had recognized from the outset of the study the need to pursue a multivariate analysis, in effect, to construct an index of biological age. Although the rationale for this approach was based on past, generally unsuccessful efforts to devise multivariate indices of biological age (for reviews see [48, 49]), we were ready with a fresh new approach to this challenge. The main recognition was that reliance upon individual candidate biomarkers would not provide evidence of a "global" effect on aging, that is, affecting several organ and physiological systems. To assist in this effort, we were fortunate to be contacted by Eitaro Nakamura from the Kyoto University, Japan, who expressed an interest in spending a sabbatical at NIA. Eitaro had already published a couple of papers using multivariate analysis to construct indices of physical fitness and biological age (e.g. [50]).

After Eitaro's arrival at the GRC in 1992, we reconsidered the logic behind developing an index of biological age in rhesus monkeys. For this first effort we relied upon the growing dataset being generated from the routine blood chemistry and hematology collected during quarterly exams. Building off the story presented in the first report for the study [28], we could take advantage of the 5 years of longitudinal data that had accumulated. Without rehashing the details of our approach, we can relate that we carefully defined the major objectives that could be directed toward identifying individual candidate biomarkers of aging and using these to construct a biological age score [51]. The expressed criteria for a candidate biomarker of aging were as follows: (1) it should reveal significant correlations with chronological age (CA) when viewed both cross-sectionally and longitudinally; (2) the individual differences observed in a biomarker should be stable across time, e.g. the variability observed should be genuine rather than due to random measurement error. Thus, from the battery of dozens of measurements, we found that 6 variables met these criteria. The next step was to submit these variables to a principle component analysis to examine their underlying relationships. To our satisfaction, we found that the selected variables loaded onto a single component that accounted for over 50 % of the total variance, which strongly supported their underlying relationship. With this information we could then apply the factor score coefficients from the first principle component to construct an equation to be used for constructing a biological age score (BAS) for each individual monkey. When we compared the rate of change in BAS between control and CR monkeys, we found no statistically significant difference; however, the slope of the regression of BAS onto CA appeared steeper for the control group compared to the CR group. Thus, while we were unable to detect a diet effect on the rate of aging, we had established a strategy by which additional biomarkers of aging could be identified and evaluated. In a subsequent analysis using the same strategy with 7 years of longitudinal

data, we again could not detect a significant diet effect, but we were able to confirm the logic and power of this approach [52].

A major challenge hindering progress in developing biomarkers of aging is the lack of consensus of their validation. What we proposed as a logical approach to validation was to compare the slopes of age-related change in an individual candidate computed from a regression analysis across related species, in this case, nonhuman primates [48]. The logic was as follows: If a candidate biomarker is a valid measure of the rate of aging, then the rate of age-related change in the biomarker should be proportional to differences in lifespan among related species. For example, the rate of change in a candidate biomarker of aging in chimpanzees should be twice that of humans (60 vs. 120 years maximum lifespan); in rhesus monkeys three times that of humans (40 vs. 120 years maximum lifespan). To advance this strategy, we realized that we needed to establish a huge primate aging database that would foster collaboration across various primate centers that would permit assembly of their databases on aging into a central database that could be accessed by qualified investigators. With the assistance of Joseph Kemnitz at the University of Wisconsin and Nancy Nadon of the NIA extramural staff, we began conversations with several investigators with access to such databases. Over several years, these conversations jelled into a NIA-sponsored program to construct and maintain an internet based primate aging database (iPAD), which was created under the auspices of the Wisconsin National Primate Research Center (www.ipad.primate.wisc.edu). Despite this major effort, we can regrettably report to date that little progress has been made and no consensus has been established about how to identify and validate biomarkers of aging.

Summary. Within 10 years of initiating the study, we were able to confirm evidence based on several different parameters that a CR phenotype had been produced in our monkey study. These included attenuation of skeletal growth, reduced levels of IGF-1, triglycerides, body temperature, T3, and insulin and increased energy expenditure, locomotor activity, and glucose tolerance. We had also proposed a strategy for constructing a multivariate index of biological age. What we had not accomplished within that first decade of work was to generate believable evidence that CR had attenuated the rate of aging in the monkeys. Given the young ages of most of the monkeys involved, the data emerging on morbidity and mortality was still limited with no clear picture emerging at that time.

11.6 Disappointments and Dead-Ends

We cannot provide the entire list of the assays that we attempted to develop ourselves or with the help of collaborators. As with any long-term scientific project, many efforts just do not yield success, sometimes due to problems with the assays, other times due to problems with the collaborators, and also because often due to the results, i.e. they did not support the hypothesis, in this case, were not age-sensitive.

Cortisol. A major problem we encountered early on was the measurement of plasma levels of cortisol. As increased levels of glucocorticoids were a well-established biomarker in CR rodents, we were determined to evaluate this hormonal parameter [53]. As mentioned earlier, we tried to standardize the method for blood collection, but the results from our assays yielded enormous variability. We looked into other methods for obtaining samples. For example, we looked into the possibility of presenting the monkeys with flavored cotton balls, retrieving these, and then measuring cortisol in the salvia. Again we found the data to be highly variable. Even later attempts to measure levels in hair samples also proved unreliable. The problem was partially solved by having blood samples obtained in the monkeys shipped to the ONPRC.

Nail Growth. A second disappointment was a simple measure of nail growth. This parameter had been suggested as a simple in vivo biomarker of aging that measured the rate of cellular proliferation [54]. Again, after considerable efforts standardizing an assay in which a small mark was made on the nail of the index finger, and measured weekly across several weeks, we found no reliable age sensitivity to the assay, and thus, abandoned further work.

Wound Healing. Another simple assay that we wanted to develop was wound healing [55]. The time required for a standardized wound to heal was being considered as a biomarker of aging. Similar to the presumed mechanism in nail growth, a reduced rate of wound healing was considered to reflect impaired cell proliferation in addition to reduced cytokine signaling to recruit proteins involved in the healing process. We carefully constructed a standard wound healing protocol for both rats and monkeys in which a punch wound was made on the back of the animal, and then the closure of the wound was mapped by photographic images over the course of a few weeks. While we noted significant age-related decline in the rate of wound healing in both rats and monkeys, we found no significant effect of diet. Later studies noted that rodents on CR did show superior wound-healing when returned to the control diets [56].

11.7 Increased Exposure and Publicity for the Study

Because of NIA press releases generated about the first lipid and body temperature papers in the mid-1990s, the study began to receive a great deal of publicity in the popular press. In addition to reports in many leading newspapers and news magazine, we had several interviews on national radio and television programs, including an appearance of GSR on the Today Show on NBC.

Of course, publicity can be a two-edged sword, and our brief contentment with this positive exposure for the study was soon tempered by inquiries from People for the Ethical Treatment of Animals (PETA), who became concerned about the treatment of the monkeys. One of us (GSR) spoke to representatives of this organization a few times on the phone in amicable enough terms that we felt they had

been reassured of our commitment to the highest quality animal care and welfare. Shortly later we were advised by NIH to refer all such calls to specialized administrative personnel in the future. In fact, we were asked to meet with the NIH Director of Security to assure that the Poolesville facility would be safe in the event of any PETA demonstrations. These later did occur on the main Bethesda, Maryland campus in regard to other research projects unrelated to ours, as well as at the University of Wisconsin, but Poolesville and our own study were spared, perhaps because of the relatively remote location (in retrospect, a good geographical choice at least).

11.8 Moving Forward

Because the study we are describing is still on-going, we are not providing an up-to-date history in this chapter. Rather our objective was to focus on the early years, essentially the first decade of the study. Thus, the complete history of this long-running study will remain to be written. Nonetheless, we would like to mention a few key administrative and scientific issues which helped to shape the study going forward after the first decade.

Administrative Oversight. From the beginning of the project, including approval for its inception, oversight was provided by the NIA Board of Scientific Counselors (BSC). This is a panel of distinguished scientists appointed by the Director of NIA to provide a formal review of every program within the IRP at least once every 4 years. While not strictly bound by their recommendations, the Director and Scientific Director of NIA usually abide closely to the report of the BSC. Bernadine Healy had appointed Richard Hodes as NIA Director in 1993. George Martin was the Scientific Director. Following a review of the monkey project around 1994, the BSC advised Dr. Hodes that the study should have its on oversight committee to review the project on an annual basis. Although their findings and recommendations were generally positive and constructive, the BSC apparently felt that the CR project was too large, complex, and important to be conducted without additional, specialized oversight. Consequently a Scientific Advisory Committee (SAC) was organized consisting of about five members to whom we would report annually. In turn, they would provide feedback in the form of a report to the Scientific Director and Director. We were extremely fortunate to have Byung Pal Yu appointed as first chair of the SAC. Dr. Yu had extensive experience in the CR paradigm and provided steady leadership. Following his retirement, another renowned biogerontologist and expert in the CR paradigm, Arlan Richardson, agreed to chair the SAC. Over the years, many eminent scientists volunteered their time to serve on the SAC, including David Allison, Mark Beasley, Lauri Brignolo, Harvey Cohen, Vincent Cristofalo, Robert Good, John Holloszy, George Martin, Edward Masoro, Simin Meydani, Olivia Pereira-Smith, Robert Sapolsky, Jay Segmiller, John Sorkin, Mary Lou Voytko, and Norman Wolf.

While the SAC would generate many constructive suggestions, we can cite two recommendations that would have major impact on the study. One clear recommendation emerged around the 10-year anniversary of the study, which was to abandon the objective of testing the hypothesis that CR would retard aging based on the assessment of biomarkers of aging. While we had outlined a clear strategy for developing biomarkers of aging to apply to our study, many biogerontologists had begun to lose considerable faith in this approach. This loss of faith seemed to emerge from disappointments expressed in the field regarding the failure to replicate results from initial reports touting the potential of several molecular biomarkers of aging. Given the resources that had been invested in our study and the expressed commitment by the NIA, the SAC recommended that we go forward with the study to assess morbidity, mortality, and function. They concluded that the field would have much more faith in this type of evidence than relying on a collection of individual biomarkers, whether presented individually or in some multivariable index. Related to this issue, the SAC was also concerned about the impact that continual, oftentimes invasive, assays could have on the health of the monkeys.

Given this new mandate to focus on morbidity, mortality, and function, we had to consider the issue of statistical power for the study. To this end, we convened a workshop to address statistical power that involved several eminent biogerontologists as well as biostatisticians. The result of that workshop was a consensus recommendation to double the size the study, which was around 120 monkeys at the time. The power calculations indicated that we would need this sample size for detecting a 2-year difference in median survival with a power of 80 % and alpha level of 0.05. To this end, over the period of a couple of years in the late 90s, we went through the rounds of proposing this expansion to the SAC, the BSC, and relevant committees at the NIA. We also spent an enormous amount of time tracking down monkeys that could be purchased for this expansion and considered the required housing at Poolesville. Unfortunately, after getting approval for this expansion through all the required channels, Dr. Hodes did not give his final approval for reasons apparently related to budgetary issues at the time. Thus, we were now moving forward with an objective of examining the effects of CR on mortality in a study that some might consider underpowered.

Expanding the Collaborator Base. Another major recommendation of the SAC was to expand our collaborator base to take advantage of this valuable research resource. With the assistance of Nancy Nadon, Frank Bellino, Huber Warner, and Evan Hadley from the NIA extramural staff, we were able to secure a new pool of money from the Planning and Contracts Committee to construct a "Request for Applications." This RFA called for proposals to utilize the NIA study to address important hypotheses related to the CR paradigm. Beginning in 2002, a competitive process was organized to solicit proposals that were reviewed by a Special Emphasis Panel for which we had no formal approval authority. We could comment on the feasibility of each proposal regarding demands on our resources, but we did not rate the science. After a long process, the following proposals and collaborations (and attendant projects) were established and were formally launched around 2004:

1. Patricia Kramer, University of Washington: *Osteoarthritis and Calorie Restriction*
2. Janko Nikolich-Zugich, Oregon Health Sciences University: *Caloric Restriction and Immune Senescence*
3. Charleen Moore, University of Texas Health Science Center, San Antonio: *Calorie Restriction Effects on Chromosomal Aberrations*
4. John Novak, University of Kentucky, and Mark Reynolds, University of Maryland. *Aging: Effects on Infection, Inflammation and Disease*
5. Mary Zelinski-Wooten, Oregon Health Sciences University, and Mary Ann Ottinger, University of Maryland: *Ovarian Aspects of Caloric Restriction*

Most of these projects evolved into productive collaborations and generated important reports of the findings. We will not comment on these at this time.

Statistical Support. Another recommendation by the SAC was that we should have stronger biostatistical support. To this end, collaboration was secured with David Allison, who was at the time at Columbia University and then later moved to the University of Alabama Birmingham, where he established the Section on Statistical Genetics. Along with Mark Beasley from his section, we began to rely greatly on their assistance for statistical analysis. David and Mark were exceptionally innovative biostatisticians, but we were greatly aided by David's extensive background and knowledge of obesity which had evolved into a keen research interest and career in biogerontology, including CR.

Cooperation with the University of Wisconsin. Given similar objectives and the role of Richard Weindruch in the early years our study, we maintained a close working relationship with UW. Besides Rick Weindruch and Bill Ershler, the other PIs there included Joseph Kemnitz and Ricki Colman. Additionally, David Allison and Mark Beasley also provided statistical support for this study. Over the years we held joint meetings involving staff from both studies to discuss ideas for collaboration, which yielded several reports.

The UW study has been described in detail elsewhere [57, 58]. The major differences between the two studies were that the UW study employed a purified diet and was started in cohorts of adult monkeys (8–14 years) in which their food intake had been monitored prior to the onset of CR. Of course, a major scientific difference that emerged over the past few years is that UW investigators have reported that monkeys on CR in their study show a statistically significant increase in survival compared to their control group [59, 60]; whereas, NIA investigators have reported that no statistically significant CR effect on survival is currently observed nor is likely to be observed in the future [47]. Possible explanations of the reported difference in survival effects were addressed in the recent UW publication [58]. As other reports are currently being assembled to further explore this question, we will not address this issue in this chapter. What we can say is that our clear impression of how best to explain the differences in survival observed in the two studies is to focus on the composition of diet provided to the UW monkeys and how

the body composition and disease profile they have reported differ dramatically from that observed in the NIA study.

Changes in the Scientific Staff. Major additions to the scientific staff occurred in early 2000s as well, which made the RFA studies more manageable. First, another of the authors, Julie A. Mattison (JAM), was recruited as a post-doctoral fellow. JAM had trained with Andrzej Bartke at Southern Illinois and thus was very familiar with the biology of aging and the CR paradigm. Rising quickly through several responsibilities, JAM essentially became the project manager upon the departure of Mark Lane in 2002. Second, another author, Rafael de Cabo (RC), was recruited from Purdue University as a post-doctoral fellow, first in the laboratory of Mark Lane and then in the laboratory of DKI after Mark's departure in 2002. RC became immediately involved in many CR studies, primarily focused first on rodent models, but later became very involved in the primate study. Mary Ann Ottinger joined the study in 2002 while on sabbatical from the University of Maryland and brought needed expertise in studying the reproductive axis in female monkeys. GSR retired from NIA in 2000, but has maintained contact with the study since then. One of the primary reasons for his departure was to head up a start-up biotech in Maryland, GeroScience, Inc., that was focused on developing CR mimetics. DKI retired from NIA in 2006 and joined the faculty of the Pennington Biomedical Research Center at Louisiana State University, and has also maintained contact with the study. Following the departure of DKI from NIA in 2006, the study has been capably supervised by JAM and RC, the latter having advanced to Chief of the Translational Gerontology Branch.

11.9 Epilogue

Our objective for this chapter was to document events and decisions that guided development of the first formal trial evaluating the effects of CR on aging in a primate species. As such, we have not provided an exhaustive review of all the studies conducted and findings reported as this would exceed the page limits under which we are operating. Instead, what we have described relates generally to the years of planning preceding the study and the first decade of work after its initiation. We have also described other important activities that affected the operation of this long-running study. We trust there will be opportunities in the future to fully document and summarize the findings, in particular, how the different results, pertaining to effects of CR on mortality obtained between the University of Washington and NIA, might be explained.

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References

1. Young VR (1979) Diet as a modulator of aging and longevity. Fed Proc 38(6):1994–2000
2. Masoro EJ, Yu BP, Bertrand HA, Lynd FT (1980) Nutritional probe of the aging process. Fed Proc 39(14):3178–3182
3. Osborne TB, Mendel LB, Ferry EL (1917) The effect of retardation of growth upon the breeding period and duration of life in rats. Science 45:294–295
4. McCay CM, Crowell MF, Maynard LA (1935) The effect of retarded growth upon the length of life and upon the ultimate body size. J Nutr 10:63–79
5. McCay CM (1958) Nutritional experiments on longevity. J Am Geriatr Soc 6(3):171–181
6. Pope F, Lunsford W, McCay CM (1956) Experimental prolongation of the life span. J Chronic Dis 4(2):153–158
7. Harrison DE, Archer JR (1989) Natural selection for extended longevity from food restriction. Growth Dev Aging 53(1–2):3
8. Phelan JP, Rose MR (2006) Caloric restriction increases longevity substantially only when the reaction norm is steep. Biogerontology 7(3):161–164
9. Shanley DP, Kirkwood TB (2006) Caloric restriction does not enhance longevity in all species and is unlikely to do so in humans. Biogerontology 7(3):165–168
10. Leto S, Kokkonen GC, Barrows CH (1976) Dietary protein life-span, and physiological in female mice. J Gerontol 31(2):149–154
11. Barrows CH Jr, Kokkonen G (1978) The effect of various dietary restricted regimes on biochemical variables in the mouse. Growth 42(1):71–85
12. Goodrick CL (1978) Body weight increment and length of life: the effect of genetic constitution and dietary protein. J Gerontol 33(2):184–190
13. Goodrick CL, Ingram DK, Reynolds MA, Freeman JR, Cider NL (1982) Effects of intermittent feeding upon growth and lifespan in rats. Gerontology 28:233–241
14. Levin P, Janda JK, Joseph JA, Ingram DK, Roth GS (1981) Dietary restriction retards the age-associated loss of rat striatal dopaminergic receptors. Science 214:561–562
15. Joseph JA, Whitaker J, Roth GS, Ingram DK (1983) Lifelong dietary restriction affects striatally-mediated behavioral responses in aged rats. Neurobiol Aging 4:191–196
16. Weindruch R, Walford RL (1988) The retardation of aging and disease by dietary restriction. Charles C Thomas, Springfield
17. Roth GS (2005) The truth about aging, can we really live longer and healthier? Windstorm Creative, Port Orchard
18. Mehta LH, Roth GS (2009) Caloric restriction and longevity: the science and the ascetic experience. Ann NY Acad Sci 1172:28–33
19. Short R, Williams DD, Bowden DM (1987) Cross-sectional evaluation of potential biological markers of aging in pigtailed macaques: effects of age, sex, and diet. J Gerontol 42(6):644–654
20. Ortmeyer HK, Bodkin NL, Hansen BC (1994) Chronic calorie restriction alters glycogen metabolism in rhesus monkeys. Obes Res 2(6):549–555
21. Kealy RD, Lawler DF, Ballam JM, Mantz SL, Biery DN, Greeley EH, Lust G, Segre M, Smith GK, Stowe HD (2002) Effects of diet restriction on life span and age-related changes in dogs. J Am Vet Med Assoc 220(9):1315–1320
22. Ludwig FC, Masoro EJ (1983) The measurement of biological age. Exp Aging Res 9:219–255
23. Baker GT 3rd, Sprott RL (1988) Biomarkers of aging. Exp Gerontol 23(4–5):223–239
24. Ingram DK (1988) Key questions in developing biomarkers of aging. Exp Gerontol 23:429–434
25. Adelman R, Saul RL, Ames BN (1988) Oxidative damage to DNA: relation to species metabolic rate and life span. Proc Natl Acad Sci USA 85(8):2706–2708
26. Monnier VM, Cerami A (1981) Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. Science 211(4481):491–493
27. Nagy I, Floyd RA (1984) Electron spin resonance spectroscopic demonstration of the hydroxyl free radical scavenger properties of dimethylaminoethanol in spin trapping

experiments confirming the molecular basis for the biological effects of centrophenoxine. Arch Gerontol Geriatr 3(4):297–310
28. Ingram DK, Cutler RG, Weindruch R, Renquist DM, Knapka JJ, April MA, Belcher CT, Clark MA, Hatcherson CD, Marriott BM, Roth GS (1990) Dietary restriction and aging: the initiation of a primate study. J Gerontol A Biol Sci Med Sci 45:B148–B163
29. Barnard D, Knapka J, Renquist D (1988) The apparent reversal of a wasting syndrome by nutritional intervention in Saquinus. J Lab Anim Sci 38:42–56
30. Lane MA, Reznik A, Tilmont EM, Lanir A, Ball SS, Ingram DK, Cutler RG, Roth GS (1995) The influence of aging and dietary restriction on bone metabolism in male rhesus (*Macaca mulatta*) monkeys. J Nutr 125:1600–1610
31. Cocchi D, Cattaneo L, Lane MA, Ingram DK, Cutler RG, Roth GS (1995) Effects of long-term dietary restriction in the somatotrophic axis in adult and aged monkeys. Neuro Endocrinol Lett 17:181–186
32. Lane MA, Ingram DK, Ball SS, Roth GS (1997) DHEAS: a biomarker of primate aging slowed by calorie restriction. J Clin Endocrinol Metab 82:2093–2096
33. Urbanski HF, Downs JL, Garyfallou VT, Mattison JA, Lane MA, Roth GS, Ingram DK (2004) Effect of caloric restriction of the 24-hour plasma DHEAS and cortisol profiles of young and old male rhesus monkeys. Ann NY Acad Sci 1019:443–447
34. Urbanski HF, Mattison JA, Roth GS, Ingram DK (2013) Dehydroepiandrosterone sulfate (DHEAS) as an endocrine marker of aging in calorie restriction studies. Exp Gerontol 48 (10):1136–1139
35. Verdery RB, Ingram DK, Roth GS, Lane MA (1997) Caloric restriction increases HDL2b levels in rhesus monkeys (*Macaca mulatta*): a mechanism for extending the life span by preventing atherosclerosis. Am J Physiol 273:E714–E719
36. Lane MA, Baer DJ, Rumpler WV, Weindruch R, Ingram DK, Cutler RG, Roth GS (1996) Dietary restriction lowers body temperature in rhesus monkeys, consistent with a postulated anti-aging mechanism in rodents. Proc Natl Acad Sci USA 93:4159–4164
37. Roth GS, Lane MA, Ingram DK, Mattison J, Elahi D, Tobin J, Muller D, Metter EJ (2004) Biomarkers of caloric restriction may predict longevity in humans. Science 297:881
38. Ruggiero C, Metter EJ, Melenovsky V, Cherubini A, Najjar SS, Ble A, Senin U, Longo DL, Ferrucci L (2008) High basal metabolic rate is a risk factor for mortality: the Baltimore Longitudinal Study of Aging. J Gerontol A Biol Sci Med Sci 63:698–706
39. Schrack JA, Knuth ND, Simonsick EM, Ferrucci L (2014) "IDEAL" aging is associated with lower resting metabolic rate: the Baltimore Longitudinal Study of Aging. J Am Geriatr Soc 62:667–672
40. Weed JL, Lane MA, Roth GS, Speer D, Ingram DK (1997) Activity measures in rhesus monkeys on long-term calorie restriction. Physiol Behav 62:97–103
41. Roth GS, Handy AM, Mattison JA, Tilmont EM, Ingram DK, Lane MA (2002) Effects of dietary caloric restriction and aging on thyroid hormones of rhesus monkeys. Horm Metab Res 34:378–382
42. Heilbronn LK, de Jonge L, Frisard MI, DeLany JP, Larson-Meyer DE, Rood J, Nguyen T, Martin CK, Volaufova J, Most MM, Greenway FL, Smith SR, Deutsch WA, Williamson DA, Ravussin E, Pennington CALERIE Team (2006) Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA 295:1539–1548
43. Ceresini G, Ceda GP, Lauretani F, Maggio M, Bandinelli S, Guralnik JM, Cappola AR, Usberti E, Morganti S, Valenti G, Ferrucci L (2011) Mild thyroid hormone excess is associated with a decreased physical function in elderly men. Aging Male 14:213–219
44. Baranowska B, Wolinska-Witort E, Bik W, Baranowska-Bik A, Martynska L, Broczek K, Mossakowska M, Chmielowska M (2007) Evaluation of neuroendocrine status in longevity. Neurobiol Aging 28:774–783
45. Cutler RG, Davis BJ, Ingram DK, Roth GS (1992) Plasma concentrations of glucose, insulin, and percent glycosylated hemoglobin are unaltered by food restriction in rhesus and squirrel monkeys. J Gerontol 47:B9–B12

46. Lane MA, Ball SS, Ingram DK, Cutler RG, Engel J, Read V, Roth GS (1995) Diet restriction in rhesus monkeys lowers fasting and glucose-stimulated glucoregulatory endpoints. Am J Physiol Endocrinol Metab 31:E941–E948
47. Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, Longo DL, Allison DB, Young JE, Bryant M, Barnard D, Ward WF, Qi W, Ingram DK, de Cabo R (2012) Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature 489(7415):318–321
48. Ingram DK, Nakamura E, Smucny D, Roth GS, Lane MA (2001) Strategy for identifying biomarkers of aging in long-lived species. Exp Gerontol 36:1025–1034
49. Ingram DK (2011) Biomarkers of aging: from marking time to moving forward. Public Policy Aging Rep 20:18–27
50. Nakamura E, Moritani T, Kanetaka A (1989) Biological age versus physical fitness age. Eur J Appl Physiol Occup Physiol 58:778–785
51. Nakamura E, Lane MA, Roth GS, Cutler RG, Ingram DK (1994) Evaluating measures of hematology and blood chemistry in male rhesus monkeys as biomarkers of aging. Exp Gerontol 29:151–177
52. Nakamura E, Lane MA, Roth GS, Ingram DK (1998) A strategy for identifying biomarkers of aging: further evaluation of hematology and blood chemistry data from a calorie restriction study in rhesus monkeys. Exp Gerontol 5:421–443
53. Sabatino F, Masoro EJ, McMahan CA, Kuhn RW (1991) Assessment of the role of the glucocorticoid system in aging processes and in the action of food restriction. J Gerontol 46(5): B171–B179
54. Williams DD, Short R, Bowden DM (1990) Fingernail growth rate as a biomarker of aging in the pigtailed macaque (*Macaca nemestrina*). Exp Gerontol 25(5):423–432
55. Roth GS, Kowatch MA, Hengemihle J, Ingram DK, Spangler EL, Johnson LK, Lane MA (1997) Effect of age and caloric restriction on cutaneous wound closure in rats and monkeys. J Gerontol A Biol Sci Med Sci 52A:B98–B102
56. Reed MJ, Penn PE, Li Y, Birnbaum R, Vernon RB, Johnson TS, Pendergrass WR, Sage EH, Abrass IB, Wolf NS (1996) Enhanced cell proliferation and biosynthesis mediate improved wound repair in refed, caloric-restricted mice. Mech Ageing Dev 89(1):21–43
57. Kemnitz JW, Weindruch R, Roecker EB, Crawford K, Kaufman PL, Ershler WB (1993) Dietary restriction of adult male rhesus monkeys: design, methodology, and preliminary findings from the first year of study. J Gerontol 48(1):B17–B26
58. Ramsey JJ, Colman RJ, Binkley NC, Christensen JD, Gresl TA, Kemnitz JW, Weindruch R (2000) Dietary restriction and aging in rhesus monkeys: the University of Wisconsin study. Exp Gerontol 35(9–10):1131–1149
59. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R (2009) Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 325(5937):201–204
60. Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R, Anderson RM (2014) Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat Commun 5:3557

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