Phytochemicals

Health Promotion and Therapeutic Potential

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Edited by

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This book is dedicated to the memory of Dr. James A. Joseph, a coauthor of Chapter 10, who passed away during the writing of the manuscript. Dr. Joseph was a particularly engaging presenter at the conference in 2008, speaking on the mitigation of oxidative stress and inflammatory signaling by berry and walnut polyphenols. During his career, he contributed significantly to the field, specifically in the area phytochemicals and neuroscience. His research has increased our understanding of the diet's impact on cognitive aging and his promotion of the benefits of a diet rich in color left a greater impact on the American consumer. Dr. Joseph was a valued friend and colleague to many and, although he is missed, the legacy of his work has left a lasting impact.

Contents

Preface

This book is the seventh in a series that represents the emerging science with respect to plant-based chemicals. Phytochemical research continues to expand tremendously as advancing technology allows for greater identification and characterization of these chemicals. While promising scientific advancements have been made in this field, numerous areas of opportunity still exist, surrounding topics such as bioavailability, efficacy, genomics, and synergistic mechanisms. The preceding volumes of this book, comprising of *Phytochemicals: A New Paradigm* (1998), *Phytochemicals as Bioactive Agents* (2000), *Phytochemicals in Nutrition and Health* (2002), *Phytochemicals: Mechanisms of Action* (2004), *Phytochemicals: Nutrient–Gene Interactions* (2006), and *Phytochemicals: Aging and Health* (2008), discuss the foundation and influential factors behind phytochemical research over the past few decades.

Increasing knowledge around the various protective effects of phytochemicals has sparked a vast interest in further understanding their highly complex biological mechanisms. Alongside the efforts to refine our knowledge, additional framework has been established as more discoveries are being made and technology becomes more sophisticated. Furthermore, fundamental phytochemical research is being expanded in an effort to find opportunities for clinical benefits and lifestyle recommendations. This current book in the series evolved from papers delivered at the Seventh International Phytochemical Conference, "Phytochemicals: Health Promotion and Therapeutic Potential," held in October 2008 on the Nutrilite Health Institute campus in Buena Park, California. This conference was a collaborative effort with the Department of Human Nutrition and Food Science at California State Polytechnic University, Pomona, and included presentations from leading scientists in the areas of phytochemicals with the following associations: bone and joint health, brain health, obesity and metabolism, skin health, and chronic disease prevention. Presenters later contributed chapters for this compilation along with additional invited authors whose research focuses on the biological activities and clinical outcomes associated with phytochemical consumption.

As an introduction, Chapter 1 describes some of the major research that contributed to the widespread interest in phytochemicals and health promotion. Venzon and Izzy detail a wide variety of epidemiological studies investigating the inverse relationship between plant-based diets and incidence of age-related chronic diseases, including cardiovascular disease and cancer. This notion is fundamentally supported by very large and influential population studies presented over the last decade. Undoubtedly, an abundance of evidence exists supporting the notion that fruit and vegetable intake is highly correlated with a variety of health benefits, and there is evidence that some of the benefit is related to the phytochemicals present in plants. Venzon and Izzy illustrate the characterization and biological activity of a number of these phytochemicals with regard to health promotion and disease prevention.

Chapters 2 and 3 are based on the variety of evidence surrounding a specific class of phytochemicals called polyphenols. In Chapter 2, Ebeler discusses polyphenols with respect to the health benefits associated with red wine. Though controversial, data have demonstrated that moderate wine consumption can have protective effects against cancer, prompting the question of whether these benefits are seen with alcohol consumption, in general, or only with specific types of alcohols. Ebeler describes a multitude of evidence pointing to the nonalcoholic constituents of wine as the basis of these protective outcomes. A variety of phenolic compounds, in this case originating from grapes, lend powerful antioxidant activity to the body. Research has demonstrated that these strong antioxidant properties contribute to critical biological activities as highlighted in this chapter, with DNA protection and, ultimately, cancer protection being the main point of emphasis. Ebeler further discusses points of controversy around the topic as well as various *in vitro* and *in vivo* studies that lead to the progression of our existing knowledge. A specific subcategory of polyphenols, anthocyanins, is the subject of Chapter 3. Interest in anthocyanins, a class of flavonoids contributing to many of the red, blue, and purple hues seen in plants, is exponentially growing, particularly for their effects against cardiovascular disease. In this chapter, Novotny describes epidemiological studies investigating the relationship between anthocyanins and cardiovascular disease that have yielded some inconsistent findings. However, she discusses several lines of research that clearly depict a cardioprotective benefit with anthocyanin intake. These phytochemicals can serve numerous roles related to heart health, including improvements of inflammatory markers and blood lipid levels. The evidence discussed throughout this chapter continues to shed light on the importance of adding a variety of phytochemical-rich plants into the diet.

Timmermann and Funk detail the increasing recognition of the health benefits of naturally occurring plant components in Chapter 4. This growing passion is manifesting around the globe as complementary and alternative medicine (CAM) and is becoming highly esteemed. Natural remedies and herbal products are particularly sought after, thus stimulating a vast interest in advancing the science in the field. Areas of particular interest are centered on broadening the range of natural therapies, optimizing bioavailability, safety, and efficacy, and refining methodologies for chemical characterization. Timmermann and Funk focus on ginger and turmeric, as these compounds offer unique medicinal properties related to inflammation, ulcers, and cancer among others. These phytochemicals also pose challenges in isolation and standardization of their bioactive components. The authors depict methods of chemical analysis that have shown potential in addressing these challenges and offer insight on their biosynthesis and potential as anti-inflammatory agents.

Chapter 5 outlines a recent study that has shifted phytochemical research toward a distinctive and unique application. This study summarizes a novel approach for identifying plant-based ingredients that have the potential to maintain bone health. Two specific mechanisms have been targeted with this approach—bone anti-resorptive (AR) and bone formation (BF). Fast, Chandra, Lin, Murray, and Gellenbeck reveal their extensive efforts in characterizing phytochemicals that can target these functions via associated *in vitro* bioassay models. Through this development, the authors

were able to distinguish plant concentrates that demonstrated notable effects, which were then examined further to develop a better understanding of optimal combinations and ratios for maximum bone health benefit with regard to AR or BF. This research presents the innovative framework for continuing to advance phytochemical research across broader and more exceptional capacity.

Chapters 6 through 9 discuss various aspects of phytochemicals related to sports, obesity, and metabolic disorders. Lila and Cheng present the traditional therapeutic benefits associated with certain classes of phytochemicals in Chapter 6. The adaptogenic, often regarded as "nonspecific," functions of phytochemicals have been historically documented in that consumption of certain plants can rejuvenate the body and restore internal homeostasis, promoting an overall feeling of well-being. While this information may date back through centuries of traditional folk medicine, more recent attention has been aimed at understanding the fundamental mechanisms behind the adaptogenic properties of plants. Lila and Cheng provide an in-depth analysis of various strategies used in classifying and understanding adaptogenic properties of phytochemicals. Chapter 7 covers the benefits of phytonutrient consumption, but the authors pay particular attention to shifts in diet and lifestyle over time, contributing to an array of obesity-related chronic disease. Junco and Slaga explore the numerous factors that have influenced the shift from a life of favorable energy balance and heavy fruit and vegetable intake common to the Paleolithic era toward the current consumption pattern known as the "Westernized diet." This progression toward poor dietary habits and decreasing physical activity is undeniably causing the astronomical rise in obesity and related diseases, namely, hypertension, diabetes, cardiovascular disease, and cancer. The authors illustrate the risks of obesity and a variety of consequential mechanistic factors that lead to such chronic disease. In Chapter 8, Prior provides a comprehensive overview of the research on anthocyanins and obesity. While the same positive results are not found in studies with whole berries, the author reviews studies indicating that some purified anthocyanin preparations slowed the progression of obesity and decreased triglyceride levels in animal models. Prior also discusses both *in vivo* and *in vitro* studies investigating the impact of anthocyanins on levels of leptin, insulin, and inflammatory cytokines and points out that the differences in activity of the various anthocyanin preparations may be as a result of their differences in chemical structures. In Chapter 9, Lee, Acquarone, and Mitchell focus on one particular flavonol, quercetin, and its increasing popularity in the application of sports science. Quercetin has been vastly studied for its beneficial effects on cardiovascular health, contributing additional functions such as anti-inflammatory and antioxidant protection. Some research suggests that these mechanisms, along with potential psycho-stimulatory activity, lend to improved athletic performance with quercetin intakes, and the authors present a careful review of the studies and proposed mechanisms contributing to these benefits.

Phytochemicals and brain health is the focus of Chapters 10 and 11. In Chapter 10, Miller, Shukitt-Hale, and Joseph explore the benefits of berries and nuts on the aging brain. With the increase in the aged population, there is an associated increase in the incidence of age-related cognitive decline. Brain aging is a product, in part, of responses to oxidative stress, chronic immune responses, and inflammation, and these have all been associated with age-related neurodegeneration. With these

processes being impeded and sometimes reversed by phytochemicals, it is plausible that the deleterious effects of aging on the brain could be reduced with increased phytochemical intakes. The authors discuss epidemiological evidence in support of this but find that clinical evidence points to the benefits of the whole food rather than individual phytonutrient supplementation. As such, the authors provide an in-depth discussion of *in vitro*, *in vivo*, and clinical trials with fruit and nut supplementation and the demonstrated benefits of these foods on the aging brain. Chapter 11 continues the focus on cognitive decline in aging populations with a look at *Bacopa monnieri* and *Centella asiatica*, both referred to historically as "Brahmi." Calabrese and Sowmyanath note that these herbs have been used traditionally in Ayurvedic medicine to enhance memory and intelligence, among other benefits. The authors present studies that have contributed to the growing body of knowledge that supports the use of these botanicals for improving cognitive function. They also present their proposed mechanisms of action as well as known attributes of safety and dosing.

Chapter 12 shifts gears and presents methodologies for assessing bioavailability of carotenoids with additional insight into a fruit not commonly known in the Western world. Vuong examines *Momordica cochichinensis* Spreng, a dark orange or red fruit common to many Asian countries. This fruit is known as one of the richest sources of lycopene and beta-carotene, and Vuong presents both *in vivo* and human studies to assess bioavailability as well as evidence indicating a strong antioxidant capacity of the fruit aril extract. Vuong furthers the discussion by exploring accelerator mass spectrometry (AMS) as a method for performing bioavailability studies of carotenoids. This instrumentation method, widely known for its use in carbon dating, has gained considerable interest for pharmacokinetic studies due to its exceptional sensitivity for measuring 14C in samples. With levels of detection far below the natural abundance of 14C, this instrument is a valuable analytical tool for performing isotopic tracer studies with 14C-labeled nutrients. Isotopic labeling and biological tracing via AMS allows the compound of interest to be distinguished from endogenous sources of the nutrient such that more accurate bioavailability data can be obtained. Additionally, the technique can be used to provide sensitive insight into metabolite identification. Vuong discusses this technique as well as its application and results from carotenoid bioavailability studies.

The goal of this book is to provide insight into some of the latest work in the field of phytochemicals as they pertain to health promotion and chronic disease prevention. We have presented evidence that some phytochemicals have protective effects with increased consumption. Our hope is that this book will stimulate increased interest in research regarding these compounds and their biological activities. While a multitude of knowledge is already available, opportunities still exist to deepen our understanding of the biochemistry and clinical benefits of these nutrients, as well as the application of these findings to therapeutic alternatives.

Colleen Carkeet
Samantha M. Izzy

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Editors

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Dr. Carkeet received her BSc (1998) and MSc (2000) in chemistry from Idaho State University. While there, she worked with the U.S. Geological Survey and the U.S. Department of Energy to study the geochemistry of the Big Lost River drainage basin. She also taught several courses in general and analytical chemistry, as well as multiple short courses in methods of instrumental analysis.

In 2004, Dr. Carkeet received her DPhil in agricultural and environmental chemistry with a specialization in analytical chemistry from the University of California, Davis. At Davis, she studied the pharmacokinetics of carotenoids and vitamin B12 and was issued a U.S. patent for an assay to diagnose vitamin B12 malabsorption as it relates to pernicious anemia. She also taught courses in instrumentation methods for wine analyses.

Following her graduate work, Dr. Carkeet spent two years as a postdoctoral fellow at the U.S. Department of Agriculture's Beltsville Human Nutrition Research Laboratory in Beltsville, Maryland. Her work involved analytical method development for the identification or urinary metabolites of anthocyanins. She also performed a clinical study to investigate the dose–response and metabolism of these phytonutrients from strawberries.

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Dr. Randolph has coedited books that include *Phytochemicals: Aging and Health*, *Phytochemicals: Nutrient–Gene Interactions*, and *Phytochemicals: Mechanisms of Action*, published by CRC Press. He has also coauthored 40 original papers with other distinguished researchers.

Dr. Randolph is a coauthor of the 2008 John M. Kinney Award Outstanding Paper in Nutrition and Metabolism and has earned patents for methods and kits for reducing oxidative stress–induced damage as well as nutrigenetic anti-inflammatory botanical formulation. He is a member of the American Society for Biochemistry and Molecular Biology, the American Chemical Society, and the American Society for Nutritional Sciences and is a fellow of the American College of Nutrition.

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Dr. Venzon began her doctoral work on nutrition and the brain at Washington State University. After she transferred to the University of Southern California (USC), she received a prestigious doctoral grant from the National Institutes of Health, was selected as the neuroscience graduate student of the year, and received her PhD in neuroscience in 2005. Dr. Venzon served as a postdoctoral fellow in the Department of Preventive Medicine at the USC Keck School of Medicine. She obtained several grants there to study the brain pathways responsible for hunger and satiety in female adolescents through the use of functional magnetic resonance imaging. Dr. Venzon has authored several peer-reviewed publications and book chapters on the neural processing of food rewards.

Dr. Venzon is a member of the Academy of Nutrition and Dietetics, the Society of Sports and Cardiovascular Nutritionists, the Society for the Study of Ingestive Behavior, and the Society for Neuroscience. In addition, she is a credentialed dietetics practitioner with a certification in weight maintenance in adults and adolescents.

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1 Fruit, Vegetables, and Phytochemicals in Human Health and Disease

Dawna Salter Venzon and Samantha M. Izzy

CONTENTS

INTRODUCTION

A long history and a wide variety of epidemiology research programs indicate a strong and interesting relationship between dietary intake of specific kinds of food and human health and well-being. One oft-repeated finding is the reduction in risk of specific kinds of chronic disease that coincides with increased dietary intakes

of plant foods, in particular fruits and vegetables. Some of the strongest and most consistent findings support a specific decrease in risk for cardiovascular disease, including a lessened chance of mortality from heart attack and stroke, with increased dietary fruit and vegetable intakes (Joshipura et al. 1999, 2001). While the links between dietary intake and overall mortality from cancer remain somewhat nuanced, convincing data exist for the notion that increased fruit and vegetable intake can decrease the incidence of specific kinds of cancer, particularly in the stomach or lung (International Agency for Research on Cancer 2003a; Larsson et al. 2006; Lunet et al. 2005; World Cancer Research Fund 2007). There also exists emerging evidence supporting a decreased risk of chronic degenerative diseases. Diets containing increased amounts of plant-based foods have been investigated as to their effects on age-related diseases of the eye, showing promising results. Diets high in plants, including those such as berries or green leafy vegetables, appear to support a variety of functions, including cognitive function (Galli et al. 2002; Joseph et al. 1999, 2009), decrease in risk for developing Type II diabetes (Bazzano et al. 2008; Harding et al. 2008), improvement in symptoms associated with arthritis (Pattison et al. 2004), and can be expected to help limit elevations to blood pressure as one ages (John et al. 2002). Overall, the picture appears to become increasingly apparent as data from larger and more comprehensive epidemiological studies are being revealed, and the mass of these studies continues to grow.

PROSPECTIVE COHORT STUDIES

Prospective cohort studies are those that follow a specified group of similar individuals over time in order to determine how certain behavioral factors can affect the rates of a certain outcome. Because scientists cannot deliberately expose individuals to suspected risk factors to evaluate chronic disease, researchers must follow large groups of individuals for extended periods of time in order to discern statistically significant linkages between dietary practices and chronic, age-related disease. If the group, or cohort, is large enough and being followed for an adequate period of time, then the outcome of these studies can have tremendous power in identifying risk factors related to certain health outcomes for the general human population. While prospective cohort studies are extremely useful in studying human health outcomes, they remain relatively rare because they require intense amounts of time and monetary resources.

Prospective Cohort Epidemiology Studies and Cardiovascular Disease

Two of the first and largest prospective studies are the "Nurses' Health Study" (Joshipura et al. 1999) and the follow-up "Health Professional's Study (Joshipura et al. 2001)." The first, the Nurses' Health Study, is considered to be the matriarch of women's health studies, and at initiation in 1976, it was the largest single cohort study of women, with over 100,000 enrolled and followed for 14 years. The Health Professional Follow-up Study was an all-male counterpart to the Nurses' Health Study. It was initiated in 1986 and followed an excess of 50,000 men aged

40–75 years old for 8 years. Both studies offered a wealth of information with respect to dietary risk factors related to human disease. Within these large cohort studies, specific subsets of healthy individuals with no diagnosis of previous disease were selected and evaluated for fruit and vegetable intake and cardiovascular disease. The information gleaned from these studies was the first of its kind to document the benefit of fruit and vegetable intake on cardiovascular disease outcomes. Ultimately, these findings support the notion that increased intake of fruits and vegetables can decrease one's chance of dying from coronary heart disease or ischemic stroke. The study results indicated that simply increasing fruit or vegetable consumption by one serving per day was associated with 4% lower risk of mortality from coronary heart disease and a 6% lower risk of ischemic stroke. These effects were the strongest with intakes of green, leafy vegetables and those fruits and vegetables that were rich in vitamin C (Joshipura et al. 2001).

These initial findings have been repeatedly supported over the past 10 years by more recent large cohort studies. One such prospective cohort study (Sauvaget et al. 2003a) evaluated the intake of fruits and vegetables on the outcome of stroke in a Japanese population (Hiroshima/Nagasaki Life Span Study, Table 1.1). In this cohort study, fruit and vegetable intake was evaluated in over 50,000 Japanese men and women who were followed for 18 years. The Hiroshima/Nagasaki Life Span Study was the first study to look at mortality from different types of stroke—dividing the outcomes by ischemic stroke, hemorrhagic stroke, and total stroke. Figure 1.1 demonstrates a synopsis of the data from the Hiroshima/Nagasaki Life Span Study. Japanese men who ate one serving per day of green-yellow vegetables or fruits demonstrated a significant decrease in risk of total stroke when compared to those who only ate one serving per week (Table 1.1). Likewise, for Japanese women, the daily consumption of green-yellow vegetables or fruits decreased the risk of death by total stroke by 30%. The statistically significant protection associated with daily intake of fruits and vegetables was observed in both men and women and accounted for cerebral infarction as well as intracerebral hemorrhage. This remained true even

TABLE 1.1
Results of a Prospective Cohort Study Which Evaluated Fruit and Vegetable Intake and Relative Risk of Stroke in a Japanese Population

Multivariate Adjusted Relative Hazards of Total Stroke	Men		Women	
	Servings/Week		Servings/Week	
	0–1	7+	0–1	7+
Green-yellow vegetables	1.0	0.74	1.0	0.81
Fruit	1.0	0.65	1.0	0.75

Sources: Data from Sauvaget, C. et al., *Stroke*, 34, 2355, 2003a; Sauvaget, C. et al. *Br. J. Cancer*, 88, 689, 2003b.

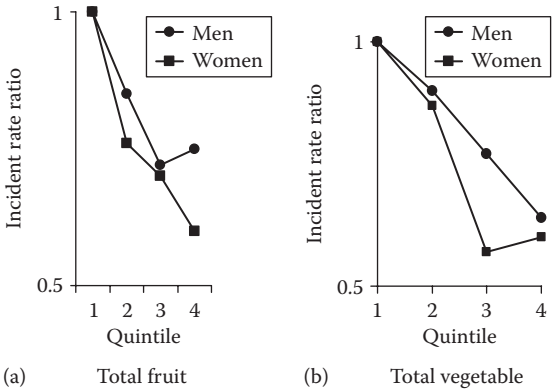

FIGURE 1.1 Results from a prospective cohort study demonstrating a strong, inverse relationship between (a) fruit and (b) vegetable consumption and incidence of cardiovascular disease. Data were calculated by evaluating the end point of coronary syndrome, which consists of unstable angina pectoris, myocardial infarction, and cardiac arrest. (Data from Hansen, L. et al., *Br. J. Nutr.*, 104(2), 248, 2010.)

after the data were controlled for age, body mass index, smoking history, alcohol use, education level, medical history, and the amount of animal products consumed.

Hansen et al. in 2010 presented data from a prospective cohort study that evaluated 57,000+ men and women between the ages of 50 and 64 from Denmark (Hansen et al. 2010). Results (Figure 1.1) again strongly document that fruit and vegetable intake is inversely related to cardiovascular disease by looking at the end point of "coronary syndrome," which was defined as a composite of unstable angina pectoris, myocardial infarction, and cardiac arrest.

CROSS-SECTIONAL EPIDEMIOLOGY STUDIES

Because cohort studies are labor, time, and resource intensive, researchers often use cross-sectional epidemiological approaches to support inferences between a dietary cause and resultant illness. A cross-sectional research study involves observing a population at a specific point of time and, to allow for more rapid evaluation of relationships or correlations, may utilize data sets from routinely collected information. These types of studies are generally considered less conclusive than cohort studies because confounding factors from previous points in time are often not identified. However, if designed properly they can be quite useful in providing additional supporting details for other cohort studies that have identified strong cause and effect relationships.

Cross-Sectional Epidemiology Studies and Cardiovascular Disease

One notable study, the Multi-Ethnic Study of Atherosclerosis (MESA), is an interesting cross-sectional population-based study that lends more precise information between habitual food consumption and those coronary events identified in the large cohort studies discussed earlier (Nettleton et al. 2008a). Here, Nettleton et al. used

a novel and creative nutritional epidemiological approach to investigate cardiac risk among White, African American, Hispanic, and Asian men and women in the United States aged 45–84 without cardiovascular disease at baseline. The researchers were able to identify dietary habits that are more likely to raise the risk of very early indicators of *potential* cardiovascular disease by incorporating systematically defined sets of dietary "patterns" rather than analyzing individual foods or nutrients. The authors relied on previous research to categorize and group these foods and further defined these various food groups by nutrient and phytochemical characteristics. Food intake was analyzed against these established criteria, and food groups were assigned as positive, negative, or neutral. Plant foods were assigned a positive score while some foods that are associated with a more industrialized or "westernized" food pattern were assigned a negative score, as illustrated in Figure 1.2. Individuals in the study were then given a total score based on their particular dietary pattern, which was evaluated both continuously and by quintile. After adjustments for demographics and lifestyle characteristics, the study identified several key findings. First, the healthy dietary pattern score was inversely associated with markers of inflammation that indicate early risk of atherosclerosis, including C-reactive protein, interleukin-6, homocysteine, and fibrinogen. This score was also inversely related to the intima-media thickness (IMT) of the common carotid artery, an established marker of subclinical atherosclerosis. Second, the dietary pattern score was associated with improved markers of vascular integrity, notably the urinary albumin-to-creatine ratio (ACR). ACR has been shown to be independently associated with cardiovascular risk, reflecting microvascular dysfunction as an early indicator of atherosclerosis. Third, the dietary pattern was associated with improved levels of high-density lipoprotein cholesterol, triglyceride levels, and a better marker of fasting insulin, all of which are risk factors for developing cardiovascular disease (Nettleton et al. 2008a).

In summary, several lines of research, using both large comprehensive cohort studies and innovative cross-sectional epidemiological approaches, support the notion

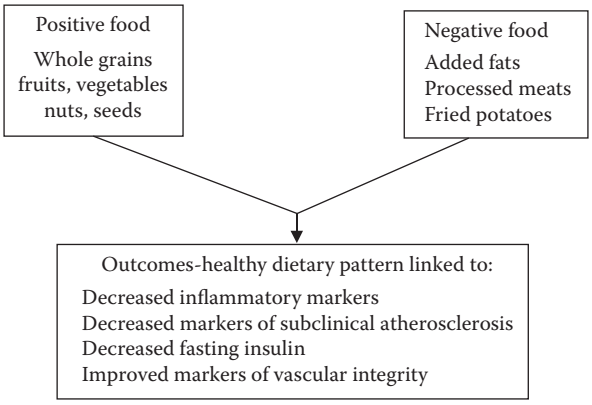

FIGURE 1.2 MESA assigns food groupings with a score based on nutrient and phytochemical characteristics resulting in a "simple healthy dietary pattern." (Data from Nettleton, J.A. et al., *Am. J. Clin. Nutr.*, 88, 185, 2008a.)

that an increase of fruits and vegetables, at the level of at least five servings per day, decreases the incidence and mortality of cardiovascular disease and stroke. Increasing other plant-based foods such as nuts, seeds, and whole grains also appears to increase such protective effects as suggested by the MESA study (Nettleton et al. 2008b).

EPIDEMIOLOGY IN OVERALL CANCER INCIDENCE OR MORTALITY

In contrast to what research has shown with cardiovascular disease, the protective effect of a plant-based diet has not been as firmly established in preventing *overall* cancer or *overall* mortality. Intervention studies linking diet with specific cancers exist, but epidemiology studies have yet to firmly establish strong and repeated findings that link dietary intake to overall cancer or mortality. This is most likely due to a lack of statistical power among the research that has been conducted up until recent years. Prior to the year 2009, there were only 6 prospective studies with over 10,000 individuals that investigated the overall incidence of cancer or mortality as a result of cancer. Further, the results of these studies have largely been mixed in supporting the hypothesis that increased dietary intake of plant-based foods lends protective effects from overall cancer or mortality risk.

Shibata et al. (1992) investigated a cohort of 11,000+ residents of a retirement community who were followed for about 7 years. Women in this study population who consumed more than 10 servings per day of vegetables and fruits demonstrated a statistical decrease in risk of overall cancer diagnosis when compared to those who consumed less than 6 servings. Women who ate more than 4.5 servings of fruits per day also appeared to garner a protective effect over those who ate less than 2.5 servings per day. Interestingly, these findings did not carry over to the men in this study, as no significant protective effects were found in the male population. In 2003, Sauvaget et al. reported decreased incidence of cancer with increased dietary intake of fruits and vegetables after evaluating 40,000 Japanese male and female individuals (Sauvaget et al. 2003b). The results demonstrated that daily fruit intake decreased the risk of overall cancer mortality by 12% compared to those individuals who ate fruits once per week or less. Further, daily intake of yellow or green vegetables decreased the risk of overall cancer mortality by 8% over those individuals who consumed these types of vegetables once per week or less. Benetou et al. (2008) investigated a cohort of nearly 26,000 individuals who were followed for nearly 8 years evaluating overall incidence of cancer. An inverse association between the incidence of cancer and an increased intake of fruits and vegetables was noted. Specifically, those women who ate over 7 servings per day of fruits and vegetables had a decreased risk of receiving a cancer diagnosis, and over 10 servings of fruits and vegetables per day was associated with significantly more protection from incident cancer. Notably, as also seen in the study by Shibata et al. (1992), this protection from dietary produce intake was found to be significant only in women and not in men.

In contrast to the studies discussed earlier, other existing cohort studies have not shown any protective effects linked with dietary fruit or vegetable intake. Two rather large studies have been published demonstrating a lack of relationship between fruit and vegetable intake and protective effects against cancer: one published in 2007 on a Japanese cohort of nearly 78,000 men and women (Takachi et al. 2008)

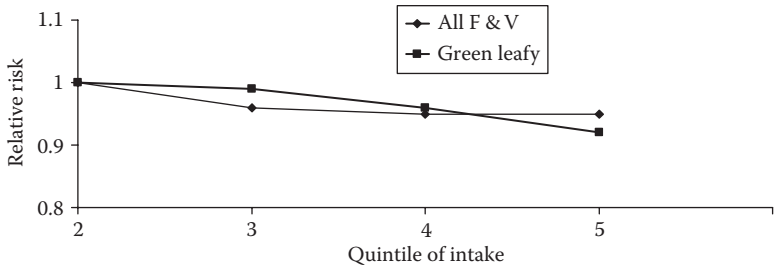

FIGURE 1.3 Fruit and vegetable intake and the relative risk of major chronic disease. Men and women in highest quintile of fruit and vegetable intake had decreased risk of major chronic disease. (Data from Hung, H.C. et al., *J. Natl, Cancer Inst*, 96, 1577, 2004.)

and another prospective study published in 2009 that enrolled over 566,000 retired American men and women. The first and most influential cohort study showing no effect between dietary intake and cancer was published in 2004 (Hung et al. 2004). In this study, the primary outcome evaluated was "major chronic disease," defined as "cardiovascular disease, cancer, or nontraumatic death, whichever came first." As illustrated in Figure 1.3, increased dietary intake of any type of fruits and vegetables had a slightly lower risk of major chronic disease, across quintiles from 1.5 servings to 8 servings per day. As well, there was noted a significant protective effect of leafy green vegetables, with a decrease in relative risk of major chronic disease as intakes of these types of vegetables increased from 0.2 servings to about 1.5 servings per day (Figure 1.3). However, while there was an association with "major chronic disease," there was no association between total fruit and vegetable intake and cancer incidence. Thus, the significant inverse association between total fruit and vegetable intake and major chronic disease was attributed primarily to the reduction in cardiovascular disease rather than cancer incidence. Additionally, the data within this large study demonstrated several other important findings: (1) While there was a slight protective effect against overall major chronic disease with greater than 8 servings of fruits and vegetables per day, protective effects were also noted if at least one of those servings was composed of leafy green vegetables; (2) participants in the study meeting the median intake of at least 5 servings of fruits and vegetables per day had a 28% decreased risk of cardiovascular disease compared to those participants who ate less than 1.5 servings per day; (3) if participants included even one serving of leafy green vegetables per day, risk of cardiovascular disease was decreased by 11%. Overall this negative finding toward cancer prevention held much weight, being that it originated from a very large and well-run cohort study and provided other valuable information regarding protective effects of increased fruit and vegetable consumption.

DIETARY RECOMMENDATIONS FOR CANCER PREVENTION

The results of the previously described studies were influential toward overall dietary recommendations of fruit and vegetable intake in regard to effective prevention of cancer. Indeed, the general picture presented by the outcomes of large

prospective cohort studies is somewhat mixed when evaluating the relationship between the diet and overall cancer incidence. Because of this, over the past 20 years, the "story" in regard to cancer prevention and fruit and vegetable intake has slowly become more conservative. In 1990, the World Health Organization (WHO) suggested four portions per day (400 g) to prevent cancer and other chronic disease (WHO 1990), and several years later, the World Cancer Research Fund claimed convincing evidence for a protective effect for fruit and vegetable intake in respiratory and digestive cancers (World Cancer Research Fund 1997). However, by 2003, the recommendations began to soften, with the WHO suggesting that fruits and vegetables "probably," but not "convincingly," reduce cancer risk (Diet, Nutrition, and the Prevention of Chronic Diseases, WHO Technical Report 2003). Moreover, the International Agency for Research on Cancer (IARC) reclassified the evidence as limited (International Agency for Research on Cancer 2003b) based on results of the forthcoming prospective studies presented by Hung et al. (2004). By 2007, the World Cancer Research Fund downgraded the strength of evidence for the protective effects of fruit and vegetable intake (World Cancer Research Fund 2007). This muddled picture of official health recommendations for cancer prevention stems from several factors, including such things as the lack of large prospective studies or the various ways that fruits and vegetables are categorized. For instance, apples, citrus, leafy greens, cruciferous, potatoes, or fruit juice can all be placed into either category of fruit or vegetable, or disregarded as such depending on the study design. Further complication arises from different studies evaluating different outcomes, for example, cancer mortality, cancer incidence, or overall chronic disease.

EUROPEAN PROSPECTIVE INVESTIGATION INTO CANCER AND NUTRITION

The European Prospective Investigation into Cancer and Nutrition (EPIC) study published in 2010 (Boffetta et al. 2010) is one of the largest and most recent prospective cohort studies adding detail to the picture being painted on the nature of the protective effects of plant-based foods in one's diet. This study enrolled over 400,000 individuals throughout Europe including participants from Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, as well as the United Kingdom. After following these participants for nearly 8 years, the study found a small but significant protective effect against overall cancer risk as dietary intake of fruits and vegetables rose. Figure 1.4 presents a graphical demonstration adapted from Boffetta et al. (2010) showing the decreased risk, as measured by hazard ratio, for overall fruit intake alone, vegetable intake alone, or a combination of the two. Increasing fruit or vegetable intake alone resulted in some decreased risk in cancer incidence from quintile 1 to quintile 4; however, when evaluating the intakes of fruits and vegetables combined, a statistically significant inverse trend with cancer risk is demonstrated. Although details around the health benefits of fruit and vegetable intake in regard to cancer prevention remain yet to be filled in entirely, the general landscape appears to indicate a definite health benefit of increasing one's dietary intake of plant-based foods. The vast quantity of epidemiological

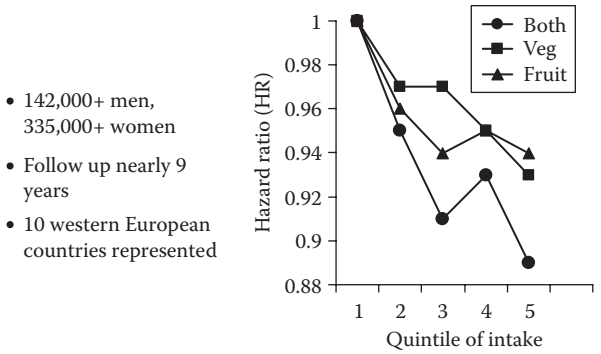

FIGURE 1.4 The EPIC revealed a small, but significant, inverse relationship between fruit and vegetable intake and overall cancer risk. (Data from Boffetta, P. et al., *J. Natl, Cancer Inst*., 102(8), 529, 2010.)

data suggests overall that an increase of these foods in the diet will afford health protection over the premature development of chronic degenerative disease including incidence of cancer. The health professionals of most industrial nations are busily trying to convince their populace to boost the intake of such foods with somewhat limited success. Additionally, many lesser-developed countries are undergoing a nutrition transition to a more industrialized or "westernized" diet. While these cultures may not be consuming significantly less quantities of fruits and vegetables than before, they may be incorporating greater intakes from processed, high-energy content foods such as those with elevated simple sugar and fat contents, lower nutrient density, and greater portions of animal meats. Since ultimately the gross quantity of fruit and vegetable intake was inadequate before transition, and continues to be inadequate, the shift to a more "westernized" diet adds another negative variable to the equation. These types of diets—high in animal meats, fat, sugar, and refined foods—appear to be an independent factor in further contributing to inflammation, obesity, diabetes, and other chronic disease. Therefore, it is even more important that health professionals and organizations continue diligent efforts to promote higher intakes of plant-based foods. Today, it is widely believed that the combinations of bioactive compounds that are uniquely found within fruits, vegetables, and other plant foods are responsible for lending the significant health benefits demonstrated in individuals that consume more of these types of foods. In this regard, it may be beneficial to investigate ways to boost intake of these bioactive components through dietary supplementation as a means for chemoprevention in human populations.

PHYTOCHEMICALS: BIOACTIVE COMPONENTS OF PLANT-BASED DIETS

Increasingly, scientific evidence supports the notion that the bioactive compounds found within fruits and vegetables, called phytochemicals, are the components of plants that promote health in humans. As worldwide dietary habits continue to progress to more westernized patterns, further increasing the intake of these protective bioactive compounds is highly warranted.

There are large classes of identified and characterized phytochemicals. In fact, there are estimated to be over 5000 compounds that have been characterized and quite possibly thousands more that have not been identified as of yet. Hundreds of these bioactive compounds have been identified as having a beneficial role toward human health. A single plant food is likely to contain thousands of different phytochemicals, and a key question remains as to whether any of these bioactive compounds, if isolated, have the same benefits as when the compound is consumed within a whole food. A few of the more studied compounds include carotenoids, organosulfur compounds such as isothiocyanates, and various isoflavonoids such as phytoestrogens. Isothiocyanates are compounds found in certain classes of vegetables, called cruciferous, and have been strongly implicated in cancer prevention. Isoflavonoids, in particular genistein and daidzein, are compounds found predominately within the soybean and have been linked to protection from specific kinds of cancer, namely, prostate and breast. Carotenoids are considerably one of nature's most widespread pigments and have received much attention, as they contribute provitamin activity as well as function as antioxidants.

Carotenoids

Carotenoids are the pigments responsible for yellow and some red colors of fruits and vegetables. There are probably more than 600 carotenoid compounds that have been identified, but to date, only about 7 can be measured in the blood and tissue and are recognized for providing health benefits to humans. Carotenoids can provide vitamin A activity within the body after being converted from provitamin A. However, there are no recommended dietary allowances for these compounds because they are not yet considered essential for human health. Nevertheless, it does appear that various carotenoids have biological actions that may be important for preventing or delaying certain kinds of chronic disease.

Carotenoids are potent antioxidants with a strong ability to quench free radicals (Krinsky 2001; Paiva and Russell 1999), and, through this mechanism, carotenoids are thought to play an important role in preventing premature cardiovascular disease, particularly by protecting lipoproteins from being oxidized (Voutilainen et al. 2006). Carotenoids are also thought to have cancer-preventive properties by inhibiting abnormal cellular growth through both improvement of cell-to-cell communication and protection of DNA within the cell (Krinsky and Johnson 2005). Finally, carotenoids appear to lend support to the immune system, and recent literature provides evidence suggesting that insufficient carotenoid levels may be a component in the etiology of inflammation that occurs alongside obesity. As such, carotenoids may play a critical role in the cardiovascular and glucose health of obese individuals (Markovits et al. 2009).

Plasma Carotenoid Levels and Chronic Disease

An extensive review by Krinsky and Johnson in 2005 documented that, in general, diets high in carotenoid-rich fruits and vegetables are strongly linked with a decreased risk of chronic disease. Observational studies have also shown that plasma carotenoids are inversely associated with cardiovascular disease, in particular ischemic

stroke (Hak et al. 2004). This is especially true of plasma lycopene levels, as well as levels of lycopene present in adipose stores (Markovits et al. 2009). Further, plasma lycopene has been inversely associated with prostate cancer (Giovannucci et al. 1995; Wu et al. 2004), and plasma levels of β-carotene have been shown to be inversely associated with lung cancer (van Poppel 1993). Furthermore, several studies have shown preliminary evidence that blood levels of β-carotene are inversely correlated with incident of Type 2 diabetes (Arnlov et al. 2009; Hozawa et al. 2006).

Carotenoid-rich diets have shown promising evidence with respect to preventing or slowing the progression of cataracts and age-related macular degeneration (AMD). Because both lutein and zeaxanthin are carotenoids that accumulate within the macula of the eye, much research has been devoted to investigating diets rich in these phytochemicals for eye health. Accumulating evidence is promising, and, in fact, the analysis from the Eye Disease Case Control (EDCC) study group provides evidence that individuals with high blood levels of lutein/zeaxanthin have significantly lowered risk of severe AMD (Antioxidant status and neovascular age-related macular degeneration. Eye Disease Case-Control Study Group 1993).

Interventional Trials with β-Carotene

While much research supports the notion of carotenoid-rich diets as being beneficial to human health, intervention studies have not been as conclusive. In fact, large, randomized intervention trials where participants were given high-dose β-carotene showed possible harm in relation to cancer among smokers and asbestos workers (Omenn et al. 1996). Additionally, minimal evidence from strong studies exists in supporting high doses of supplemental β-carotene as beneficial against development or progression of cardiovascular disease. In contrast, the Age-Related Eye Disease Study (AREDS) has shown promising results with supplementation of vitamin C, E, β-carotene, and zinc, as demonstrated by a 25% reduction in risk of progression to advanced AMD over 5 years. Ultimately, due to the conflicted nature of the results of interventional studies, it is currently recommended that supplemental β-carotene be from natural sources and kept within moderate levels.

Isothiocyanates

Isothiocyanates are another type of phytochemical, uniquely found in cruciferous vegetables such as broccoli, cabbage, cauliflower, brussel sprouts, Chinese cabbage, and some salad greens such as watercress. Isothiocyanates originate as glucosinolates in plants and, under specific conditions, are converted to isothiocyanates by an enzyme with which they coexist called myrosinase. Under normal conditions, glucosinolates and myrosinase are separate physical entities; however, under stressed conditions, for example, when damage occurs to the plant cells by means of chewing or smashing, myrosinase is released and catalyzes the conversion of glucosinolate to isothiocyanate (Fenwick et al. 1983; van Poppel et al. 1999). Isothiocyanates appear to function on two levels, by both activating phase 2 enzymes and inhibiting phase 1 enzymes. Isothiocyanates elicit the induction of phase 2 detoxification enzymes (such as glutathione S-transferase) that elevate the cell defenses against oxidative damage and help promote the removal of carcinogens (Juge et al. 2007). When a carcinogen

enters the human body, it undergoes what is called phase 1 metabolism, a process that is catalyzed by cytochrome P450 enzymes. Isothiocyanates suppress phase 1 enzymes causing the carcinogenic compounds to be less able to react within the body (van Poppel et al. 1999). The net effect of the phytochemical is thus to be chemopreventive (Shapiro et al. 2001; Talalay and Fahey 2001). Some myrosinase activity occurs in the human intestinal microflora, but studies show that consumption of cooked cruciferous vegetables significantly decreases the exposure to isothiocyanates compared to consumption of equal portions of the same raw vegetables (Conaway et al. 2000; Rouzaud et al. 2004), as exposure to heat while cooking inactivates the myrosinase enzyme and can decrease the yield of isothiocyanates (Conaway et al. 2000).

Epidemiological Studies on Protective Effects of Cruciferous Vegetables

Evidence for the protective effects of cruciferous vegetables comes from large epidemiological studies done in the Unites States, Europe, Shanghai, and Singapore. These epidemiology studies demonstrated a strong relationship between the increased intake of cruciferous vegetables and a reduction of cancer risk at certain sites including lung, breast, colon and rectum, prostate, and bladder. While the research linking isothiocyanate-producing vegetables with health promotion is strong, there remain some inconsistencies within the lines of research. It is now a widely held notion that the level of protection lent by intake of cruciferous vegetables seems to be highly linked with an individual's genotype, specifically of the glutathione transferase gene (Lin et al. 2009). Approximately 50% of the population, regardless of race, has a deletion within this gene. These "nulls" appear to have less benefit in terms of cancer protection with the inclusion of isothiocyanate-producing vegetables, when compared to those in the population that carry the gene. This is a unique area of active research and can provide interesting direction for linking specific phytochemical-rich foods with genotypes in the future.

Another factor that has lent some inconsistency to the data is the fact that normal cooking procedures can deactivate myrosinase, thus decreasing isothiocyanate exposure by 60%–90% after consumption (Conaway et al. 2000; Rouzaud et al. 2004). Two recent case-controlled studies investigated the difference between intakes of raw cruciferous vegetables against those same vegetables cooked, as they relate to incidence of primary bladder or lung cancer over a period of 16 years. Figure 1.5 illustrates data adapted from Tang et al. (2008), showing a protective effect of cruciferous vegetables against the incidence of both lung and bladder cancer, as the dietary intake increases. Importantly, this protection was statistically apparent with as little intake as—five to seven servings of cruciferous vegetables per month. When smoking practice of the participants (current or prior) was an added variable, the inverse association was even stronger.

Isoflavones/Phytoestrogens

Several lines of research strongly suggest that including plant foods rich in phytoestrogens can lend significant health benefits to the human. Phytoestrogens are so named because the compounds are naturally found in foods of plant origins—namely, soybeans—and structurally resemble natural estrogen (Dixon 2004). Phytoestrogens

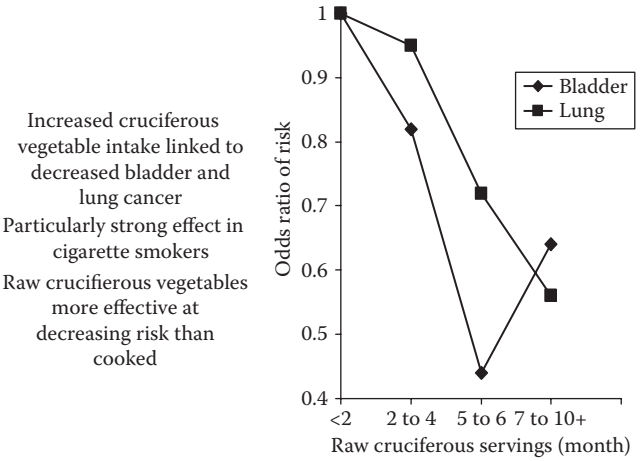

FIGURE 1.5 Isothiocyanate intake as it relates to risk of specific cancers of bladder and lung. (Data from Tang, L. et al., *Cancer Epidemiol. Biomarkers Prev.*, 17, 938, 2008; Tang, L. et al., *Cancer Epidemiol. Biomarkers Prev.*, 19, 1806, 2010.)

appear to bind to the estrogen receptor and effectively displace the 17-beta-estradiol from the alpha-receptor. Isoflavones can also bind the beta-receptor where they trigger transcriptional pathways, particularly repression of transcription (Makela et al. 1995a,b; Penttinen-Damdimopoulou et al. 2009).

Phytoestrogens can be categorized under various groups of compounds including flavonoids, lignans, coumestans, and stilbenes. At present, most scientific research has been around the subgroup of flavonoids known as isoflavones, which include genistein and daidzein. Daidzein can further be converted in the intestine to another type of isoflavone, equol (Cederroth and Nef 2009). Much of the research on phytoestrogens was first initiated upon observation that populations with high intakes of isoflavone-rich soybeans had significantly lower incidence of estrogen-related cancers, including breast and prostate. There has been much research on the potential benefit of dietary isoflavone intake and the prevention of estrogen-related cancers, and, although the conclusions on these preventive aspects are currently lacking, studies do indicate a link between isoflavone intake and human health. For instance, studies have found that higher intakes of soy isoflavones can help postmenopausal women maintain a lower body mass index, help reduce plasma concentrations of total and LDL cholesterol, and improve bone density measures.

Plasma Phytoestrogens and Prostate Cancer

Travis et al. (2009) presented data from a prospective study that evaluated 150,000 men from 10 European countries, a population that does not traditionally include high amounts of isoflavone or phytoestrogen in their diets. Plasma samples of these men were analyzed for the three isoflavones genistein, daidzein, and equol, providing a direct measurement of phytoestrogen concentration in the circulation. Having a direct measurement of plasma isoflavones was particularly powerful in this

circumstance for two reasons. First, direct measurement allowed for the capture of exposure from all sources of isoflavones. This was perhaps a much more accurate representation over other conventional means of estimating nutrient intake such as evaluating habitual food consumption and estimating isoflavone components of those foods. Second, the plasma isoflavones were further separated into measurements of genistein, daidzein, and equol. This lends for a deeper analysis, as each isoflavone is absorbed differently across the gut, metabolized differently by intestinal flora, and ultimately has different biological activity in the body. The authors also found a significant trend in regards to plasma genistein levels and prostate cancer risk across quintiles, even after being adjusted for confounders. The amount of plasma genistein in those with prostate cancer was a low 1.8 ng/mL, but only slightly higher at 2.1 ng/mL in control subjects. A slight trend was observed in plasma daidzein levels between the groups, although it was not significant. The plasma levels of equol did not appear to lend any protective effects; however, it is important to note that only a small percentage of the population has the ability to convert the daidzein to equol, and this complicates epidemiological findings such as those reported here (Atkinson et al. 2005). Other studies have found equol as being highly protective, so this is an area that requires more research in the future. Nevertheless, the observations in this study by Travis et al. indicates even small amounts of isoflavones in the diet can improve outcomes for preventing estrogen-related cancers in men.

Fruit–Vegetable–Soy Dietary Patterns and Breast Cancer

There has been some epidemiological evidence suggesting an inverse relationship between isoflavone intake and breast cancer, but it has been somewhat inconsistent (Trock et al. 2006).

Recently Butler et al. (2010) conducted a prospective cohort of postmenopausal women from Singapore that sheds important light on this inconsistency. The authors here took a creative experimental approach by identifying and comparing two dietary patterns among the population and classifying these patterns as protective or detrimental. Patterns identified and assessed consisted of fruit, vegetable, and soy (FVS) versus meat-dim sum (MDS). The authors found a very significant trend of protection in the FVS group and, on the contrary, found no apparent effects on health with the MDS dietary pattern. When examined individually, vegetables, isoflavones, and soy foods all showed some trend at decreasing risk of breast cancer in this population, but the combination of these foods provided significantly more protection. Similar correlations have been found in studies evaluating postmenopausal breast cancer risk with a soy–vegetable pattern in both Asian American and Japanese women (Wu et al. 2008, 2009). Thus, it is reasonable to suggest that the combination of isothiocyanates from cruciferous vegetables and soy isoflavones may offer greater a benefit for decreasing breast cancer risk.

PHYTOCHEMICALS AS SUPPLEMENTS AND FUNCTIONAL FOODS

There exists an increasing amount of evidence from a multitude of research that strongly supports the notion that fruit and vegetable intake is protective for health. Likewise, these health benefits have been strongly linked with the phytochemical

contents within the fruit or vegetable. While there is no parallel replacement for a healthful diet rich in fruits and vegetables, consumers have a strong desire for functional foods and supplements that can offer similar or even greater health benefits, convenience, safety, as well as some evidence of efficacy. To meet this need, some supplement providers have become quite proficient in providing these types of products. Typically, in order to achieve such functional targets, foods are identified that are high in a certain type of phytochemical that has been linked to some aspect of human health. This is followed by purifying the identified phytochemical into a concentrated form. These compounds can then be subjected to *in vitro* bioassays that correspond to some component of human health. This information is then used to create scientifically based products targeted to provide unique and important health benefits. This is an important process that supports beneficial health outcomes for a multiplicity of populations, but continued efforts to progress the science to lend better and more effective health benefits are highly warranted.

Continued scientific efforts to understand the biochemistry of these compounds are justified as the absorption, metabolism, and distribution of phytochemicals within the human body are highly complex. The complexity is also magnified when taking into consideration the extraordinary numbers and different kinds of phytochemicals within plants. As we understand more about how these phytochemicals work within the body, we can continue to produce marketable products for even better outcomes. For any compound to be utilized for health benefits, it first must be ingested, absorbed into the body, metabolized, and circulated to the target organ or system. Many phytochemicals undergo further metabolism both before and after processing in the liver, and their respective metabolites can provide additional health effects. Identified are three main opportunistic areas that can offer potential in improving and magnifying the health benefits of phytochemical-rich foods or supplements: synergy, bioavailability, and efficacy. Identifying various phytonutrients, which, in particular combinations and proportions, can compliment the function of one another, can maximize synergistic and additive benefits. As the understanding of the metabolic process of these nutrients becomes more sophisticated, identifying a means to increase the body's ability to absorb the active components can lead to more efficient nutrient utilization. Finally, the continued efforts at targeting phytochemical research toward various aspects of human health can offer promising opportunities around delay and prevention of age-related chronic disease.

Opportunities in Synergy, Bioavailability, and Efficacy

Single, isolated, and purified phytochemicals provide convenient and effective means to provide specific benefits for a targeted effect. However, continued epidemiological research suggests that a combination of certain phytochemicals, in a justified manner, can actually improve and strengthen such biological benefits. For example, the data suggest that cruciferous vegetables provide health benefits through isothiocyanates. However, the ability to obtain isothiocyanates from plants in a consistent manner can pose some challenges. The right combination of enzymes must be available to effectively yield isothiocyanates, individuals must actually incorporate raw, cruciferous vegetables in their diet, and perhaps even genotype can be a limitation in effectively

converting the nutrients. In this instance, it would make sense to design a functional food or supplement that can provide a combination of phytochemicals designed to maximize the conversion of glucosinolate to the functionally active isothiocyanate in the body. Many opportunities exist to maximize health benefits individuals receive from strategic and scientifically based combinations of phytochemicals.

Variations within both gut microbiota and genotypes of individuals can greatly affect the available health benefits of dietary phytochemicals, whether obtained through phytochemical-rich foods or supplements. Great opportunities exist to leverage this information in an effort to maximize and equalize the health benefits from supplemental phytochemicals. For example, it is estimated that only 30% of people have the ability to convert the isoflavone daidzein to equol, and there are strong lines of evidence that suggest equol is highly beneficial for human health. Utilizing strategies to maximize bioavailability of active forms and metabolites, as well as targeting phytochemical forms and amounts to meet individual genomic needs are areas that may represent great potential.

When working in the area of prevention, for example, avoiding or delaying disease, it can be difficult to produce evidence-based strategies with which efficacy can be adequately measured. Therefore, identification of early biomarkers that are suggestive of developing or progressing disease is critical for this model. In addition, identifying the correlation between improvements of these biomarkers, ultimate health outcomes, and respective phytochemical supplementation can be critical to improving measures of efficacy.

SUMMARY

Evidence over the past decade has shown a strong inverse relationship between plant foods and development of cardiovascular disease, including both stroke and coronary events. In contrast, the relationship between fruit and vegetable intake and cancer prevention has not been as firmly established and has resulted in conservative recommendations for chemoprevention. Now, several large prospective cohort studies are demonstrating the benefits of increased consumption of fruits and vegetables on overall cancer risk, lending significant strength to the notion that plant-based diets can decrease chronic disease. Despite this rapidly growing body of evidence, people throughout the world continue to transition to diets higher in fat and simple carbohydrates, potentially diluting the powerful and protective effects of increased fruits or vegetables.

Likely the protective and therapeutic nature of a high fruit- and vegetable-containing diet is derived from the synergistic mechanisms of bioactive phytochemicals found naturally within plants. Many commonly consumed phytochemicals such as carotenoids, isothiocyanates, and phytoestrogens have specific health-promoting benefits. Because of this, much important work has been placed into the identification, capture, and standardization of phytochemicals for use in plant-based supplements and functional foods. Nevertheless, significant scientific challenges persist in identifying the nature of phytochemical synergies, maximizing bioavailability, and measuring efficacy within the human body—posing a tremendous area of opportunity for continuing research.

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2 Wine and Cancer

Susan E. Ebeler

CONTENTS

INTRODUCTION

Many epidemiological studies over the past 20 years have indicated that moderate wine consumption is associated with a decreased incidence of cancer (Grønbaek et al. 1995, 1998, 2000; Macfarlane et al. 1995; Benedetti et al. 2009; Chen et al. 2009). The relationship between wine consumption and incidence of cancer, particularly breast cancer, remains controversial however, with some studies showing either no effect or a decreased risk of breast cancer associated with moderate wine consumption (Longnecker et al. 1995; Zhang et al. 1999; Bessaoud and Daures 2008; Newcomb et al. 2009) and other studies indicating an increased breast cancer risk even with moderate consumption (Viel et al. 1997; Smith-Warner et al. 1998; Allen et al. 2009). Moderate wine consumption is defined by the U.S. Department of Agriculture Dietary Guidelines (USDA 2010) as up to one 5 oz glass of wine (12% ethanol) per day for women and up to two 5 oz glasses of wine per day for men.

Some epidemiological studies have tried to address the question of whether the type of alcoholic drink affects cancer incidence and whether ethanol alone may have different effects than wine or other alcoholic beverages. In most cases, ethanol alone, particularly at high levels of consumption, has been shown to increase the incidence of most cancer types, particularly cancers of the liver and oral cavity (Longnecker 1995; Baan et al. 2007). Numerous mechanisms have been suggested for the effects of ethanol on cancer development; however, no absolute mechanistic processes have been established (Longnecker 1995; Poschl and Seitz 2004). For example, ethanol may act as a promoter by altering cell proliferation and regeneration or by altering hormone levels. In addition, ethanol consumption can displace other nutrients in the diet and alter nutrient metabolism. Importantly, ethanol can

affect the carcinogenicity of other compounds via effects on Phase I and Phase II metabolism. These effects on metabolism may explain the synergistic effects on cancer risk associated with ethanol consumption when combined with smoking (Poschl and Seitz 2004; Vineis et al. 2004; Hashibe et al. 2007; Allen et al. 2009). In contrast to these overall deleterious effects, it has been proposed that ethanol may increase absorption of some beneficial nutrients and vitamins; however, this has not been consistently shown *in vivo* (Donovan et al. 1999, 2002; D'Archivio et al. 2010).

Much evidence now points to the nonalcoholic components of wine as being important for their cancer- and tumor-preventative properties (Grønbaek et al. 2000; Grønbaek 2009). As noted recently, compared to beer and spirits consumption, moderate wine consumption has been associated in epidemiological studies with a significantly decreased risk of mortality from all cancer types (Figure 2.1, Grønbaek 2009). Animal studies have supported these observations. For example, Clifford et al. (1996) observed that the alcohol-free solids of red wine, when consumed as part of a healthy diet, significantly delayed tumor onset in a transgenic mouse model of neurofibromatosis (Clifford et al. 1996). Reductions in chemically induced tumors (e.g., mammary and colorectal) have also been observed in rats following consumption of polyphenol extracts from wine (Wilson et al. 1999; Caderni et al. 2000; Dolara et al. 2005; Femia et al. 2005). Wine is a rich source of phenols that have been shown to have numerous chemopreventive properties (Singleton 1981;

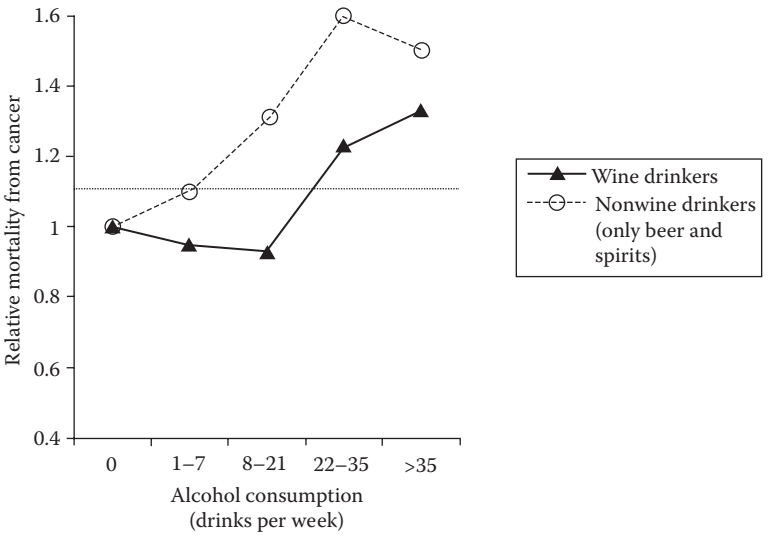

FIGURE 2.1 Relative risk of death from cancer according to type of alcohol. (Copenhagen Centre for Population Studies). (From Grønbaek, M., Becker, U., Johansen, D., Gottschau, A., Schnohr, P., Hein, H O., Jensen, G., and Sørensen, T.I.A.: Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. *Ann. Intern. Med.* 2000. 133. 411–419. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Redrawn with permission; Grønbaek, M. The positive and negative health effects of alcohol and the public health implications. *J. Intern. Med.* 2009. 265. 407–420. 2009. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Adapted with permission.)

Middleton et al. 2000). Ebeler et al. (2002) observed that the main monomeric polyphenol in wine, catechin (and/or its metabolites), significantly delayed tumor onset in the transgenic mouse model of neurofibromatosis. Wines with higher catechin levels appeared to be more effective in delaying tumor development; however, other wine phenols probably also played a role in the observed effects of wine solids on tumor development (Ebeler et al. 2002).

The polyphenols in wine are potent antioxidants, and while this activity has been recognized for decades (Singleton and Esau 1969), Frankel et al. (1993) were among the first to propose that this antioxidant activity may be a mechanism by which polyphenols exert their health protective effects. Many other potential mechanisms for the cancer chemopreventive effects of wine polyphenols have now been proposed and evaluated using *in vitro*, cell culture and animal studies; these mechanisms include effects on cell proliferation, apoptosis, inhibition and/or activation of enzymes or signal transduction pathways, hormone antagonism or inhibition, and enhancement of plasma urate levels (Singleton 1981; Middleton et al. 2000; Ferguson 2001; Nijveldt et al. 2001; Schmitt and Stopper 2001; Yang et al. 2001, 2008; Nichenametla et al. 2006; He et al. 2008; Modun et al. 2008; Pezzuto 2008; Virgili and Marino 2008; Petti and Scully 2009; Androutsopoulos et al. 2010; Fresco et al. 2010).

It is possible that dietary polyphenols act at multiple levels and their biological effects may be a result of a complex interplay of multiple cellular responses (Virgili and Marino 2008). While a full discussion of all of the proposed mechanisms is beyond the scope of this review, we will focus on recent results from our laboratory that support multiple roles for wine polyphenols acting directly or indirectly to influence DNA damage and repair processes. Since the biological effects of wine appear to be dependent on the phenol composition, we also provide a brief overview of factors influencing the phenol composition of wines, as well as factors influencing phenol bioavailability and metabolism.

WINE PHENOL COMPOSITION

In grapes, most phenols are associated with the skins and seeds (Adams 2006; Jackson 2008). During processing, the stems are removed from the berries and the grapes are typically gently crushed. For white wine production, pressing of the crushed grapes separates the skins and seeds from the juice, yielding a juice that is low in phenolics. The juice then undergoes alcoholic fermentation with yeast (typically *Saccharomyces cerevisiae*) to yield the final white wine. During red wine processing, the skins are not pressed prior to fermentation; the crushed grapes, or must, are fermented with skin and seed contact through all, or a portion of, the alcoholic fermentation. As a result, compared to white wines, finished red wines contain higher concentrations of total phenols due to the extraction of polyphenols from the skins and seeds during fermentation (Table 2.1). Estimates of total polyphenol concentration in red wine can be as high as 1500–3500 mg/L (Singleton 1981; Ritchey and Waterhouse 1999).

The phenols in grapes occur as both monomeric and polymeric (i.e., tannins) compounds and can be further separated into chemical classes based on structure (Figure 2.2). Among the monomeric phenols, the nonflavonoid phenolic acids

TABLE 2.1
Phenol Composition of Typical Table Wines from *Vitis vinifera* Grapes

Class[a]	White Wine	Red Wine
Nonflavonoids, total	**160–260**	**235–500**
Cinnamates, derivatives	130–154	150–165
Hydrolyzable tannins[b]	0–100	0–260
Other[c]	1–15	5–60
Flavonoids, total	**25–35**	**705–1060**
Catechins (flavan-3-ols)	15–25	150–200
Flavonols	tr[d]	10–50
Anthocyanins	0	20–200
Soluble tannins, derivatives	5–10	450–550
Other flavonoids, derivatives	Unknown	60?–75?
Totals	**19–290**	**955–1300**

Source: Adapted from Singleton, V. L., Grape and wine phenolics: Background and prospects, in *Grape and Wine Centennial Symposium Proceedings*, University of California, Davis, CA, A. D. Webb (Ed.), pp. 215–227, 1982.

Estimated in mg Gallic Acid Equivalents/L (mg GAE/L).

[a] General phenol classes given in bold.

[b] Hydrolyzable tannins occur if the wine is stored in oak cooperage.

[c] Other nonflavonoids include low-volatility benzene derivatives, tyrosol, resveratrol, and volatile phenols.

[d] tr, trace.

predominate in white wines. Flavonoids (also called proanthocyanidins), which are predominant in skins and seeds, can be further divided into classes based on substitution of the C-ring in the flavonoid structure (Figure 2.2). Substitution on the B-ring defines class members. Red grapes and wines are distinct from white grapes in that they contain anthocyanins, which contribute to the red color. Polymeric compounds are formed biochemically in the skins and seeds and chemically during fermentation and aging. The flavonoids combine in various substitution patterns to form oligomers and polymers, with varying degrees of galloylation also occurring. Due to the many possible combinations of proanthocyanidins present in grapes, the possible polymeric structures are very complex and have not been fully characterized (Herderich and Smith 2005; Adams 2006). The main phenols in red and white wines are summarized in Table 2.1. The actual phenol composition of a grape or wine will be dependent on the grape variety, growing conditions (including location, climate, soil, and viticultural practices), grape maturity at harvest, the winemaking and storage conditions (skin contact time, fermentation and storage temperatures, pressing conditions, filtration, and oak storage), and the age

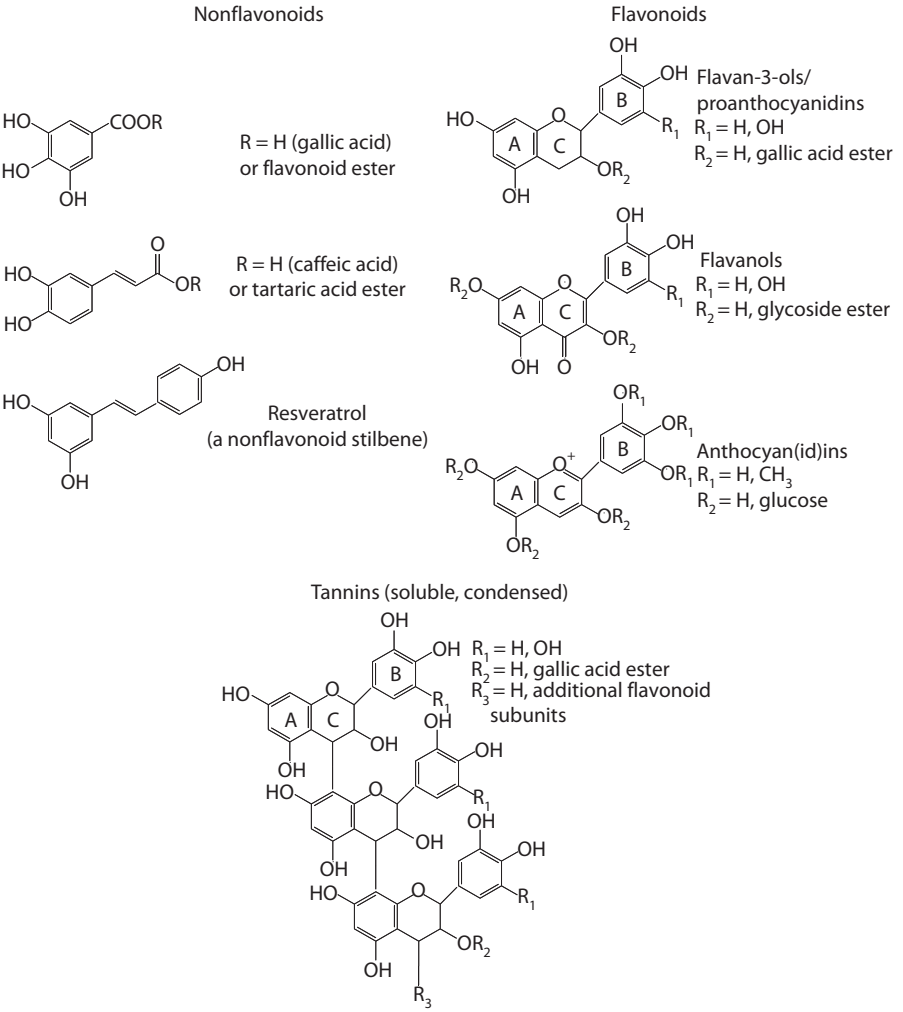

FIGURE 2.2 Basic structures of phenols found in grapes and wines.

of the wine or vintage (Boulton et al. 1996; Sacchi et al. 2005; Stockley and Høj 2005). An excellent overall review of plant polyphenols and their chemical properties has recently been published (Quideau et al. 2011).

The wine polyphenol resveratrol has received much attention for its potent cancer chemopreventive activity and resveratrol analogs with pharmacologic potential have been developed (Gatz and Wiesmüller 2008; Pezzuto 2008; Udenigwe et al. 2008). Although grapes and wines are an important dietary source of resveratrol, levels in grapes and wines are typically low (<5 mg/L; Goldberg et al. 1995; Lamuela-Raventos et al. 1995; Pervaiz 2003), particularly when compared to concentrations of other polyphenols (Table 2.1). As a result, the overall complexity of the polyphenol composition of grapes and wine makes it difficult to ascribe the health effects of

wine to any individual wine component. This is consistent with Femia et al. (2005) who observed that diets containing a total phenol extract from red wine, and not specific phenol fractions, were most effective in inhibiting chemically induced colon carcinogenesis in rats.

MECHANISMS OF TUMOR- AND CANCER-PREVENTIVE EFFECTS OF WINE COMPONENTS

As noted earlier, many mechanisms for the health protective effects of wine have been proposed. One of the most commonly proposed mechanisms is that the antioxidant activity of the wine polyphenols can help to prevent the damaging effects of endogenous and exogenous reactive oxygen species in the body (e.g., hydroxyl radical, hydrogen peroxide, superoxide, singlet oxygen, hypochlorite, nitric oxide, and peroxynitrite). Oxidative damage to cellular proteins, lipids, and DNA has been implicated as playing an important role in many degenerative diseases including coronary heart disease, neurodegenerative diseases, and cancer (Ames et al. 1993; Dhalla et al. 2000; Sayre et al. 2001; Dalle-Donne et al. 2006). Therefore, the presence of dietary components that can help to prevent these damaging effects may be beneficial. However, any evaluation of the biological effects of polyphenols must consider the low (200 nM–1 μM) doses of these compounds that have been observed in plasma and other tissues following ingestion, as well as the somewhat higher (mM) levels that may be present in the stomach, intestine, and colon (Abd El Mohsen et al. 2002; Ebeler et al. 2002; Rice-Evans 2004; Lambert et al. 2006; Halliwell 2007). A brief discussion of factors influencing bioavailability and metabolism of dietary polyphenols *in vivo* is presented in a later section of this review.

Effects on Oxidative DNA Damage

Oxidative modifications to DNA lead to formation of modified bases (e.g., 7,8-dihydro-8-oxo-2′-deoxyguanosine [8-oxo-dG, 8-OH guanine]), damage to the sugar moiety, and/or formation of DNA–protein crosslinks (Dizdaroglu 1991). These modifications can be highly mutagenic as, for example, the 8-OH guanine adduct can lead to G:C→T:A transversion. Using an *in vitro* assay, we screened selected flavonoids for their ability to prevent formation of different oxidative DNA adducts following hydrogen peroxide challenge. The modified DNA bases monitored included dihydrothymine, 5-hydroxy uracil, 5-hydroxy methyl uracil, 4,5-diamino-5-formamidopyrimidine (Fapy-adenine, the open imidazole ring structure), and 8-OH guanine. Interestingly, our results showed that very low levels (0.1 μM) of the wine flavonoids, catechin, quercetin, and cyanidin significantly decreased 8-OH guanine formation but not formation of any of the other modified bases (Min and Ebeler 2008). Also of importance was the observation that higher levels of the flavonoids did not always result in similar reductions in oxidative DNA adduct formation, and in some cases, flavonoids at concentrations of 1–100 μM actually induced adduct formation. The results indicate that the ability of flavonoids to prevent oxidative DNA damage depends on the flavonoid being studied, its concentration, and the

actual end-product being measured. Although 8-OH guanine has been widely studied as a marker of oxidative stress, measuring only one adduct may not present the full picture of the effects of dietary components on oxidative damage.

The aforementioned results are consistent with those observed in a separate *in vitro* study where selected flavonoid anthocyanins and their aglycone anthocyanidins induced single-strand breakage, or scission, of plasmid DNA (Webb et al. 2008a). We hypothesized that under the conditions of our assay, designed to mimic physiological pH (i.e., pH 7.5) and redox conditions (reaction buffer containing 1 mM dithiothreitol as a reductant), the anthocyani(id)ins form quinones that autoxidize to produce hydrogen peroxide (Singleton 1987). The hydrogen peroxide then degrades to hydroxyl radical in the presence of trace amounts of transition metals (e.g., Fe^{+3}), and the hydroxyl radical serves as the actual DNA cleavage agent (Sakano et al. 2004). While this increase in DNA damage may be initially considered negative, it has also been shown that anthocyan(id)ins are inhibitors of cell growth and can induce apoptosis, cell cycle perturbations, and differentiation in a variety of cell models (Kamei et al. 1995; Koide et al. 1997; Gasiorowski et al. 2001; Katsube et al. 2003; Forester and Waterhouse 2010). Therefore, the induction of DNA damage by anthocyan(id)ins and other polyphenols may initiate cellular responses that ultimately result in inhibition of tumor/cancer cell growth.

While *in vitro* assays can provide critical insight into mechanisms of action under controlled conditions, they do not take into account polyphenol metabolism *in vivo*. Therefore, we have also evaluated the effects of the flavonoid quercetin on DNA oxidation in an *in vivo* cell culture model using Caco-2 (human colon cancer) cells (Min and Ebeler 2009). In this study, we observed that quercetin at low concentrations (1 μM) had no effect on DNA single-strand breakage, while a higher quercetin concentration (100 μM) increased single-strand breakage. On the other hand, under oxidative stress conditions induced by hydrogen peroxide treatment, both concentrations of quercetin significantly decreased DNA single-strand breakage compared to control oxidant challenge without quercetin addition. As with the *in vitro* studies, these results point to complex effects of polyphenols on cellular processes, and importantly, the effects may be dependent on the concentrations studied as well as the redox status of the cell.

Intercalation and Topoisomerase IB Inhibition

Based on the studies mentioned earlier, we further evaluated the effects of polyphenols on cellular processes, including studies aimed at understanding how polyphenols can interact with DNA and DNA-maintenance enzymes. Using a screening assay developed in our laboratory (Webb and Ebeler 2003), we focused on intercalative binding of polyphenols to DNA and effects of polyphenols on the activity of the topoisomerase IB (topo 1) enzyme.

Intercalators, compounds with the ability to insert into a DNA strand, have been studied for their anticancer effects (see review by Wheate et al. 2007). We observed that polyphenols can intercalate into DNA, but the intercalation activity is strongly dependent on the structure of the polyphenol studied (Webb and Ebeler 2004; Webb et al. 2008a). Flavones and flavonols, which have a sufficiently planar

structure to insert within the DNA strands, had the greatest intercalating activity of the polyphenols studied. Among the most potent intercalating agents was quercetin, a common flavonol in grapes and wines. This intercalating activity was relatively weak overall however, and in our studies, the intercalating activity of the polyphenols was ~100 times weaker than the potent intercalator ethidium bromide. Interestingly, the anthocyan(id)ins did not have intercalating activity in our assay conducted under physiological pH conditions (pH 7.5). Although previous studies (Mistry et al. 1991; Sarma and Sharma 1999; Habermeyer et al. 2005) have observed that anthocyanins intercalate into DNA and RNA under mildly acidic conditions where the anthocyanins exist in the planar flavylium cation form, at higher pHs the quinone form would predominate and would be less likely to insert into the DNA.

Intercalation of compounds into DNA has also been associated with inhibition of topoisomerase enzymes involved in DNA maintenance and repair. Topoisomerase IB (topo I) regulates supercoiling of chromosomal DNA and plays a role in chromosome transcription, recombination, segregation, condensation, and repair. Topo I acts through a multistep process: the enzyme first binds to the DNA at the cleavage site, the DNA strand then breaks via nucleophilic attack of a tyrosine residue of topo I to the DNA phosphodiester at the cleavage site, and this is followed by unwinding of the DNA and relegation before the enzyme is released. Topo I inhibitors have been widely studied as antitumor drugs; these inhibitors block the DNA religation step and the resulting strand breakage leads ultimately to cell death (Dias and Bailly 2006). Using the same *in vitro* assay that assessed intercalation activity, we evaluated the ability of 34 polyphenolic compounds to act as topo I inhibitors. Flavones and flavonols that exhibited the strongest intercalation activity also inhibited (or poisoned) topo I; however, there was not a clear correlation between the degree of intercalation and the degree of poisoning ($R^2 = 0.213$) (Webb and Ebeler 2004). In addition, some non-intercalative flavonoids were also capable of inhibiting topo I. In general, there was a dose–response relationship between inhibition/poisoning and polyphenol concentration.

Induction of DNA Repair

The studies mentioned earlier indicate that the anticancer effects of polyphenols could be via inhibition of DNA maintenance and repair enzymes, resulting in cell death. An alternative hypothesis, however, is that low levels of DNA damage induced by polyphenols (e.g., through oxidation, intercalation, and/or topoisomerase inhibition) could result in a DNA-damage response that elicits DNA repair. The polyphenols could also act directly to induce DNA repair. Therefore, we used an *in vivo* cell culture system to evaluate the effects of the polyphenol, quercetin, on the mRNA expression of the DNA repair enzyme, human 8-oxoguanine DNA glycosylase (hOGG1) (Min and Ebeler 2009). This enzyme recognizes the oxidized adduct 8-oxoguanine opposite cytosine and cleaves it, leaving an abasic site. As noted earlier, quercetin alone induced some oxidative DNA damage in Caco-2 cells; however, it also induced expression of the repair enzyme hOGG1 especially at the higher quercetin concentration studied (100 μM). This indicates that the quercetin-induced

DNA damage may directly elicit a repair response in the cells. When quercetin was added to the cells following oxidant challenge with hydrogen peroxide however, even low concentrations (1 μM quercetin) significantly decreased the extent of DNA damage within the cells. In addition, quercetin treatment at 100 μM induced expression of hOGG1 mRNA, and the higher level of expression was maintained for a longer time than in cells that were not treated with quercetin. As a result, quercetin may protect DNA and enhance genome stability by acting to prevent the oxidative DNA damage that occurs under oxidative stress conditions, as well as by modulating DNA repair. Gao et al. (2006) have observed similar effects for the polyphenol naringenin, found in citrus. These studies are consistent with others showing that dietary antioxidants can have important effects on regulation of DNA repair processes (Cooke et al. 1998; Abalea et al. 1999; Torbergsen and Collins 2000; Collins and Harrington 2002; Astley et al. 2004; Silva et al. 2008).

Our results also show that there is a complex interplay between effects of dietary components on DNA oxidation, damage, and repair. Zhou and Elledge (2000) have proposed that DNA damage response and repair are interacting networks: DNA damage can lead to apoptosis and cell death; however, it can also control repair processes. Therefore, it is likely that the cancer chemopreventive effects of polyphenols found in wine act by multiple mechanisms that depend on the structures of the polyphenols present, their concentrations, and the cellular environment.

BIOAVAILABILITY AND METABOLISM CONSIDERATIONS

While *in vitro* and cell culture studies are invaluable for elucidating potential mechanisms of action of dietary polyphenols, they do not take into account their overall bioavailability and metabolism in the whole organism. Factors influencing bioavailability and metabolism of polyphenols have been previously reviewed (Scalbert and Williamson 2000; Yang et al. 2001, 2008; Forester and Waterhouse 2009; Nardini et al. 2009; D'Archivio et al. 2010). An overview of selected studies focusing on metabolism of wine polyphenols is provided here.

Polyphenols can exist in wines as glycoside conjugates or as "free" aglycones (Figure 2.1). During digestion, polyphenols are typically deglycosylated either by endogenous enzymes or by microflora in the gut. Additional enzymatic and chemical degradation reactions have also been shown to occur in the gut, for example, anthocyanins can be degraded to 3-*O*-methylgallic acid, syringic acid, 2,4,6-trihydroxybenzaldehyde, protocatechuic acid, and phloroglucinaldehdye (Forester and Waterhouse 2008; Kay et al. 2009). The aglycones and/or degradation products then pass through the intestines, where, following absorption, they are typically further metabolized via methylation, sulfation, and/or glucuronidation. For example, in humans, the predominant metabolites of catechin in plasma and urine following consumption of red wine were sulfated and glucuronidated forms of catechin and 3′-*O*-methylcatechin (Donovan et al. 1999, 2002). Due to the relatively rapid metabolism, circulating levels of the free aglycones are typically very low while the predominant metabolites will be dependent on the polyphenol studied and its dose (Scalbert and Williamson 2000; Spencer et al. 2008). Importantly, the structure of the metabolite can influence its persistence *in vivo*; for

example, sulfated and methylated metabolites of catechin were eliminated faster than unmethylated metabolites following consumption of red wine (Donovan et al. 1999, 2002). The kinetics of polyphenol metabolism as well as knowledge of tissue distribution may have important implications for understanding biological activity but have not been extensively studied.

When mice were fed increasing concentrations of catechin (0–4 nmol catechin/kg diet) in a nutritionally adequate diet, the time to tumor onset was delayed in a dose-dependent manner; however, increasing the amount in the diet to 8 nmol catechin/kg diet did not result in further delay in average tumor onset (Ebeler et al. 2002). Levels of plasma catechin and metabolites also appeared to reach a maximum when catechin levels were 4 nmol/kg diet with little further increase at 8 nmol catechin/kg diet (Ebeler et al. 2002). As noted by Scalbert and Williamson (2000), there appear to be mechanisms for maintaining circulating plasma levels at low levels when polyphenols are consumed at concentrations typical of a normal diet. However, further work is needed to understand these mechanisms and the factors influencing absorption, metabolism, and elimination of polyphenols when they are present in the diet at normal levels over long periods of time.

Most studies of polyphenol metabolism have focused on the monomeric polyphenols, while metabolism of dimers, oligomers, and polymers is less well understood. It is generally thought that the large molecular weight polymers are not well absorbed, although gut microflora may degrade them to various low molecular weight and monomeric phenolic compounds (Scalbert and Williamson 2000; Koleckar et al. 2008). Recently, Appeldoorn et al. (2009) observed that procyanidin dimers found in wine could be absorbed intact from the small intestine in rats. However, the extent of absorption was only 5%–10% that of the monomer, epicatechin, and was dependent on the dimer structure. The dimers were not conjugated or methylated following absorption. Trimers and tetramers were not absorbed; however, the presence of tetramers enhanced the absorption of some, but not all, dimers (e.g., absorption of dimer, B2, was enhanced, but absorption of dimer, A2, was not affected when a mixture of tetramers was present).

Total intake of dietary polyphenols is estimated at ~1 g/day and corresponding polyphenol concentrations in the stomach, intestine, and colon can thus be relatively high (mM levels); however, overall absorption and bioavailability of polyphenols is low and circulating levels in the plasma are typically <1 μM (Scalbert and Williamson 2000; Halliwell 2007). In rats, urinary metabolites of polyphenols accounted for ~10% of the red wine polyphenols consumed (Gonthier et al. 2003). Numerous factors can affect the bioavailability and metabolism of polyphenols, including the structure of the polyphenol, the quantity ingested, matrix effects and interactions with other compounds, and individual differences in metabolism (Kim et al. 2000; Scalbert and Williamson 2000; D'Archivio et al. 2010). Knowledge of the factors influencing the bioavailability, metabolism, and tissue distribution of polyphenols is critical for full understanding of the biological effects of wine on cancer and tumor development. However, a complete understanding of polyphenol metabolism is complicated by current limitations in analytical methodologies for detecting and identifying the numerous metabolites that may be present, particularly those present at low concentrations in target tissues.

INTERPRETATION OF EXPERIMENTAL STUDIES

While epidemiological studies can point to important relationships between wine consumption and cancer risk, detailed mechanistic studies are necessary to fully relate specific components of the diet to observed biological effects. Numerous *in vitro*, cell culture, and animal studies have proposed multiple mechanisms by which wine polyphenols could exert health-protective benefits. However, obtaining an integrated view of these mechanisms is difficult due, at least in part, to the diversity of polyphenol structures in grapes and wines as well as to a limited understanding of their chemistry and solution behavior *in vivo* (Webb and Ebeler 2004; Webb et al. 2008b). Factors such as pH, ionic strength, redox conditions, trace metals, etc., can all influence the reactivity of polyphenols in biological systems and may not always be considered in experimental studies. Further, polyphenols exist in equilibrium between ionized, unionized, and aggregated forms, and these forms can significantly influence their chemistry and biological activity (Webb and Ebeler 2004).

While the epidemiology linking moderate wine consumption with reduced cancer risk remains somewhat controversial, it is clear that the polyphenol components of wine may play a role in preventing degenerative diseases. An improved understanding of the relationship between moderate wine consumption and cancer will require advances in many areas. For example, improved analytical methods are needed to allow accurate characterization of the complex polyphenol composition of wines as well as the metabolites that occur *in vivo* following consumption. In addition, an improved understanding of how the polyphenol composition and other components of whole foods (e.g., wine) influences bioavailability and metabolism of individual polyphenols is needed, along with studies that evaluate the chemopreventive mechanism-of-action of these complex polyphenol mixtures. Finally, an improved understanding of the interplay between multiple reaction mechanisms is needed in order to take into account both direct and indirect effects of wine polyphenols on cellular responses to DNA damage.

Recent studies are showing that polyphenols in wine can act to prevent oxidative DNA damage at low physiological levels. In addition, although polyphenols may also cause DNA damage in some cases, this may result in an overall induction of multiple DNA repair processes. Based on results from these and a range of *in vitro* and *in vivo* studies, it may be possible in the future to identify dietary interventions targeted toward enhancing genome maintenance and repair and ultimately leading to improved health and reduced incidence of degenerative diseases such as cancer.

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3 Anthocyanins and Heart Disease

Janet A. Novotny

CONTENTS

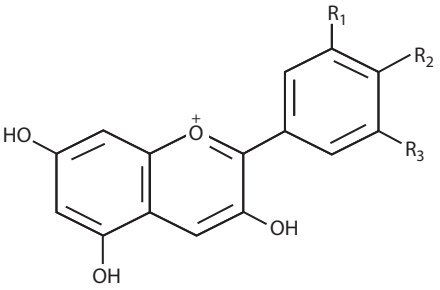
INTRODUCTION

Anthocyanins are red, blue, and purple pigments distributed throughout nature. The pigments in flowers are present to attract pollinators. In fruits, anthocyanins attract animals, which eat the fruits and thus allow distribution of seeds. In leaves, anthocyanins act as sunscreen, protecting the plant from excessive light damage. In our diet, anthocyanins add color to our plates and provide a variety of health benefits.

One potential health benefit of dietary anthocyanins is protection against cardiovascular disease (CVD). CVD was listed in the most recent National Vital Statistics Report (Heron et al. 2009) as the leading cause of death in the United States, accounting for 26% of all-cause mortality. The overall cost of CVD in the United States has been estimated at 316.4 billion dollars annually (Lloyd-Jones et al. 2010). Thus, there is great motivation to identify lifestyle changes that could impact the incidence of heart disease. Inclusion of anthocyanin-rich foods into the diet may be one lifestyle trait that could provide benefits for cardiovascular conditions. Evidence for beneficial effects of anthocyanins with respect to heart disease includes epidemiology, animal studies, cell studies, and clinical studies, but there are also a few studies that have failed to show a relationship between anthocyanins and factors related to CVD. Some of these studies were characterized by weak study designs, while others suggest that the relationship between anthocyanins and CVD may be complex and influenced by many factors, some of which may be difficult to identify.

ANTHOCYANIN TYPES AND SOURCES

Anthocyanins are flavonoids comprised of anthocyanidin backbones (Figure 3.1) with various sugar and acyl side groups attached. More than twenty anthocyanidin backbones have been identified, but only six are widely found in fruits and vegetables, and those six anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin,



Anthocyanidin	R_1	R_2	R_3
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Malvidin	OMe	OH	OMe
Pelargonidin	H	OH	H
Peonidin	OMe	OH	H
Petunidin	OMe	OH	OH

FIGURE 3.1 Structure of anthocyanidin.

TABLE 3.1
Anthocyanin Contents of Fruits and Vegetables

Fruits	Anthocyanin Content (mg/100 g)	Vegetables	Anthocyanin Content (mg/100 g)
Elderberry	1375	Red cabbage	322
Black raspberry	687	Red radish	100
Black currant	476	Eggplant	86
Blueberry	365	Red onion	49
Blackberry	245	Black beans	45
Cranberry	140	Red beans	7
Black plum	125	Red lettuce	2
Cherry, sweet	122		
Concord grape	120		
Red raspberry	92		
Nectarine	7		
Peach	5		
Apple	1–12		

Source: Data from Wu, X. et al. *J. Agric. Food. Chem.*, 54(11), 4069, 2006.

petunidin, and malvidin. Common sugar attachments include xylose, arabinose, rhamnose, galactose, and glucose. The sugar attachments can be further substituted with aliphatic, hydroxybenzoic, or hydroxycinnamic acids, most commonly acetic, malonic, *p*-coumaric, and caffeic acids, and less commonly oxalic, succinic, malic, *p*-hydroxybenzoic, gallic, vanillic, syringic, protocatechuic, ferulic, and sinapic acids. The various combinations of anthocyanidins, sugars, and acylations result in over 600 anthocyanins identified in nature.

In the diet, anthocyanins are primarily found in fruits, especially berries. Good sources of anthocyanins include foods like strawberries, blueberries, blackberries, raspberries, red cabbage, and beans. Table 3.1 lists sources of dietary anthocyanins and their content. A serving of a high-anthocyanin food can deliver several hundred milligrams of anthocyanins. Mean anthocyanin consumption per person has been estimated to be 12.5 mg/day in the United States (Wu et al. 2006), 2.9 mg/day in Australia (Johannot and Somerset 2006), 7.6 mg/day in Belgium (Mullie et al. 2007), and 47 mg/day in Finland (Ovaskainen et al. 2008).

EPIDEMIOLOGICAL ASSOCIATIONS BETWEEN ANTHOCYANINS AND HEART DISEASE RISK

Most epidemiological studies related to anthocyanins and disease have focused on flavonoids, the polyphenol category that includes anthocyanins, rather than anthocyanins themselves. Epidemiological studies have produced mixed results with respect to the association between flavonoids and risk for CVD. Some studies have suggested that flavonoids provide protection against CVD, while others have found no association. A study conducted in the Netherlands in which men and women were interviewed about their diet suggested that dietary flavonoids were inversely associated with fatal myocardial infarction (Geleijnse et al. 2002). In another population-based study of elderly men in the Netherlands, there was also an inverse relation between flavonoid intake and myocardial infarction and an inverse relation between flavonoid intake and mortality from coronary heart disease (Hertog et al. 1993). Data from the same study suggested that flavonoid intake may reduce incidence of stroke (Keli et al. 1996). In Finland, men and women consuming high levels of flavonoids had decreased risk of coronary mortality (Knekt et al. 1996). Data from the Seven Countries Study, a large epidemiological study to investigate the impact of lifestyle on coronary heart disease and stroke, suggested that average flavonoid intake was inversely associated with coronary heart disease mortality (Hertog et al. 1995). Looking specifically at anthocyanins, the Iowa Women's Health Study surveyed almost 35,000 postmenopausal women to find significant inverse associations between anthocyanins and coronary heart disease incidence and between anthocyanins and CVD mortality (Mink et al. 2007).

Several studies have also been published that have failed to uncover a relationship between dietary flavonoids and CVD. In a population of Finnish men, different classes of flavonoids were examined to find no association between anthocyanins and CVD (Mursu et al. 2008). Two very large prospective studies called the Women's Health Study and the Nurses' Health Study showed no association between flavonoid intake and CVD, including myocardial infarction, stroke, and CVD death (Sesso et al. 2003;

Lin et al. 2007). In a large prospective study of men, flavonoid intake did not reduce risk for myocardial infarction among the whole population, but there was a trend for lower coronary mortality rates among men that had previously had coronary heart disease (Rimm et al. 1996). Specific examination of strawberry intake also showed no association with various markers of CVD risk (Sesso et al. 2007).

While the results of large cohort studies have been mixed, there is sufficient evidence to suggest a link between flavonoids and CVD. These positive associations have prompted further research into flavonoids and different subclasses of flavonoids, including anthocyanins, with animal models and human interventions.

EVIDENCE FROM ANIMAL STUDIES

Evidence from animal studies has generally been supportive of a role of anthocyanin-rich products for protecting against CVD. Several animal studies have suggested that anthocyanins can improve plasma or serum lipid profile. Hamsters fed mulberry extract for 12 weeks had lower serum triglyceride, lower total cholesterol, and lower LDL to HDL (low-density lipoprotein to high-density lipoprotein) cholesterol ratio (Peng et al. 2011). Mice fed a high-fat diet with powders from freeze-dried blueberry or freeze-dried strawberry added to the water had serum cholesterol and triglyceride levels that were lower than those of mice consuming unsupplemented water (Prior et al. 2009). Rabbits fed ground black rice, which is black due to its rich anthocyanin content, had increased HDL cholesterol compared to rabbits supplemented with ground white rice (Abdel-Moemin 2011); the primary difference in the diets was the anthocyanin content, suggesting the anthocyanins (or their metabolites) were the active components in these findings.

Several anthocyanin studies have been conducted with apo-E-deficient mice. The apo-E-deficient mouse is a popular animal model of atherogenesis because of its propensity to spontaneously develop atherosclerotic lesions. Apolipoprotein E is an apolipoprotein that binds to a receptor on liver and peripheral cells and is important for removal of lipid from the blood stream. In one study, the diet of apo-E-deficient mice was supplemented with bilberry extract for 16 weeks, and the result was a significant inhibition of plaque development. These researchers found no change in oxidative stress status nor lipid profile; thus, they concluded that other mechanisms, such as anti-inflammatory effects, may be responsible (Mauray et al. 2009). Reduced plaque formation was also found in a study of apo-E-deficient mice fed black rice extract for 20 weeks (Xia et al. 2006). In this study, both serum triglycerides and serum total cholesterol were lower for the mice fed black rice extract. Decreased plasma total cholesterol was also observed in apo-E-deficient mice fed bilberry extract for 2 weeks (Mauray et al. 2010). In this experiment, global gene expression showed upregulation of genes related to bile acid synthesis and cholesterol uptake by liver, as well as downregulation of proinflammatory genes. A key metabolite of anthocyanins called protocatechuic acid has also been found effective against processes involved in progression of CVD, thus opening the possibility that the parent anthocyanin compounds and/or their metabolites may both have roles in cardiovascular protection. When apo-E-deficient mice consumed protocatechuic

acid for 20 weeks, aortic I-CAM and V-CAM expression was reduced, nuclear factor κB activity was inhibited, and plasma I-CAM and V-CAM levels were lower (Wang et al. 2010). These processes are important, as I-CAM and V-CAM are adhesion molecules whose level is positively correlated to cardiovascular events, and nuclear factor κB modulates immune response.

EVIDENCE FROM CLINICAL INTERVENTION STUDIES

While evidence from animal studies for the role of anthocyanins in lowering risk factors or inhibiting mechanisms related to CVD has been for the most part positive, evidence from clinical studies has been mixed. Some clinical studies have suggested a positive effect of anthocyanins for reducing CVD risk, while others have shown no influence on markers of coronary artery disease. Some of the studies with null results have been characterized by poor study design (e.g., interventions too short to affect the mechanisms in question). Generally, the clinical studies have had a broad range of outcome variables, including blood lipids, platelet reactivity, markers of inflammation, and vascular reactivity.

Studies of the influence of anthocyanins on blood lipids have had mixed results. Two studies have shown effects of anthocyanins on blood lipids, the most common measure of CVD risk. In one study, middle-aged volunteers consumed mixed berries twice daily for 8 weeks. The berry treatment was comprised of bilberries, lingonberries, black currants, strawberries, chokeberries, and raspberries. Serum HDL cholesterol concentrations increased significantly after berry consumption (Erlund et al. 2008). When 120 dyslipidemic adults consumed 160 mg anthocyanins from bilberry and black currant twice daily for 12 weeks, their LDL cholesterol concentrations decreased and their HDL cholesterol concentrations increased (Qin et al. 2009), both of which would be associated with reduced risk for cardiac events. The anthocyanin treatment also inhibited cholesteryl ester transfer protein (CETP). CETP is a protein found in the blood that exchanges cholesteryl esters and triglycerides between lipoproteins. CETP affects coronary disease by transferring cholesteryl esters from HDLs to very-low-density lipoproteins and LDLs. Increased activity of CETP, which can be caused by a genetic mutation, is associated with development of atherosclerosis. Thus, the inhibition of CETP by anthocyanins would provide protection against atherosclerosis. Note that these subjects were dyslipidemic at the beginning of the study, which may have contributed in the ability for the anthocyanins to be effective. In contrast, postmenopausal women ingesting elderberry extract providing 500 mg anthocyanins per day for 12 weeks showed no improvement in total cholesterol, HDL cholesterol, or LDL cholesterol (Curtis et al. 2009). In another study with young volunteers consuming elderberry juice powder (400 mg containing 10% anthocyanins) for 2 weeks, there was no improvement in plasma cholesterol or resistance of LDL to become oxidized (Murkovic et al. 2004). Cranberry juice consumption for 2 weeks did not alter HDL cholesterol, LDL cholesterol, total cholesterol, or triglycerides (Duthie et al. 2006). However, the latter two studies should be interpreted with caution because 2 weeks is generally considered insufficient to significantly affect blood lipids. Overall, the studies with sufficient intervention length suggest that anthocyanin-rich preparations are protective against CVD.

Clinical studies have suggested that anthocyanin-rich preparations can reduce inflammation. Inflammation may impact CVD in multiple ways. Inflammation may facilitate the development of deposits on the vessel walls, or it may cause rupture of atherosclerotic plaques, thus causing a cardiac event. Consumption of 330 mL/day of bilberry juice for 4 weeks resulted in significant decreases in several markers of inflammation, including C-reactive protein, interleukin-6, interleukin-15, and interferon gamma (Karlsen et al. 2010). Similarly, a supplement of 300 mg/day of anthocyanins isolated from bilberries and black currants reduced several indicators of inflammation, including interleukin-4, interleukin-8, and interleukin-13 in adult volunteers after 3 weeks (Karlsen et al. 2007). In contrast to these positive results, elderberry extract ingested by postmenopausal women for 12 weeks did not improve markers of inflammation, including interleukin-6 and C-reactive protein (Curtis et al. 2009). It is possible that the bilberry compounds are more effective at reducing inflammation than the elderberry compounds, though the data are too sparse to draw that conclusion with confidence. More studies are needed to determine which anthocyanin-rich products are effective for reducing inflammation. Anthocyanins may also affect platelet function and clotting in humans. Platelets are cells in the blood that are involved in clotting and can also play a role in heart attacks, strokes, and peripheral vascular disease. Fibrinogen is a plasma protein involved in the clotting cascade. In CVD, abnormal clotting occurs that can result in heart attacks or stroke; thus, the dietary interventions that disrupt the clotting cascade are considered favorable. A clinical study with berries showed interference with clotting. Each day for 8 weeks, middle-aged volunteers consumed a variety of berries, including bilberries, lingonberries, black currant or strawberry puree, or chokeberry or raspberry juice. Platelet function was inhibited by the intervention, though platelet activation, coagulation, and fibrinolysis did not change (Erlund et al. 2008). Platelet reactivity was not affected when postmenopausal women consumed elderberry extract for 12 weeks (Curtis et al. 2009). While the positive effect of mixed berries on platelet function is encouraging, more data are needed to understand the potential role of anthocyanins in modulating platelet function.

CONCLUSION

In summary, while there are a few clinical studies that failed to show a relationship between anthocyanin intake and biomarkers for CVD processes or risk, there are also many studies, both with animals and with humans, suggesting that anthocyanins exhibit protective effects. These protective effects are broad and include improvement of blood lipids, reduction of inflammation, and possibly inhibition of platelet function. While the null results, when considered with the positive results, may seem contradictory, these different results are probably due to differences in study design, including dose, length of intervention, target population, specific anthocyanins used for treatment, and other uncontrolled factors. Nonetheless, when taken as a whole, the body of evidence for anthocyanins in inhibiting mechanisms involved in the progression of CVD and reducing CVD risk factors is generally positive, and it is likely that anthocyanin-rich dietary components offer protection from CVD in addition to wonderful colors on our plates.

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4 Multidisciplinary Studies of Anti-Inflammatory Botanicals
Ginger and Turmeric

Barbara N. Timmermann and Janet L. Funk

CONTENTS

INTRODUCTION

Complementary and alternative medicine (CAM) therapies are used worldwide and are rapidly growing in popularity in the United States. CAM interventions include herbal remedies, megavitamins, minerals, and diet products, the majority of which come from natural sources (McHughes and Timmermann 2005). Indeed, natural products are the CAM therapy most frequently used in the United States (Barnes et al. 2008a).

In 1994, Congress passed the Dietary Supplement Health and Education Act (DSHEA), with the mission to increase research and information about dietary supplements for the purpose of educating the public and improving health care (Institute of Medicine of the National Academies 2005), specifically targeting botanical products that were purported to have health-related benefits to be marketed as foods, dietary supplements, or new botanical drug products (Chen et al. 2008). Some of these phytotherapeutics have been proposed to be both more effective and less toxic than standard pharmaceutical options. However, evidence in support of some of these claims is tenuous at best and sometimes involves products that have not been properly characterized, standardized, or evaluated for human consumption (Swanson 2004).

Since the complexity of some natural products may include multiple compounds that are primarily bioactive, as well as others that enhance delivery or efficacy of those primary components, in-depth research is necessary to elucidate and evaluate each constituent. These studies are often complicated, as complex products cannot be subjected to the same methods of investigation as isolated, purified compounds (Chen et al. 2008). While challenging, natural products research provides important insights into the strength and safety of botanical supplements and can also aid in the understanding of diseases on a molecular or mechanistic basis (Barnes et al. 2008b).

The National Center for Complementary and Alternative Medicine (NCCAM 2012) of the National Institutes of Health (NIH) was established in 1999 with the mission of exploring current CAM therapies in the context of rigorous science, including the current and potential uses of botanical supplements. Through funding and training opportunities, NCCAM continues to advance scientific research in this field, with the purpose of expanding current therapeutic options, in addition to educating both physicians and the general public on the benefits and risks of alternative therapies. Among their investigations are pharmacology; bioavailability; and absorption, distribution, metabolism, and excretion (ADME) studies for biologically based interventions. This includes optimizing dosage levels, validating results of treatments, and obtaining preliminary data for safety and efficacy, including early phase clinical investigations, to determine mechanisms of action (NCCAM 2009).

The NIH Office of Dietary Supplements (ODS), an office specifically charged with strengthening knowledge and understanding of dietary supplements, including but not limited to botanicals, has partnered with NCCAM in an attempt to improve U.S. botanical research efforts by funding the Botanical Research Centers Program. These Centers, which have been competitively assigned to major research institutions in the United States for 5 year periods since 1999, are expected to advance the scientific base of knowledge about botanical safety, efficacy, and biological action, with each center focusing on different botanicals and/or disease states (Swanson and Liu 2008). The following work represents a highlight of results from the collaborative research efforts of one such center, the Arizona Center for Phytomedicine Research (AzCPR), which was established at the University of Arizona in 2000. The multidisciplinary work of this center focused on the safety and efficacy of several botanicals alleged to have anti-inflammatory activities and to be specific in the treatment and prevention of rheumatoid arthritis (RA), which is the focus of our investigations, or other chronic inflammatory diseases.

Various preparations of rhizome of turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale* Rosc.) are widely used food additives of the tropical and subtropical Zingiberaceae family and have become increasingly popular for modern medicinal use, based on their traditional benefits described in ayurvedic and other traditional medical systems. Curcumin, as a major and important constituent of the turmeric rhizome, exhibits many of these medicinal properties, including anti-inflammatory, antiulcerogenic, and antitumor activities, among others (Aggarwal and Shishodia 2006, Choi et al. 2006, Maheshwari et al. 2006). [6]-Gingerol, as a major component of the ginger rhizome, has also been shown to possess physiological and pharmacological activities, which include anti-inflammatory, analgesic, and cardiotonic effects (Jiang et al. 2006d). However, each of these chemicals is but one of hundreds present in botanical preparations extracted from these plants and used medicinally. To scientifically address questions of efficacy and biological action, the composition of the botanical at hand is a critical, complex, and often overlooked part of the scientific puzzle that adds significant value to the assessment of medicinal benefit and safety. Therefore, spanning research efforts from botany and agronomy to analytical chemistry and preclinical trials, the AzCPR initiated a full spectrum of scientific inquiry to assess these botanicals: the chemistry of active compounds or mixtures of compounds; their efficacy, safety, and mechanisms of action; and their pharmacodynamics, pharmacokinetics, and oral bioavailability. The results reviewed here provide an example of the extraordinary knowledge that can be gained when modern scientific methods are applied to the study of ancient botanical remedies and the past and future potential for the federally mandated and directed Botanical Research Centers Program to significantly impact and improve public health.

CHEMICAL ANALYSIS BY MASS SPECTROMETRY

Research efforts to characterize the safety and efficacy of any natural product, in order to be clinically useful and subject to replication, must necessarily begin with a careful identification and description of the product to be tested. For multicomponent botanicals, this requirement is complicated by the complexity and natural variety of these products, as well as a lack of standardized methodologies for characterizing their content. In 2001, the ODS established the Dietary Supplements Methods and Reference Materials Program to specifically promote analytical method development and standardization, as well as the validation of botanical and other reference materials for use by academic institutions, regulatory agencies, industries, and clinical testing (Saldanha et al. 2004). In this regard, the AzCPR directly addressed these goals by developing validated analytical methods and using these to standardize ginger and turmeric samples prior to analysis of their biological properties.

A living collection of over 100 accessions of different members in the Zingiberaceae was established for the purpose of these studies. Samples obtained from commercial sources, agricultural fields, and botanical museums provided fresh materials for taxonomic identification and phylogenetic, morphological, and genetic studies, including microarray analysis and validation of phylogenetic trees. Taxa within the collection included medicinal species of diverse

Zingiberaceae, including various species of *Curcuma*, *Zingiber*, *Hedychium*, *Alpinia*, and *Kaempferia*, enabling a multitude of insights and discoveries regarding chemical characterization and bioactivity analyses of these botanicals as validated phytomedicines.

To investigate whether or not the claims of medicinal activity for ginger and turmeric were indeed valid, it was first necessary to characterize fully the samples to be investigated prior to *in vivo* testing for antiarthritic efficacy and mechanism of action. Several complementary methods were used to achieve this goal, including (1) analytical mass spectrometry (MS), (2) gas chromatography coupled with MS (GC-MS), (3) liquid chromatography coupled with MS (LC-MS), (4) high-pressure liquid chromatography (HPLC), and (5) LC-electrospray ionization tandem mass spectrometry (ESI/MS/MS). Analytical MS is a tool that provides critical support for the characterization and identification of natural products and other complex chemical structures. Prior to the advent of MS, these types of analyses were difficult, time-consuming, and even inaccurate at times. Additionally, samples of natural products in powdered biomass or solvent extract forms proved impossible to use for DNA extraction and chemotaxonomic phylogenetic profiling analysis (Jiang et al. 2006d). GC-MS is useful for compounds that are volatile or thermally labile and is often used with success. However, the utilization of LC-MS in the analysis of biomedical plant samples additionally enables the identification of nonvolatile compounds, such as those with relatively long carbon chains (Jiang et al. 2005). LC-MS continues to provide insights, both into the composition of botanicals and their uses and into the utilization of MS in many forms as a valuable analytical tool for natural products chemistry and metabolic studies.

Ginger

Our first metabolic profiling studies of medicinal ginger (*Z. officinale*) allowed us to differentiate between the compounds present in each analyzed sample of frozen fresh ginger rhizome (Jiang et al. 2006d). Extractions with methyl *t*-butyl ether or methanol were followed by GC-MS analysis, enabling the identification of more than 60 components. These plants, obtained from diverse populations, had been grown under the same conditions in the greenhouse. This qualitative GC-MS analysis revealed that, upon removing the environmental variables, the ginger samples exhibited similar metabolic fingerprints with respect to their volatile components (Jiang et al. 2006d). In contrast, other *Zingiber* species were significantly divergent, and many of the compounds identified were unique to few or only one of the species. By using these compound markers to aid in differentiation between various accessions, it was demonstrated that GC-MS could be used to identify species of *Zingiber* or contaminants from other species or plant sources that could be present in wild samples (Jiang et al. 2006d).

While GC-MS provided a means for qualitative analysis of *Z. officinale* samples, HPLC was used for assessing the constituents quantitatively. These results support the contention that ginger from different sources is likely to possess different properties both in flavor and pungency and potentially in terms of bioactivity and

health benefits. Identification of genes involved in regulating the production of these compounds is a major goal of future research in this area (Jiang et al. 2006d).

In subsequent studies, LC-MS was utilized as a means of identifying those compounds for which GC-MS was ineffective, due to low compound volatility and thermal instability. However, it was quickly realized that MS on a single-dimensional level was insufficient for the identification of all known and unknown compounds present in the ginger samples. Preliminary studies using LC-ESI/MS/MS, however, showed potential as a powerful option for specific analysis of these gingerols and related compounds (Jiang et al. 2005). Using both negative and positive ionization ESI-MS/MS coupled to diode array detection instrumentation (with maximum ultraviolet [UV] absorption for gingerols at 280 nm and a shoulder at 230 nm; those with extended conjugation exhibited a wavelength of 425 nm as well), fragmentation data were collected for the purpose of characterizing the *Z. officinale* group of compounds. Using mass spectra, UV spectra, and chromatographic characteristics, 3 new acetylated gingerdiols of 33 total gingerol-related compounds were identified (Figure 4.1) (Jiang et al. 2005). The chemical structures identified included gingerols, methylgingerols, gingerol acetates, shogaols, paradols, gingerdiols, mono- and diacetyl gingerdiols, and dehydrogingerdiones; all were detected as protonated molecular ions or ammonium/sodium adduct ions in (+)ESI-MS. (+)ESI-MS/MS spectra were obtained for all compounds, with fragmentation behavior used to confirm molecular structures (Jiang et al. 2005).

Many of these compounds were only detected by the MS detector, suggesting that LC-MS analysis is both highly specific and highly sensitive to this particular group of compounds (Jiang et al. 2005). It was also noted that selective ion chromatograms from MS and MS/MS analyses can be used to distinguish compounds with very close HPLC retention times, which is especially useful in analyses of crude extracts without previous fractionation (Jiang et al. 2007). HPLC/ESI-MS/MS coupled to diode array detection, therefore, was proven to be an efficient and effective tool for the identification of the gingerol-related compounds.

Turmeric

Turmeric (*C. longa*), another member of the Zingiberaceae known to possess medicinal properties, was also studied with the intent of identifying its components and determining levels of activity. Curcuminoids including curcumin, demethoxycurcumin, and bisdemethoxycurcumin have been identified as the major diarylheptanoids responsible for many of the medicinal properties of this species, which include anticancer, anti-inflammatory, and anti-HIV activities (Aggarwal and Shishodia 2006, Choi et al. 2006, Maheshwari et al. 2006). Our initial studies used single-dimension MS with only positive ionization to collect data, an insufficient means for detecting and identifying compounds within the samples or producing detailed structural information (Jiang et al. 2006c). It was hypothesized that the use of both positive and negative LC/ESI-MS/MS might (1) enable the identification of additional compounds in the *Curcuma* species that might also contribute to the medicinal qualities of these natural products and (2) provide detailed structural information for these yet unknown compounds.

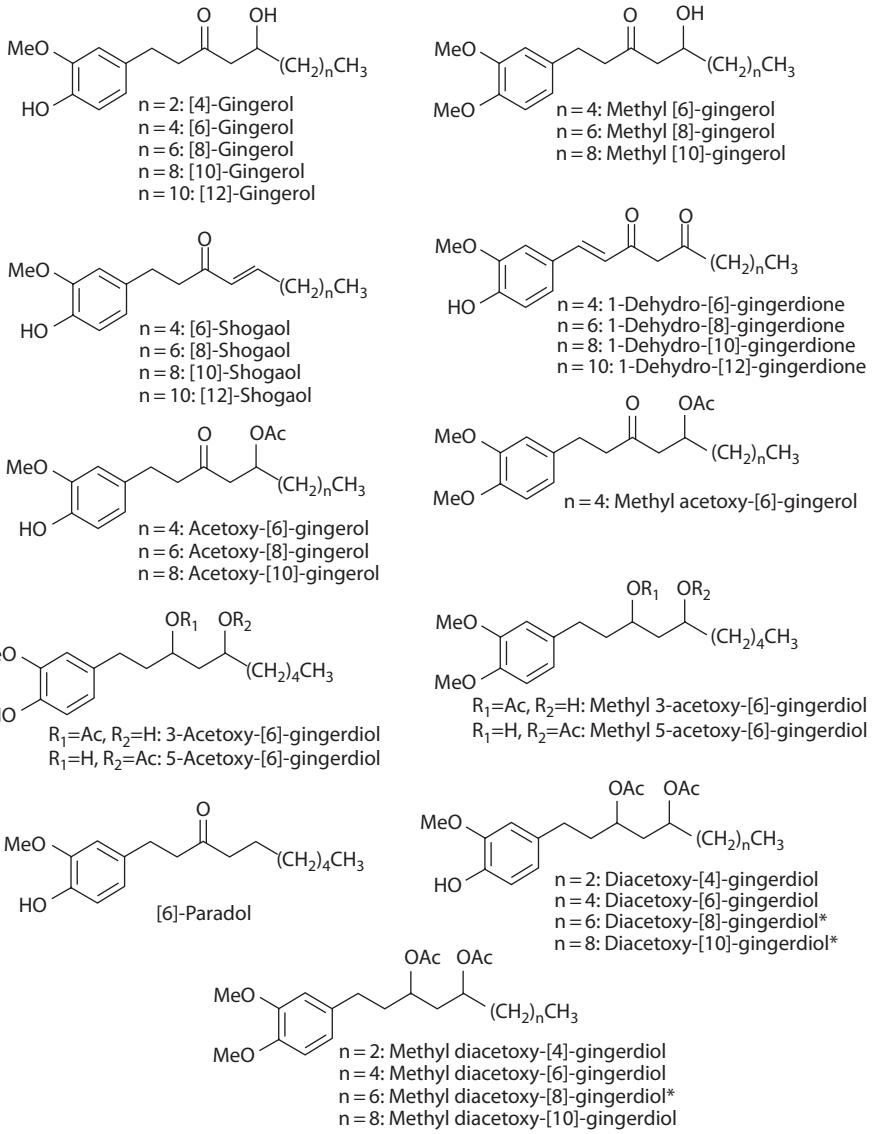

FIGURE 4.1 Chemical structures of characterized gingerol-related compounds, isolated from ginger rhizome. Note: * indicates new compound. (From Funk, J.L., Turmeric, in *Encyclopedia of Dietary Supplements*, 2nd edn., Chapter 87, Informa Healthcare, New York, 2010.)

The LC/ESI-MS/MS analytical methods that had proven successful for ginger analysis now enabled the content determination of even low levels of specific metabolites within the turmeric samples, and in-line diode array detection allowed for significant improvements in accuracy of identification and quantitation. In particular, (–)ESI-LC/MS proved successful in identifying diarylheptanoids from

methanolic extracts of fresh frozen turmeric rhizome samples; in contrast to the preferred positive mode analysis used for the gingerols, it provided simpler chromatograms for interpretation. (+)ESI-LC/MS was once again used for advanced structural analysis and determination. Through the chromatographic and mass spectral data from these studies, 13 diarylheptanoids were conclusively identified, 7 of which had not previously been identified from the turmeric rhizome (Figure 4.2) (Jiang et al. 2006c).

Subsequent work was completed toward the understanding of the fragmentation behavior of bisdemethoxycurcumin, demethoxycurcumin, and curcumin, for the

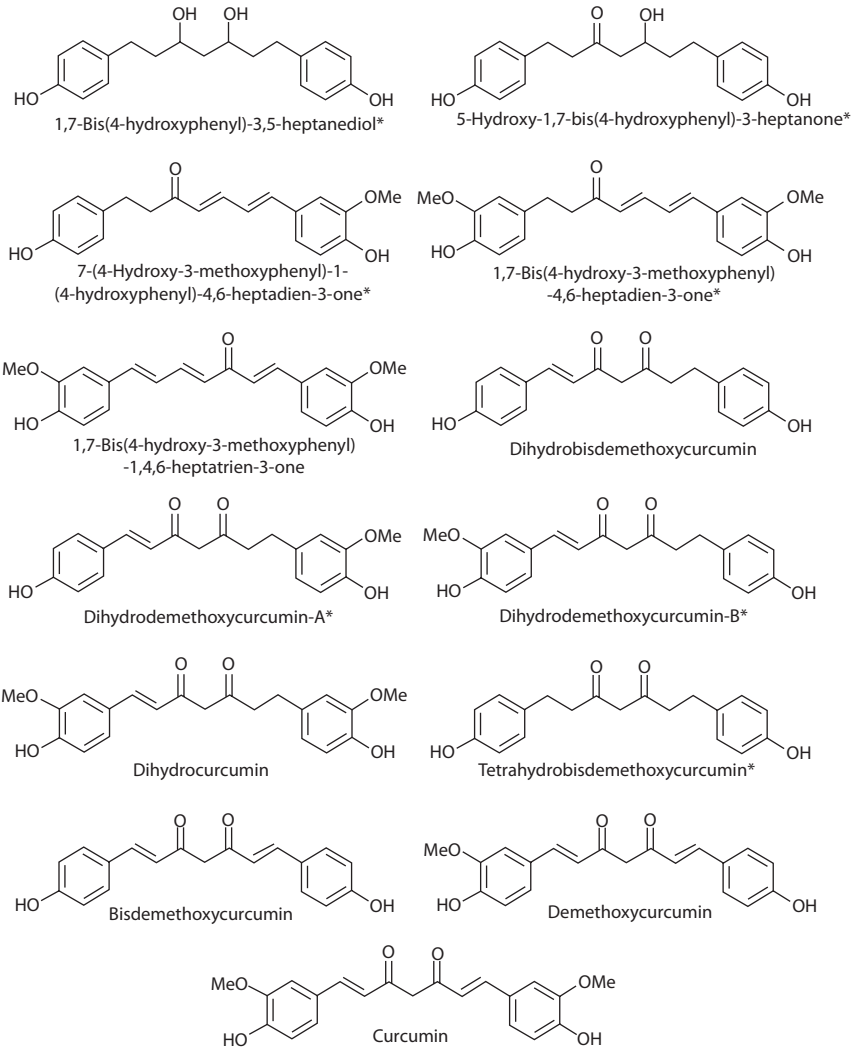

FIGURE 4.2 Diarylheptanoids identified from turmeric. Note: * indicates compound not previously identified from turmeric rhizome. (From Jiang, H. et al., *Rapid Commun, Mass Spec*., 19, 2957, 2005.)

purpose of being able to identify unknown curcuminoids in other samples of turmeric or related plants. Again using methanolic extracts of turmeric samples, both positive and negative ionization ESI were utilized for tandem and multidimensional MS experiments. Quadrupole ion trap instruments, high-resolution and accurate mass MS instrumentation, and sustained off-resonance irradiation (SORI) MS/MS were utilized in these studies to confirm all results (Jiang et al. 2006a).

Additionally, the synthesis of a number of phenolic monoacetates of the curcuminoids was necessary to determine which ions were present in MS analysis, particularly in light of the ability of the curcuminoids to tautomerize between their keto and enol forms. Ion traps from different MS instrumentation manufacturers were also tested with stock compounds for the purpose of ensuring that similar ionization and fragmentation methods were utilized and thus could provide reproducible results regardless of instrumentation. Through these studies, specific fragmentation profiles were identified in order to match patterns of compounds of interest for future studies of curcuminoids, using methods that can easily be translated to analysis of related compounds as well (Jiang et al. 2007). In contrast, another study revealed that differences in instrumentation conclusively *did* yield results that differentiated significantly (Jiang et al. 2006b). This work analyzed the previously described gingerols of the ginger rhizome in place of curcuminoids but followed the same ion trap LC/ESI-MS/MS methodology as had been used for the analysis of the turmeric species. Once again, the methodology proved effective in producing advanced structural data and confirmation of patterns within the species as shown with the curcuminoids. However, while consistent results were obtained for all of the compounds using the same instrument, discrepancies arose when using instruments from separate manufacturers. These problems were traced to the ability of the various instruments to produce covalent dimer adducts of the gingerols, since fragmentation patterns after that point were similar or identical. Three ion traps were compared (two separate models from one manufacturer and another from a different manufacturer), with the final conclusion that the differences in precursor ions from the gingerols could be attributed to different ion formation mechanisms during ESI in the instrument (Jiang et al. 2006b).

Summary

Thus, ion trap LC/ESI-MS/MS, coupled to diode array detection, has proven to be a powerful and efficient tool for the identification of medicinal and other compounds from plant extracts, specifically in the cases of ginger and turmeric. Utilization of this technique for analysis allows for the identification of novel compounds that may possess valuable medicinal properties and can supplement existing methods to provide advanced structural information for these and other compounds. Additionally, data from these and related studies can be used to provide evidence to elucidate the biosynthetic pathways producing desired compounds of medicinal interest. Unfortunately, as discussed, these studies have also given evidence that data may not be able to be reproduced with exact accuracy, given differences between instruments from separate manufacturers and the techniques employed with this equipment. This critical issue has yet to be resolved and presents a substantial problem for the fields of

metabolite target analysis, metabolic profiling, and metabolomics investigations, all of which would benefit greatly from the availability of LC/MS libraries.

Nonetheless, utilizing a variety of GC/MS and LC/MS techniques, standardization of botanical analysis can be inclusive of both volatile and nonvolatile compounds. As seen with ginger and turmeric, facilitating detection and identification of types and quantities of chemical constituents within samples allows for a greater degree of accuracy in studies of natural products in dietary supplements. Additionally, developments in mass spectrometric methodology will continue to enable characterized and standardized samples to be made available for preclinical evaluation and clinical trials.

BIOSYNTHESIS OF GINGEROLS AND CURCUMINOIDS

Due to their importance to human health, decades of ginger and turmeric research have included preliminary investigations into the biosynthetic production of the phenolic components in these plants that contribute to their medicinal effects. Studies have supported the early theory that the major constituents of these natural products, the gingerols and curcuminoids, are derived from intermediates in the phenylpropanoid pathway. These intermediates are condensed with molecules generated by the acetate and short- and medium-chain fatty acid pathways. Given the potential medicinal benefits of enhancing the biosynthesis and content of these phenolics in domesticated crops of turmeric and ginger, these metabolic pathways were further elucidated as part of the AzCPR collaborative research efforts.

Initial labeling studies suggested that curcumin and other diarylheptanoids are formed from two phenylpropanoids and a one-carbon unit that is most likely derived from malonate. It was also proposed that polyketide synthase-like enzymes catalyze the formation of the curcuminoid or gingerol backbone structure (Ramirez-Ahumada et al. 2006). Based on these initial discoveries, two possible biosynthetic pathways for gingerols and curcuminoids were proposed (Figure 4.3) (Ramirez-Ahumada et al. 2006). Enzymes for these proposed pathways would necessarily include one or more polyketide synthases, cytochrome P450 hydroxylases, and S-adenosyl-L-methionine-dependent *O*-methyltransferases. Studies were undertaken to isolate and identify such enzyme activities in both turmeric and ginger tissues, and to provide evidence for involvement of phenylpropanoid pathway enzymes in the production of the curcuminoids and gingerols. To this end, crude protein extracts from the leaves, shoots, and rhizomes of ginger and turmeric were assayed for activities of phenylalanine ammonia lyase (PAL), hydroxycinnamoyl-CoA transferases (HCTs), caffeic acid *O*-methyltransferase (COMT), caffeoyl-CoA *O*-methyltransferase (CCOMT), and polyketide synthases (PKS). Results supported the hypothesis that the phenylpropanoid pathway is fully operational in all of these tissues for both ginger and turmeric, as indicated by high levels of activities for all of these enzymes. However, because the question of whether the formation of 3-methoxyl groups on the aromatic rings occurs before or after the formation of the backbone remains unanswered, these studies did not allow for determination of which of the two proposed pathways is accurate (Ramirez-Ahumada et al. 2006).

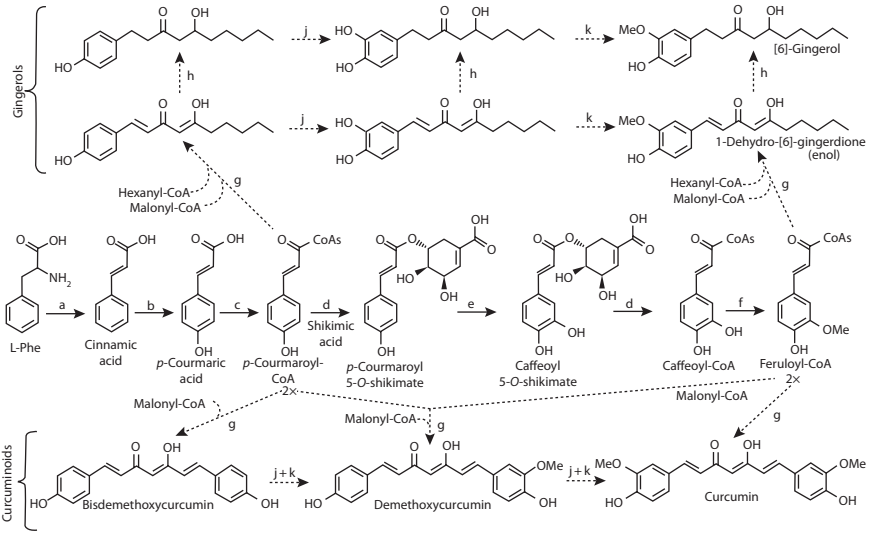

FIGURE 4.3 Proposed biosynthetic pathways for curcuminoids and gingerols. Enzymes: (a) phenylalanine ammonia lyase; (b) cinnamate-4-hydroxylase; (c) 4-coumarate:CoA ligase; (d) *p*-coumaroyl shikimate transferase; (e) *p*-coumaroyl 5-*O*-shikimate 3′-hydroxylase; (f) caffeoyl-CoA *O*-methyltransferase; (g) polyketide synthase(s); (h) reductase(s); (j) hydroxylase; (k) *O*-methyltransferase. (From Maheshwari, R.K. et al., *Life Sci*., 78, 2081, 2006.)

Additional studies identified the novel enzymatic activity of curcuminoid synthase, which is capable of forming the curcuminoids in turmeric, in concert with malonyl-CoA and hydroxycinnamoyl-CoA esters from the phenylpropanoid pathway. The resulting activity may be the consequence of a single enzyme or of multiple enzymatic steps. It is worth noting that it was necessary for both *p*-coumaroyl-CoA and feruloyl-CoA to be present in order for product to be observed in the preliminary assays (Ramirez-Ahumada et al. 2006). Alternative approaches to isolate and characterize curcuminoid synthase and gingerol synthase, as well as to identify the genes responsible for direct formation of curcuminoids and gingerols in turmeric and ginger, respectively, will (1) continue to provide insights into the natural synthetic pathways that stimulate the production of these bioactive compounds and (2) help guide future efforts to manipulate these pathways during the domestic cultivation of these medicinal plants.

EVALUATION OF ANTI-INFLAMMATORY POTENTIAL

Ginger

Once developed, the analytical abilities described earlier enabled the (1) chemical analysis and (2) biological standardization by *in vitro* screening for anti-inflammatory activity of botanicals prior to testing in animals, thus enhancing the scope of discovery when elucidating potential biological effects of these plants in complex whole systems. Detailed chemical studies of ginger included the identification and

characterization of two organically grown fresh white and yellow ginger varieties from Hawaii, as well as a commercially processed dry bulk ginger (Table 4.1). Three major gingerol homologs were isolated and determined to be [6]-, [8]-, and [10]-gingerol, with [6]-gingerol being the most abundant in both fresh and dry gingers (Table 4.1) (Jolad et al. 2005, 2004). Except for the major differences in the amounts of [6]-, [8]-, and [10]-gingerols, with yellow ginger having the highest total gingerol content, and the presence or absence of a few minor constituents, the white and yellow ginger varieties appeared to be basically similar (Jolad et al. 2005, 2004). Dried versus fresh ginger most notably differed in that the content of [6]-shogaol, the dehydration product of [6]-gingerol, was much higher in the commercial bulk dry ginger (Table 4.1). As would be anticipated, when comparing randomly selected commercial ginger dietary supplements with bulk dry ginger, [6]-shogaol, rather than [6]-gingerol, was the primary or sole constituent in those commercial ginger supplements in which gingerol homologs could be detected (n = 3 of 4 samples tested) (Timmermann et al., unpublished data).

Chemical variations noted between fresh and dry gingers reflected marked differences in their mechanisms of action when screened *in vitro* for their anti-inflammatory potential, as determined by inhibition of lipopolysaccharide (LPS)-stimulated prostaglandin E_2 (PGE_2) production from human macrophage cell lines (Table 4.1). This *in vitro* bioassay was chosen as PGE_2 inhibition had been a primary focus of the anti-inflammatory potential of gingerols in earlier research. Crude extracts of both fresh and dry ginger samples demonstrated remarkable anti-inflammatory activity, with a majority of the compounds detected in the bioactive fractions of the fresh ginger varieties also being found in the dried ginger (Jolad et al. 2005, 2004). In contrast to their potent inhibition of PGE_2 production ($IC_{50} < 0.1$ μg/mL), these organic ginger extracts were much less effective at inhibiting tumor necrosis factor (TNF)-α ($IC_{50} > 50$ μg/mL).

Individual fractions of the crude extracts of fresh and dried ginger, prepared by column chromatography, were also analyzed for bioactivity (Table 4.1). As discussed, the complex extracts or fractions containing predominantly gingerols and/or shogaols were highly active at inhibiting LPS-induced PGE_2 production ($IC_{50} < 0.1$ μg/mL), while gingerol-depleted fractions containing either terpenes or unidentified polar compounds were less effective ($IC_{50} > 1.0$ μg/mL) (Lantz et al. 2007). Extract fractions containing the gingerols were determined not to be cytotoxic; however, those fractions containing predominantly shogaols were cytotoxic at concentrations at or above 5–20 μg/mL (Lantz et al. 2007). Notably, [6]-, [8]-, and [10]-gingerols in isolation were more potent inhibitors of PGE_2 production than [6]-shogaol (Lantz et al. 2007). Based on these initial studies, it has been proposed that the length of the side chain in gingerol homologs is a determinant of both cytotoxicity and PGE_2 inhibitory activity (Jolad et al. 2005, 2004). Interestingly, some fractions that contained neither gingerols nor shogaols were still effective inhibitors in the PGE_2 assay (Table 4.1), substantiating the hypothesis that other active constituents have yet to be identified and may act additively and/or synergistically with the gingerols or shogaols in mediating anti-inflammatory activity.

COX-2, an inducible cyclooxygenase that stimulates PGE_2 production, is the target of a large class of anti-inflammatory therapeutics. In our *in vitro* studies, it was

TABLE 4.1
Isolation and Chemical and Biological Characterization of Ginger Extracts and Fractions

			Column Chromatographic Fractionation and HPLC Characterization of Extracts			Sample Composition: [6]-,[8]-, and [10]-Gingerols (1–3) and [6]-Shogaol (4),% by Weight					
Ginger Product	**Material and Extraction Method**	**Extract Yield (%)**	**Fraction Yield (%)**	**Fraction Components**	**Fraction Number**	**1**	**2**	**3**	**4**	**Terpenes**	***In Vitro* PGE_2 Inhibition, IC_{50} (μg Extract/mL)**
Crude extract—fresh white	**Fresh white ginger**	**2.0**				**28**	**3**	**5**	**0**	+	**0.1**
	DCM extraction of aqueous phase remaining after stripping MeOH used to extract fresh rhizome		16	Sesquiterpenes	1–4					+	0.1–0.3
			10	Paradols	5–7						0.1
			7	Shogaols	8–10					+	0.1
			3	Acetylated gingerol derivatives	11–12						0.1
			28	Gingerols	13–16	+	+	+			0.1
			8	Gingerdiols	18						0.1
			26	(Polar)	19–20						1.0–10

Crude extract—fresh yellow	**Fresh yellow ginger**	**0.5**				**34**	**5**	**8**	**0**	+	**0.1**
	DCM extraction of aqueous phase		12	Sesquiterpenes	1–4					+	0.3–41
			10	Paradols	5–6						0.1
	remaining after		4	Shogaols	7–8				+		0.1
	stripping MeOH used to extract		4	Acetylated gingerol derivatives	9–10						0.1
	fresh rhizome		33	Gingerols	11–13	+	+	+			0.1
			6	Gingerdiols	16						0.1
			24	(Polar)	17–18						0.3–9
Crude extract—dried	**Commercial dried ginger**	**6.4**				**10–11**	**2–3**	**3–5**	**3–6**	+	**0.1**
	DCM extraction		23	Sesquiterpenes	1–2					+	0.3–50
			22	Paradols + Shogaols	3				+		0.1
			4	Shogaols + Acetylated gingerol derivatives	4				+		0.1
			22	Gingerols	5–7	+	+	+			0.1
			2	Gingerdiols	8						0.1
			28	(Polar)	9–10						0.4–25
Gingerol-related fraction—dried	**Recombined fractions of DCM extraction of dried ginger**		**42**	**Gingerols and Acetylated derivatives, Shogaols, Paradols, Gingerdiols**	**3–8**	**21**	**7**	**10**	**9**		**0.1**
Standards											
[6]-Gingerol							+				0.1
[8]-Gingerol								+			0.1
[10]-Gingerol									+		0.1
[6]-Shogaol										+	>1.0

determined that extracts containing multiple gingerol derivatives were extremely effective at inhibiting COX-2 gene expression (Lantz et al. 2007). Isolated compounds in the gingerol class, while less effective than the extracts, were also capable of inhibiting LPS-induced COX-2 expression. In contrast, [6]-shogaol had no effect on COX-2 expression. In general, the efficacy of isolated compounds and ginger extracts in inhibiting the production of PGE_2 paralleled their efficacy in blocking COX-2 gene expression. In contrast, concentrations of crude dried ginger extract or the isolated gingerol/shoagol compounds inhibiting 50% of PGE_2 production (IC_{50}) had no effect on the enzyme activity of either COX-1 or COX-2 (Lantz et al., unpublished data). These studies, which were the first to demonstrate that extracts from ginger can alter COX-2 mRNA levels, demonstrate that multiple compounds found in ginger are capable of inhibiting production of PGE_2, an important mediator joint inflammation, and likely act by more than one mechanism to achieve this effect (Lantz et al. 2007).

Turmeric

Using analytic approaches developed for turmeric and a characterization strategy analogous to that described for ginger, the PGE_2 inhibitory effects of turmeric extracts were tested and correlated with chemical content. Crude methanolic (but not aqueous) extracts of turmeric, which contained the three major curcuminoids, were found to be capable of inhibiting LPS-induced PGE_2 production *in vitro* (IC_{50} = 0.1–1.0 μg/mL, Table 4.2) (Lantz et al. 2005). In isolation, purified curcumin was more active than either demethoxycurcumin or bisdemethoxycurcumin in this regard (Table 4.2) (Lantz et al. 2005). However, a crude turmeric extract was a more potent inhibitor of PGE_2 production than a curcuminoid-only fraction ("curcuminoid fraction," Table 4.2), suggesting synergistic and/or additive effects of curcuminoids with non-curcuminoid components in the crude extract (Table 4.2). Fractions of the crude turmeric extract, isolated through HPLC purification, showed differing biological activity in blocking PGE_2 production, ranging from IC_{50} values of less than 1 μg/mL to greater than 6 μg/mL (Table 4.2). Of these, the fraction containing the curcuminoids (Table 4.2, fraction 5) was the most potent inhibitor of PGE_2 production. Interestingly, when several major fractions containing turmeric oils were recombined, the essential oils were more potent inhibitors of PGE_2 than the curcuminoids (IC_{50} = 0.5 μg/mL), displaying a potency (1) greater than that of any individual essential oil fraction, yet (2) comparable to that of the purified curcuminoids or the crude extract (Table 4.2) (Lantz et al. 2005). This result was confirmed when a similar mixture of essential oil compounds, isolated by hexane extraction of the dried rhizome, also inhibited PGE_2 with a potency similar to that of the commercially available nonsteroidal anti-inflammatory drug (NSAID), indomethacin (IC_{50} = 0.1 μg/mL) (Lantz et al. 2005). This finding suggests that non-phenolic components in the essential oils of turmeric may act in a synergistic or additive nature to increase their anti-inflammatory potency upon administration.

The essential oil component of turmeric is a complex mixture of more than 80 terpenoids, including mono-, di-, and sesquiterpenes (Chen et al. 2007). While the

TABLE 4.2
Isolation and Chemical and Biological Characterizations of Turmeric Extracts and Fractions

			HPLC Fractionation and Characterization of Extract			Sample Composition: Curcumin (1), Demethoxycurcumin (2), and Bis-Demethoxycurcumin (3), % by Weight					
Turmeric Product	**Material and Extraction Method**	**Extract Yield (%)**	**Fraction Yield (%)**	**Fraction Components**	**Fraction Number**	**1**	**2**	**3**	**Total 1–3**	**Terpenes**	***In Vitro* PGE_2 Inhibition, IC_{50} (µg Extract/mL)**
Crude DCM/MeOH extract	**Commercial dried turmeric**										**0.9**
	(1:1 DCM/MeOH extraction)		19	(Polar)	1–3						2.2–4.7
			10		4						1.0
			32	Curcuminoids	5	+	+	+	+	+	0.9
			3		6					+	3.7
			4		7					+	7.5
			12	Sesquiterpenoids	8					+	1.7
			14		9					+	6.3
			6		10					+	6.3
			38	Recombined essential oil fractions	6–10					+	0.5
Crude extract (MeOH)	**Commercial dried turmeric** (MeOH extraction)	**9.5**		**Curcuminoids/ essential oils/polar compounds**		**21.4**	**7.2**	**5.1**	**33.7**	+	**0.1**

(*continued*)

TABLE 4.2 (continued)
Isolation and Chemical and Biological Characterizations of Turmeric Extracts and Fractions

			HPLC Fractionation and Characterization of Extract			Sample Composition: Curcumin (1), Demethoxycurcumin (2), and Bis-Demethoxycurcumin (3), % by Weight					
Turmeric Product	**Material and Extraction Method**	**Extract Yield (%)**	**Fraction Yield (%)**	**Fraction Components**	**Fraction Number**	**1**	**2**	**3**	**Total 1–3**	**Terpenes**	***In Vitro* PGE_2 Inhibition, IC_{50} (μg Extract/mL)**
Essential oil-depleted extract	**Commercial dried turmeric** (MeOH extraction of hexane marc)	**3.1**		**Curcuminoids/ polar compounds**		**25.7**	**8.7**	**6.2**	**40.6**		**0.5**
Essential oil fraction	**Commercial Dried Turmeric** (Hexane Extraction)	**3.7**		**Essential oils**						+	**0.1**
Curcuminoid fraction	**Commercial "curcumin"**			**Curcuminoids**		**74.2**	**14.9**	**4.5**	**93.6**		**2.7**
Purified Compounds											
Curcumin						+					0.5
Demethoxycurcumin							+				2.6
Bis-demethoxycurcumin								+			1.2
Indomethacin											0.1

curcuminoids in turmeric, analogous to the effects of the phenolic gingerols, inhibited COX-2 gene expression at the same concentrations that blocked PGE_2 production, the essential oils of turmeric did not, suggesting a different mechanism of action. (Lantz et al. 2005). An additional parallel between the phenolic curcuminoids and gingerols can be seen in that neither blocked COX-1 or COX-2 enzyme activity at concentrations inhibiting PGE_2 release (Lantz et al., unpublished data). Lastly, as in the ginger studies, when randomly chosen dietary supplements of turmeric, claiming to be standardized to 95% curcumin or curcuminoids, were assessed for chemical content, their total curcuminoid content was less than that indicated on the label. All supplements were mixtures of the three major curcuminoids in varying ratios and were devoid of turmeric essential oils (Funk and Timmermann 2006).

Summary

Overall, the results of these studies underscore a fundamental precept of medicinal botanical research that characterization of botanical product chemical composition, while a very complex undertaking, is central to elucidating the bioactivity and mechanism of action of plant products, ensuring the reproducibility of their biological effects and allowing the possibility of harnessing the combinatory chemistry of nature to develop medicinal products with additive and/or synergistic effects. More specifically, these studies also confirmed that both ginger and turmeric show important anti-inflammatory activities, warranting further investigation to evaluate sites of action and safety of the active compounds in the treatment of chronic inflammatory diseases (Funk and Timmermann 2006). When comparing the phenols of these related plants, the gingerols appear to be more potent inhibitors of inflammation than the curcuminoids, as measured by their PGE_2 inhibitory effects, which are similar in potency to indomethacin. For both plant products, however, crude extracts appear to be more potent than individual compounds when normalized to phenolic content. Additionally, and unexpectedly, the essential oils of turmeric appear to be as or more potent anti-inflammatory agents than its more familiar metabolite, curcumin. Directed by these data and utilizing extracts matching the chemical and biological footprint of the library of extracts screened, *in vivo* preclinical studies were subsequently undertaken at the AzCPR to test the anti-inflammatory potential of components present in these botanicals with respect to arthritis, a traditional disease target for these medicinal plants, and to identify their mechanisms of action as a critical next step in guiding the rational clinical use of these products. In so doing, we hoped to obtain the first direct scientific evidence, which was lacking at the time, to prove the antiarthritic effects of specific components of these traditionally used plants. Additionally, by uncovering the mechanisms of their effects, we hoped to identify (1) unique combinations of secondary metabolites from these plants for appropriate use in disease treatment either as a single agent or in combination with standard pharmaceuticals and/or 2) additional diseases, outside the scope of traditional use, which share a common pathogenic mechanism and therefore may also derive a benefit from botanical treatment.

BIOAVAILABILITY ASSESSMENTS OF ANTI-INFLAMMATORY BOTANICALS

In anticipation of both preclinical and clinical trials, the disposition kinetics and bioavailability of the phenolic compounds in ginger and turmeric were characterized. Sensitive, selective, and reproducible isocratic HPLC methods for the separation and quantitation of gingerols and curcuminoids in plasma samples were established, with the most sensitive assay able to detect the concentration of curcumin in plasma down to 2.5 ng/mL (Pak et al. 2006). Precise and accurate plasma quantitation was based on both intra- and inter-day validation as indicated by low values for coefficients of variation and bias. Initially, *in vivo* studies were performed with the Yucatan micro pig. This model was chosen because of physiological and anatomical similarities to the human digestive system, which, in addition to the convenient size and docile nature of the animal, have made the Yucatan micro pig an increasingly popular choice for studies of pharmacokinetics (Peter et al. 2001).

Gingerols

Characterization of the disposition kinetics of the gingerols and assessment of oral bioavailability was established using the Yucatan pig (n = 4 animals) receiving intravenous bolus doses of an extract containing the three gingerols at average doses of 0.682, 0.124, and 0.374 mg/kg for [6]-, [8]-, and [10]-gingerols, respectively. The compounds were observed to rapidly disappear from the blood following intravenous dosing with average half-lives of 10.5, 6.2, and 8.8 min for [6]-, [8]-, and [10]-gingerols, respectively. This very short half-life is associated with a large average systemic clearance for each compound (41, 33, and 40 mL/min-kg for [6]-, [8]-, and [10]-gingerol, respectively). The kinetic studies in blood and plasma indicated that gingerols are stable in these media for at least 6 h. Thus, blood does not appear to contribute to the large clearance of the gingerols.

A single example of a commercial oral capsule containing ginger was selected from among a number of commercially available products for determination of bioavailability on the basis of the presence of all three gingerols in the product. Based upon extraction and analysis of these capsules and quantitation by HPLC, the capsules contained 1.48, 0.30, and 0.23 mg of [6]-, [8]-, and [10]-gingerols, respectively, and 1.96 mg of [6]-shogaol. Animals were orally dosed with eight capsules, and blood samples taken over the subsequent 8 h were assayed for the gingerols. There were no detectable plasma concentrations of any of the gingerols or shogaol at any time during these studies. Following glucuronidase incubation, there were still no measurable concentrations of gingerols and also no detectable presence of metabolites, indicating very poor absorption. It was concluded that the gingerols present in these commercial ginger capsules have a low (<10%) absolute oral bioavailability. Oral dosing of the same animals with the original extract used intravenously in the disposition kinetics studies revealed that, while there was no evidence of intact gingerols in plasma, glucuronidase incubation gave measurable concentrations of [6]-gingerol only. It was concluded that the [6]-gingerol was absorbed to some extent but underwent first-pass metabolism prior to systemic distribution.

Curcuminoids

Similar turmeric studies were also completed in order to determine the disposition kinetics and oral bioavailability of curcumin. Following intravenous bolus dosing, curcumin had a short half-life ranging from 3.5 to 6.6 min. Corresponding systemic clearance was very high (110–237 mL/min-kg), and the apparent volume of distribution was small to intermediate in value (0.62–1.23 L/kg). In contrast to the ginger studies, a considerable portion of clearance was attributed to blood clearance, based on these experiments. Following oral solution dosing of 0.4–6.0 g of pure curcumin, the absolute oral bioavailability of curcumin was less than 1%.

Interestingly, however, a greater absolute oral bioavailability was achieved following the dosing of a commercial turmeric product, of which each capsule contained approximately 3.7 mg of curcumin. After dosing with six to eight commercial capsules, the absolute oral bioavailability of curcumin was variable, with ranges from 4% to 39%. Hydrolysis of plasma with β-glucuronidase gave a much larger increase in the area under the curve for curcumin following oral (6.70) versus intravenous (1.42) dosing, suggesting a substantial first-pass effect.

These studies of ginger and turmeric bioavailability indicate that the absolute oral bioavailabilities of the putative active components of ginger ([6]-, [8]-, and [10]-gingerols) are low (<10%) from commercial products. Systemic activity of complex extracts from these products, therefore, may reside in metabolites of the gingerols or in other phytochemicals instead. Absolute oral bioavailability of curcumin appears to be dose dependent, with the greatest observed efficiency at low oral doses. Systemic activity may reside in metabolites of curcumin or other components, such as turmeric oils. Additionally, matrix effects of complex turmeric products versus pure curcumin may enhance the bioavailability of this diarylheptanoid.

ANTIARTHRITIC EFFICACY

Ginger

In our *in vitro* anti-inflammatory screening assays, a reconstituted mixture of only those fractions containing gingerol-related compounds, but lacking the essential oils and polar components present in the crude extract, displayed the same IC_{50} for inhibition of PGE_2 production as the crude ginger extract (Table 4.1). Because the crude extract had a twofold lower content of PGE_2-inhibitory gingerols and gingerol derivatives, this suggested that the non-gingerol-related components of the crude ginger, while relatively inactive in isolation, might be synergizing with gingerol-related compounds to block this inflammatory pathway (Table 4.1). At the same time, while certain pharmacological treatments for arthritis specifically target PGE_2 production, this clearly is not the sole mechanism causing joint inflammation and destruction. Therefore, *in vivo* experiments were undertaken to determine whether ginger extracts did in fact have antiarthritic efficacy and, if so, whether the crude extract, consistent with *in vitro* screening results, was indeed more effective.

Extracts were tested using streptococcal cell wall (SCW)-induced arthritis in rats, a well-characterized model that mimics pathogenic and pathological elements of

human RA, a particularly severe form of inflammatory arthritis (Funk et al. 2006a). In this model, over the course of a month, an initial transient phase of joint inflammation is followed by chronic persistent joint swelling that is coupled with destruction of articular cartilage and bone. Early joint swelling is driven by an influx of inflammatory cells followed by the invasive tumor-like proliferation of joint synoviocytes, which under normal circumstances only form a delicate membrane circumscribing the joint space. All of these accumulated cells in the inflamed joint produce local joint-destructive proteins that quickly begin to erode the joint, leading to its destruction, just as occurs in patients with RA (Funk et al. 2006a).

Using this model of RA, experiments were undertaken to determine whether the non-gingerol components of ginger could synergize with the gingerol derivatives to block joint swelling, as suggested by the *in vitro* screening results. To this end, the effects of a crude extract of dried ginger were compared head-to-head with those of a fraction only containing gingerols and their derivatives (Table 4.3). Extract doses were normalized to gingerol content and administered intraperitoneal (ip) to eliminate any matrix or other effects on the oral bioavailability of gingerol derivatives (Cassidy 2006). Using this experimental approach, we were able to determine that the crude ginger extract was indeed more effective than the gingerol-related-only fraction in preventing joint inflammation and the destruction of articular cartilage and bone (Table 4.3). At the same time, it was evident that the antiarthritic efficacy of both ginger products was remarkable (Table 4.3), as even the gingerol-only fraction inhibited joint swelling by 62%, a degree of protection greater than that reported in this model local blockade of TNF, a common pharmaceutical target in RA (Funk et al. 2009). Indeed, even a delay in treatment with the gingerol-related fraction, until after joint inflammation was maximal, did not eliminate the joint protective effects of this gingerol derivatives–only product (Funk et al. 2009).

The pharmaceutical treatment of RA with advanced biological therapeutics, most notably TNF-blocking agents, has markedly improved disease outcome for patients with RA. However, these treatments are not without serious side effects, including the reactivation of quiescent infectious diseases, such as tuberculosis (TB), which can only remain dormant when the body is able to mount an effective granulomatous inflammatory defense (Funk et al. 2006a). Interestingly, one feature of the SCW model, in addition to the induction of RA-like joint inflammation at sites of articular SCW deposition, is a separate granulomatous inflammatory response occurring at sites of SCW deposition within the liver. When comparing the effects of crude versus gingerol-only extracts on hepatic granulomatous inflammation, a distinct difference was noted between the extracts and also in comparison to their anti-inflammatory effects in the joint (Table 4.3); only the crude extract, which also contained essential oils and polar compounds, blocked granulomatous inflammation, while the gingerol-only fraction was without effect. This finding suggests that the complex ginger extract may target a wider range of inflammatory pathways and/or processes than the gingerols alone. At the same time, the wider anti-inflammatory efficacy of the crude ginger extract may be associated with increased side effects, such as the potential for reactivating TB, analogous to the risk profile of TNF blocking pharmaceuticals.

TABLE 4.3
Composition and *In Vivo* Effects of Ginger and Turmeric Extracts and Fractions

	Extract Components			Extract Dose		Anti-Inflammatory Activity		Toxicity
Zingiberaceae Extract	Phenolics (% Curcuminoids or Gingerols)	Essential Oils	Polar Compounds	Extract Dose (mg/kg/day)	Phenol Dose (mg/kg/day)	Arthritis (% Inhibition)	Granulomatous Inflammation (% Inhibition)	Mortality (%)
GINGER					Gingerols			
Crude extract	+ (18)	+	+	148	26	97	76	18
Gingerol-related fraction	+ (37)			70	26	62	NS	12
TURMERIC					Curcuminoids			
Crude extract	+ (34)	+	+	135	46	98	100	17
Essential oil-free extract	+ (41)		+	112	46	93	(100)	6
				56	23	60	(54)	
Curcuminoid fraction	+ (94)			10	4	NS	NS	0
				24	23	55	NS	0
				4	4	68	NS	0
Essential oil fraction		+		85	ND	(97)	(78)	20
				43	ND	(100)		
				4	ND	NS	(NS)	0

NS, no statistically significant effect; ND, none detected.

At present, our laboratories are continuing to explore inflammatory pathways targeted by ginger in arthritis, with a particular emphasis on the biological effects of its non-gingerol components when administered alone or in combination with the gingerols. Our findings to date evaluating the antiarthritic effects of ginger are extremely promising, suggesting that additional clinical trials are merited to evaluate the safety and efficacy of ginger as an arthritis treatment.

Turmeric

In vitro screening to evaluate turmeric's anti-inflammatory potential revealed distinct differences when compared to its botanical family member, ginger. While ethnobotanical evidence supporting turmeric use for arthritis treatment is strong, and one of its major phenolic compounds, curcumin, has long been thought to be a major anti-inflammatory agent, the anti-inflammatory potency of the curcuminoids in the *in vitro* screening assay (Table 4.2) was 10-fold less than that of the gingerols (Table 4.1). Moreover, the most potent PGE_2 inhibitory activity of turmeric appeared to reside with its essential oils, rather than its phenolic curcuminoids (Table 4.2).

Our novel discovery of turmeric oil's anti-inflammatory bioactivity led us, in an initial study, to compare the antiarthritic effect of a crude turmeric extract containing both curcuminoids and essential oils versus a complex, but essential-oil-depleted, extract (Table 4.3). (Peter et al. 2001, Funk and Timmermann 2006, Funk et al. 2006a). As in our ginger studies, head-to-head experiments were conducted using doses normalized to curcuminoid content with extracts administered ip to eliminate any matrix effects on oral pharmacokinetics (Cassidy 2006, Antony et al. 2008). Both curcuminoid-containing extracts inhibited over 90% of joint swelling *in vivo* in animals during 1 month of treatment (Table 4.3). However, the protective effect of the crude extract was tempered by an increased mortality over this period (Table 4.3). Thus, in subsequent experiments, the dose-dependent antiarthritic effects of the essential-oil-depleted extract were evaluated and compared to those of a curcuminoid-only fraction. Surprisingly, given the fivefold lower activity of the curcuminoid-only fraction (vs. essential-oil-depleted extract) in the *in vitro* anti-inflammatory screening studies (Table 4.2), the opposite effect was documented *in vivo* with respect to antiarthritic efficacy; the curcuminoid-only fraction was fivefold more effective in blocking joint swelling than the essential-oil-depleted extract (Table 4.3). Given the complex etiology of arthritis, which clearly is not solely due to an excess of PGE_2 production, mechanistic studies were undertaken to identify signaling pathways blocked *in vivo* by curcuminoid-containing turmeric extracts. An important pharmacological target of the curcuminoids within the arthritic joint appeared to be the transcription factor, nuclear factor kappa B (NF-KB) (Funk et al. 2006a). Activation of NF-KB turns on the expression of an inter-related set of inflammatory products in arthritic joints, including chemokines responsible for attracting inflammatory cells into the joint, as well as the production of joint destructive PGE_2 via induction of COX-2 expression by synoviocytes and other cells within the joint (Funk and Timmermann 2006, Funk et al. 2006a). Thus, these studies provided the first *in vivo* proof that curcuminoids in turmeric have significant antiarthritic bioactivity, while also identifying

their mechanism of action, blockade of an early step in the inflammatory cascade (activation of NF-KB) that limits the influx inflammatory cell into the joint and the local production of joint-destructive compounds (Funk et al. 2006a).

Given our unanticipated *in vitro* evidence that turmeric's other important secondary metabolite, the essential oils, have very significant anti-inflammatory effects when used in isolation as a complex mixture, a finding unique to the oils of turmeric (vs. ginger), we also conducted separate studies to evaluate possible antiarthritic effects specific to turmeric's essential oils (Funk et al. 2010). In the SCW arthritis model, when turmeric essential oils were administered in isolation using an ip oil dose approximating that received by animals treated with the crude turmeric extract (i.e., assuming the oils comprised one-third of the crude extract weight [Table 4.2]), joint inflammation and swelling were, remarkably, almost completely suppressed (Table 4.3). Again, however, as occurred with the crude essential-oil-containing turmeric extract, the joint protective effects of the oils was accompanied by marked toxicity (Table 4.3), including high mortality with evidence of anemia, gastrointestinal bleeding, and hepatotoxicity (Funk et al. 2010). Because minimal data were available in the literature regarding the medicinal and pharmacokinetic profiles of turmeric essential oils, additional oral dosing studies were performed to determine whether the toxicity of the essential oils was limited to ip dosing. Daily oral treatment with turmeric essential oil at doses 20-fold higher than those administered ip was non-toxic, but only modestly suppressed joint inflammation (–20%) (Funk et al. 2010). This antiarthritic effect of the essential oils when administered orally can be compared with the curcuminoids when similarly administered orally and in isolation at a dose 30-fold higher than administered ip, which inhibited joint swelling by 45% (Funk et al. 2006b). Thus, the oils and curcuminoids of turmeric each appear to have independent anti-inflammatory joint protective effects when administered orally at doses that in humans would approximate 4–6 and 1.2 g/day, respectively, after correcting for body surface area (Funk et al. 2006b, 2010). Still to be explored rigorously are the potential antiarthritic effects and safety profiles of these two secondary metabolites when administered orally and in combination for prolonged periods of time using doses that greatly exceed (>10-fold) normal dietary levels of consumption. Additionally, as essential-oil-containing, but not curcuminoid-only, turmeric extracts also inhibited granulomatous inflammation, the potential for additional untoward side effects when treating arthritis with turmeric oils, such as reactivation of latent granulomatous infection diseases as was discussed for the crude ginger extracts, must also be considered. Further complicating, but also adding promise, to the idea of combining turmeric's oils and curcuminoids for arthritis treatment are recent data suggesting that the oils of turmeric can significantly increase the oral bioavailability of the curcuminoids (Cassidy 2006).

From our rigorous assessment of the *in vitro* and *in vivo* anti-inflammatory bioactivity of each of turmeric's secondary metabolites, when administered either in isolation or in combination, a number of notable conclusions can be drawn that are relevant to botanical research in general and to turmeric in particular. First, given the chemical complexity of natural extracts and the biological complexity of specific disease processes, the efficacy and potential toxicities of a given plant for treatment of a particular disease must be specifically tested using products that are both

well characterized and carefully vouchered. Second, when we employed this type of detailed scientific approach to evaluate the ethnobotanical use of complex turmeric products for arthritis treatment, we uncovered potentially very significant biological effects for the curcuminoids as well as turmeric's other major secondary metabolite, the essential oils, which, while clearly present in traditional preparations, have been almost completely overlooked in scientific studies assessing the medicinal potential of turmeric (Funk 2010).

CONCLUSIONS

The comprehensive results of studies from the AzCPR have shed significant light on our understanding of ginger and turmeric as botanical medicines. However, chemical and biological methods for standardization of botanicals still present a major challenge for scientific research regarding safety and efficacy. The development and utilization of reliable experimental techniques and methods are necessary for further validation of their biological effects, enabling alternative and possibly even more effective means of prevention and treatment for a number of diseases of great significance to nations, and individuals, throughout the world.

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5 Novel Approach for Screening Natural Plant Ingredients for Development of Nutraceutical Bone Health Supplements

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CONTENTS

INTRODUCTION

Osteoporosis is a degenerative disease that primarily affects women and its prevalence is rising worldwide. According to the National Health and Nutrition Examination Survey (NHANES) 1999–2000 report, an estimated one out of two women and one out of eight men will develop osteoporosis during their lifetime. Of the risk factors associated with osteoporosis, bone fracture is prominent. Current data for osteoporosis-related fractures show that 37% of elderly people with hip fractures die within 1 year of suffering the injury. The loss of remaining years of life is estimated to be between 20% and 60% depending on the age at which fracture occurs. In addition, comorbidities associated with hip fracture have increased since 2003, and 50% of hip fracture patients will require some type of ambulatory assistance 1 year postfracture (walking stick, walking frame, wheelchair, and bedridden).

Osteoporosis has become a global issue as the elderly are becoming a dominant subpopulation. This situation creates a tremendous economic burden as osteoporosis and its negative impact on quality of life present a substantial impact on the health care system. Finding an effective management for osteoporosis and osteoporosis-related illness is a great challenge. One potential approach for bone health management is to use a dietary regimen to strengthen bone before injury can occur. The benefit of this approach can be seen in studies showing the effect of ingesting calcium from dairy products as well as many fruits and vegetables through increases in bone density.

Similarly, the purpose of this bone health research was to develop botanical supplements to maintain bone health. Two approaches that target different mechanisms of action have been proposed. One formula aims to reduce bone resorption and the other aims to stimulate bone growth. Results described here summarize preliminary results from a novel approach leading to selection of ingredients toward the development of bone anti-resorptive (AR) and bone formation (BF) nutraceutical formulas.

These two opposing mechanisms, BF and bone resorption, directly modulate bone strength and bone mineral density. These activities are controlled by two distinct cell types, osteoblasts and osteoclasts. Osteoblasts build bone by synthesizing the osteoid, an extracellular matrix composed primarily of type I collagen and mineralization of the osteoid by deposition of calcium. Conversely, osteoclasts resorb bone to liberate calcium into the general circulation when the body needs calcium for other biological functions such as muscle contractions (i.e., heart beat) and transmission of

signals in the nervous system. Under normal circumstances, bone is constantly being remodeled with a balance between BF and bone resorption. This balance is lost at menopause when the rate of osteoclastogenesis, the differentiation of osteoclasts, and bone resorption become dominant over the rate of BF. Prior to menopause, estrogen helps to maintain the balance between these processes through the inhibition of inflammation that promotes osteoclastogenesis.

The AR nutraceutical formula was developed based on mechanisms known to affect osteoclasts. Osteoclastogenesis is primarily regulated by the inflammatory cytokine receptor activator for nuclear factor-κB ligand (*RANKL*) (Li et al. 2000). *RANKL* is a signaling molecule released by activated immune T cells and other bone cells in response to inflammatory mediators such as interleukin-1β that causes macrophages to differentiate into osteoclasts (Stejskal et al. 2001). Therefore, a menopausal increase in *RANKL* expression results in a greater number of mature osteoclasts. Mature osteoclasts then reduce bone strength and increase bone mineral density loss by secreting matrix-digesting enzymes (matrix metalloproteases) to break down bone's collagen/calcium–phosphate framework (Lerner 2006). Overproduction of *RANKL* has been linked to degenerative bone diseases such as rheumatoid arthritis and osteomyelitis (Haynes et al. 2001). Conversely, agents inhibiting *RANKL* activity increase bone density, volume, and strength. These agents include osteoprotegerin, a *RANKL* decoy receptor, and anti-*RANKL* monoclonal antibodies such as denosumab that block *RANKL*'s interaction with its receptor (Haynes et al. 2001; McClung et al. 2006). These materials reduce bone turnover and increase bone density.

The BF nutraceutical formula developed in this project was based on mechanisms known to affect osteoblasts. Specifically, we assessed botanical extracts and phytochemicals for their ability to activate the gene for bone morphogenetic protein 2 (BMP2), resulting in increased expression of the protein. BMP2 is known to have direct effects on osteoblast cell differentiation and to promote bone and cartilage formation (Chen et al. 2004). In fact, recombinant BMP2 is used orthopedically, especially in circumstances when there is delayed union of fractures (Geiger et al. 2003).

FORMULA DEVELOPMENT USING *IN VITRO* BIOASSAY MODELS

The strategies for development of the AR and BF formulas were based on scientific literature for each of the two mechanisms. As mentioned earlier, the molecular target for development of the AR formula was *RANKL*. *RANKL* is produced by osteoblasts and induces the differentiation of pre-osteoclasts into mature osteoclasts. This is important in that the osteoclast is the cell that resorbs bone. More differentiated osteoclasts take away more of the existing bone structure. The expression of *RANKL* increases at menopause when estrogen levels decline and estrogen is known to suppress inflammation (Pacifici et al. 1991; Crisafulli et al. 2004). The decline in estrogen levels at menopause leads to inflammation-induced increases in *RANKL* expression, and consequently, the number of mature osteoclasts in bone (Eghbali-Fatourechi et al. 2003)

Bioassay testing for the AR formula began with screening of botanical samples for their ability to suppress the expression of *RANKL* under inflammatory conditions. For these studies, osteoblast-like MG-63 human osteosarcoma cells (ATCC, Manassas, VA) were used and were cultured in phenol red-free media to eliminate

TABLE 5.1
***RANKL* Inhibition Results after Treating Natural Ingredients**

Concentration (μg/mL)	P	GB	GT	GS	R	SG	I	DQ	SJ
Percentage in Change Against[a]									
1	14%	31%	19%	11%	74%	50%	No effect	16%	42%

Cells were stimulated with IL-1β (10 ng/mL) following treatment with botanical ingredients.
P, pomegranate extract; GB, *Ginkgo biloba*; GT, green tea extract; GS, grape seed extract; R, *Rehmannia spp*.; SG, Siberian ginseng; I, ipriflavone; DQ, Dong Quai extract; SJ, *Sophora japonica*.
[a] Values >10% indicate significant suppression of *RANKL* production.

phenol red's estrogenic effects. The cells were exposed to the botanical samples overnight. Human recombinant IL-1β was also added to induce *RANKL* expression. Following the incubation period, quantitative real-time PCR for *RANKL* messenger RNA levels was performed using DLUX primers for human *RANKL* with Superscript III Platinum reagents, both purchased from Invitrogen (Carlsbad, CA). *RANKL* expression was normalized to expression of the housekeeping gene *GAPDH*. The data in Table 5.1 summarize the results of botanical samples that showed any effect on *RANKL* expression. The most potent of the samples were preparations of *Gingko biloba*, *Rehmannia* spp., Siberian ginseng, and *Sophora japonica*. In addition, preparations of pomegranate, green tea, grape seed, and Dong Quai showed modest inhibition of *RANKL* expression activity. Interestingly, ipriflavone, a synthetic isoflavone used to prevent osteoporosis, had no effect in this assay.

In order to confirm and expand these findings, the botanical samples that demonstrated inhibition of *RANKL* gene expression activity were also tested for their ability to inhibit bone resorption in a calvarial assay (studies performed by Osteoscreen, San Antonio, TX). Briefly, murine calvarial tissues were harvested and incubated with ^{45}Ca to radiolabel the bone. Unincorporated ^{45}Ca was removed by washing and the tissues were incubated with samples and IL-1β to induce *RANKL* expression. As with the MG-63 cell culture experiments, IL-1β induces an inflammatory response similar to that which is thought to occur in the bone of postmenopausal women that results in increased bone resorption. The resulting inflammation in the calvarial tissues induces loss of calcium that is leaked into the culture media over an incubation period of 4–7 days. Radioactivity released into the medium is counted as an indication of bone loss. For the AR formula, the samples that inhibited *RANKL* expression in MG-63 cells were evaluated for their ability to prevent loss of ^{45}Ca from prelabeled tissue exposed to IL-1β. The data in Figure 5.1 demonstrate that pomegranate, green tea, and grape seed all inhibited the loss of ^{45}Ca from the calvarial tissue, while *Rehmannia* spp., *Sophora japonica*, Siberian ginseng, and *Ginkgo biloba* had little effect. Pomegranate had a dose-dependent effect, while green tea and grape seed were effective only at the highest concentration tested. In addition, the controls of ipriflavone and alendronate also both inhibited loss of calcium in a dose-dependent manner. These results suggest that phytochemicals

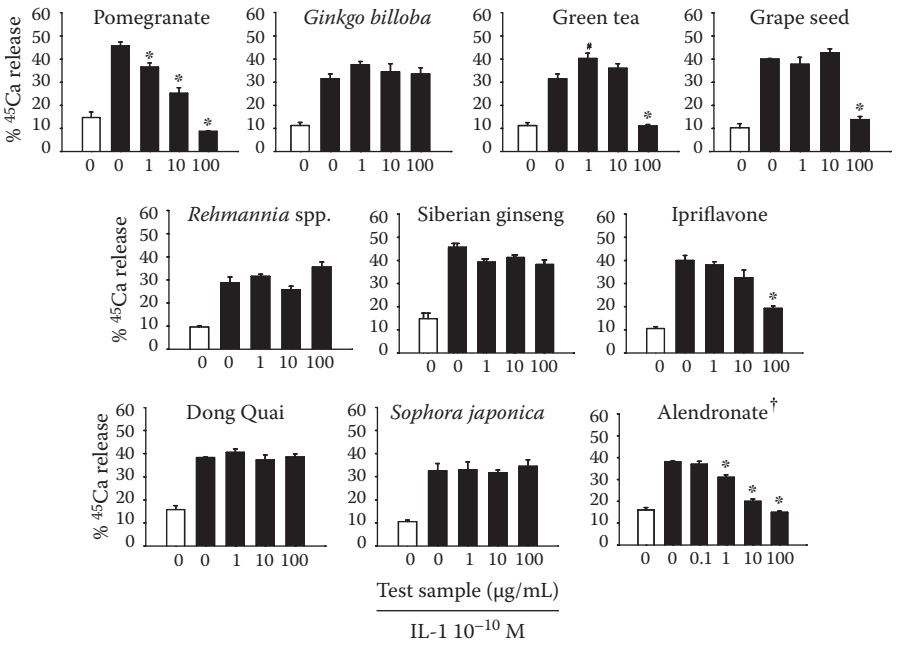

FIGURE 5.1 AR formula calvarial calcium release results after treatment with botanical samples. White bars indicate the amount of calcium release without IL-1β stimulation, and black bars indicate the amount of calcium release with IL-1β stimulation. All data were expressed as mean ± SEM. †Alendronate was used as a positive control. *Indicates significant reduction in calcium release as compared to untreated samples after IL-1β stimulation ($p < 0.05$).

present in pomegranate, green tea, and grape seed are available to the calvarial tissue, inhibit *RANKL* expression, and, consequently, prevent bone resorption under inflammatory conditions that might be present in menopausal bone.

The results from the *RANKL* expression data and the calvarial studies are summarized together in Figure 5.2. The white bars show the effect of *RANKL* expression after treatment with botanical samples. The black line at 10% on the Y-axis demonstrates the *RANKL* response of the control (IL-1β alone). The results are represented so that a positive response is an increase above the line. Gray bars show relative calcium loss from calvarial tissues in the presence of the same botanical samples. The black line at ~20% on the Y-axis demonstrates calcium release from IL-1β-stimulated control tissues. The results for these data are represented so that a positive response is a decrease below the line. From this analysis of both studies, pomegranate, green tea, and grape seed extracts (GSEs) were identified as the top AR performers as they inhibited gene expression in the cell-based *RANKL* assay, as well as showing the best performance in the calvarial resorption assay.

Based on these results, additional calvarial studies were performed to optimize a potential AR formula using combinations of two or three botanical samples. The data in Figure 5.3 show that the combination of pomegranate and grape seed in a 10:1 ratio inhibited calcium loss in a dose-dependent manner. Likewise, a combination of pomegranate, grape seed, and ipriflavone in a 43:4.3:52 ratio also inhibited loss of

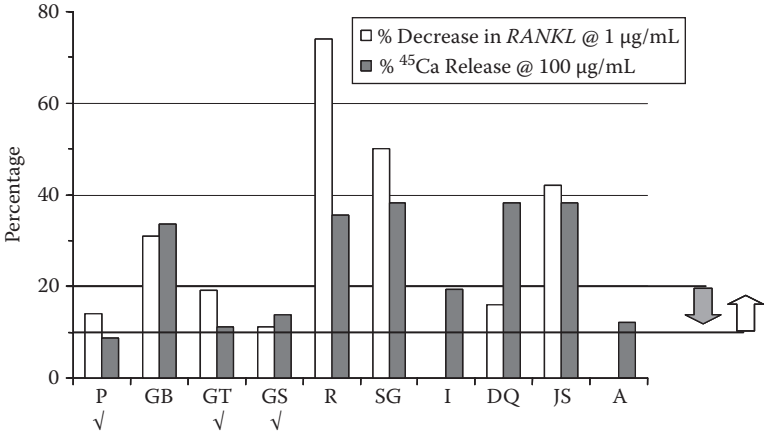

FIGURE 5.2 Summary of AR bioassay and calvarial study. P, pomegranate extract; GB, *Ginkgo biloba*; GT, green tea extract; GS, grape seed extract; R, *Rehmannia* spp.; SG, Siberian ginseng; I, ipriflavone; DQ, Dong Quai extract; SJ, *Sophora japonica*; A, Alendronate. *Note: values >10% indicate significant suppression of *RANKL* and values <20% ^{45}Ca release indicate significant suppression of calcium release. †Alendronate was used as a positive control. √Top performing ingredients.

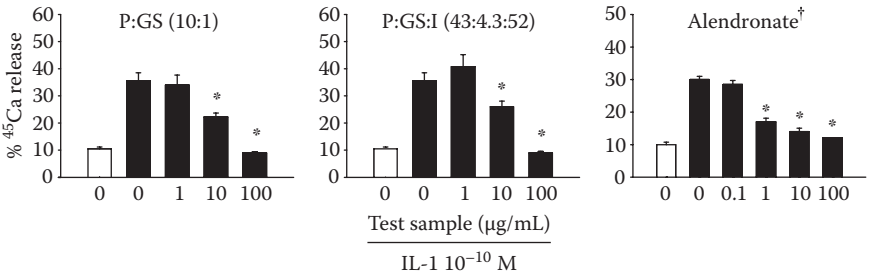

FIGURE 5.3 AR formula calvarial calcium release results after treatment with a combination of botanical samples. P, pomegranate extract; GS, grape seed extract; I, ipriflavone. White bars indicate the amount of calcium release without IL-1β stimulation, and black bars indicate the amount of calcium release with IL-1β stimulation. All data were expressed as mean ± SEM. †Alendronate was used as positive control. *Indicates significant reduction in calcium release as compared to untreated samples after IL-1β stimulation ($p < 0.05$).

calcium in a dose-dependent manner. As seen in the previous experiment, the positive control, alendronate, also inhibited calcium loss in a dose-dependent manner. Based on these results, it is suggested that a formula comprised of pomegranate and GSEs in a 10:1 ratio may prevent bone resorption *in vivo*.

The second formula (BF) was developed to promote BF based on the ability of botanical samples to induce the expression of BMP2. Following the same development strategy that was used for the AR formula, the BF formula development was based on initial bioassays followed by confirmation of activity and formula optimization using calvarial tissues. Results from initial bioassay screening of botanical samples for their ability to promote *BMP2* gene expression are shown in Table 5.2. The most

TABLE 5.2
***BMP2* Gene Expression Results**

Concentration (μg/mL)	R-1	R-2	SJ-1	SG	R-3	SJ-2	Q	I	L	3P
Fold[a]	28×	11.7×	49×	27×	6.3×	8.3×	2.5×	No effect	45×	17×

R-1, *Rehmannia* spp. extract; R-2, *Rehmannia* spp. extract; SJ-1, *Sophora japonica* 1; SG, Siberian ginseng; R-3, *Rehmannia* spp. root; SJ-2, *Sophora japonica* 2; Q, quercetin; I, ipriflavone; L, licorice; 3P, third-party proprietary ingredient.

[a] An increase in *BMP2* gene expression of twofold or greater was considered significant.

potent samples in these initial experiments were *Rehmannia* spp., *Sophora japonica*, Siberian ginseng, and licorice. In addition, purified quercetin and a third-party proprietary product were shown to be active in this assay. These samples were subsequently tested in a secondary *BMP2* assay using a stable 2T3 cell line that contained the BMP2 promoter (–2712/+165) linked to a firefly luciferase reporter gene (Ghosh-Choudhury et al. 1996). A positive response in this bioassay was one in which a sample activated the *BMP2* promoter, resulting in an increase in luminescence in treated cells.

The data in Figure 5.4 demonstrate that quercetin and ipriflavone were the most active of the samples tested in the *BMP2* promoter assay. Licorice and *Sophora*

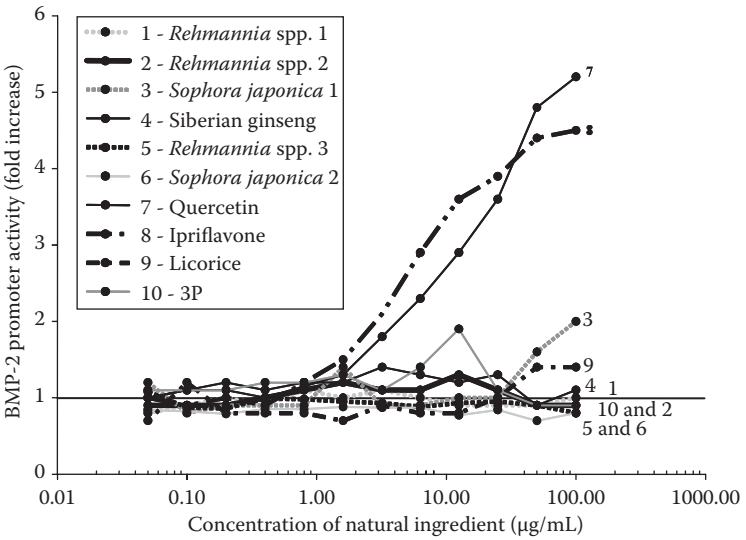

FIGURE 5.4 Effect of samples on BMP2 promoter activity. 3P, third-party proprietary ingredient. A stable cell line transfected with the murine BMP2 promoter linked to firefly luciferase was exposed to different botanical extracts. Data are expressed as fold increase in luciferase activity compared to unstimulated control cells. *Note: A value greater than 2 means that the treatment significantly activated BMP2 promoter activity. If the value is less than 2 means that treatment had no effect on BMP2 promoter activity.

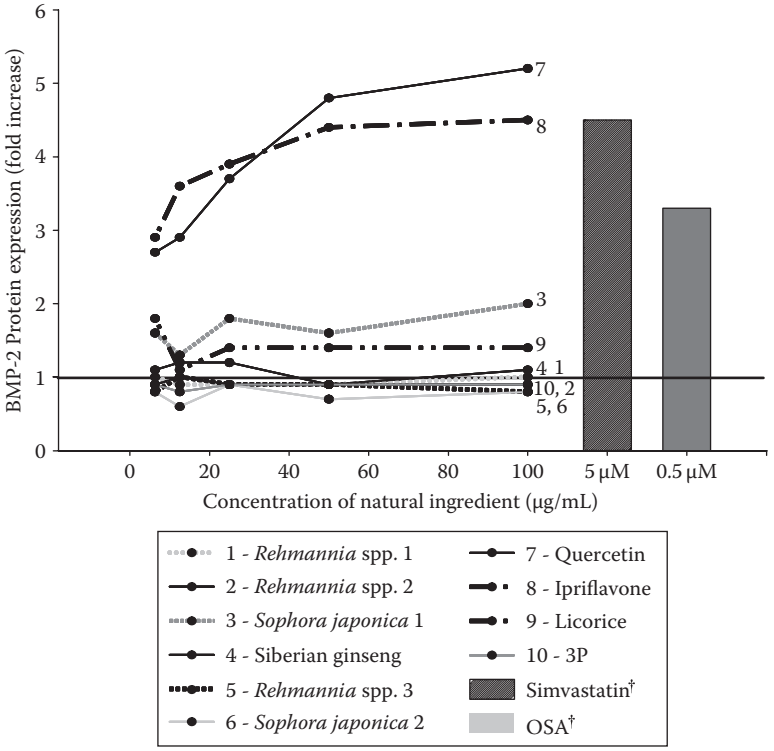

FIGURE 5.5 BMP2 protein assay results. 3P, third-party proprietary ingredient. †Simvastatin and OSA were used as positive controls. *Note: A fold increase greater than 2 was considered a significant increase in BMP2 protein expression. If the value is less than 2, this means the treatment had no effect on BMP2 protein expression.

japonica 1 also demonstrated some activity at the highest concentrations tested. The third bioassay used to screen botanical samples for BF activity was one in which BMP2 protein levels were assayed by ELISA in supernatants of MG-63 cells treated with samples. The results in Figure 5.5 show that quercetin and ipriflavone induced a dose-dependent increase in BMP2 activity. These results are similar to those seen in the BMP2 promoter assay. Controls known to promote BF were Simvastatin at 5 µM and orthosilicic acid (OSA) at 0.5 µM. Modest activation of BMP2 was also observed in cells exposed to licorice and *Sophora japonica* 1, again similar to results from the BMP2 promoter assay. The combined data from the *BMP2* gene expression, BMP2 promoter, and BMP2 protein assays suggest that quercetin, licorice, *Sophora japonica* 1, and Siberian ginseng were the most active of the botanical samples tested. Therefore, these samples were further tested for activity in the calvarial assay.

For the BF calvarial assay, botanical samples were added to the calvarial tissues and incubated for 4–7 days. At the end of the experiment, four micrometer sections of the calvaria were prepared and a morphological assessment was completed.

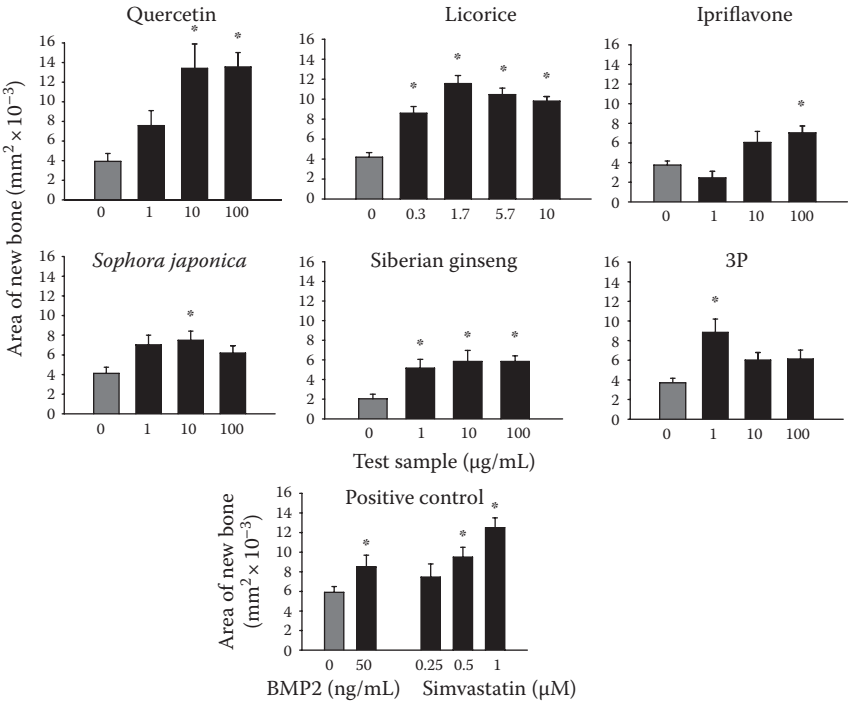

FIGURE 5.6 BF calvarial study results after treatment with botanical samples. 3P, third-party proprietary ingredient. Gray bars indicate results for the blank (untreated) samples. All data were expressed as mean ± SEM. †BMP2 and Simvastatin were used as positive controls. *Indicates results from samples treated with botanical samples that were significantly different from blank (untreated) samples ($p < 0.05$).

Digital images across sections of the neonatal murine calvaria were taken of each group. Histomorphometric analysis was performed on these calvarial images using Image Pro Plus (Media Cybernetics, Inc., Silver Spring, MD). Total bone and new bone areas (expressed as $mm^2 \times 10^{-3}$) were determined on all images across the calvarial section. The results in Figure 5.6 demonstrate that quercetin, and to a lesser extent licorice, induced a dose-dependent increase in new BF. This corroborates the result seen in the BMP2 promoter assay. Ipriflavone, *Sophora japonica*, Ginseng, and the third-party product were only modestly effective at inducing BF. The control samples, recombinant BMP2 and Simvastatin, were both effective at inducing BF.

The data in Figure 5.7 summarize all of the BF, BMP2-based, *in vitro* testing of potential ingredients for a BF formula. The gray, black, and white with dots bars show the effect of samples on BMP2 gene expression, protein expression, and promoter activation, respectively. The black line with a gray arrow on the Y-axis indicates the minimum two-fold response, and the results are represented so that a positive response is an increase above the line. White bars show relative area of new bone formed in calvarial tissues in the presence of the same botanical samples.

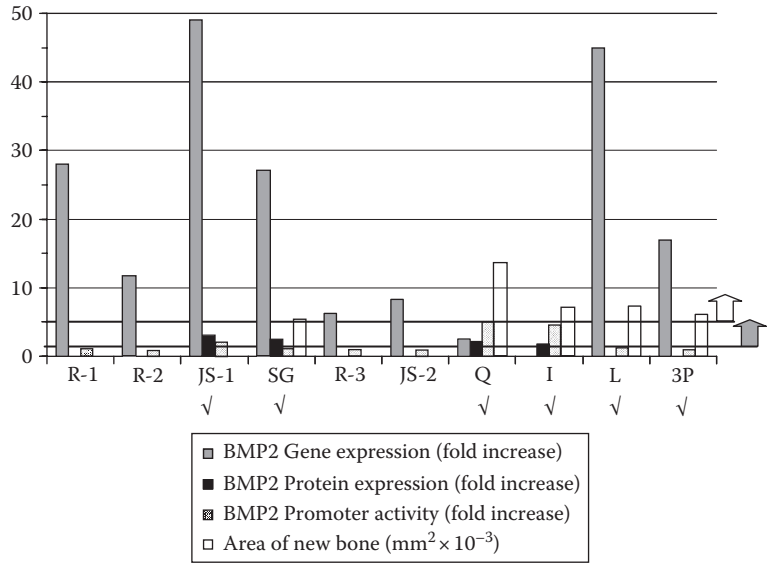

FIGURE 5.7 Summary of BF bioassay and calvarial study. R-1, *Rehmannia* spp. extract; R-2, *Rehmannia* spp. extract; SJ-1, *Sophora japonica* 1; SG, *Siberian ginseng*; R-3, *Rehmannia* spp.; SJ-2, *Sophora japonica* 2; Q, *Quercetin*; I, ipriflavone; L, licorice; 3P, third-party proprietary ingredient. *Note: Values >2 indicate significant increase of BMP2 activity and values >5 $mm^2 \times 10^{-3}$ indicate significant increase of new bone growth. √Top performing ingredients.

The black line with white arrow at ~5 on the Y-axis indicates the relative amount of bone formed in the unstimulated control tissues, and the results for these data are represented so that a positive response is an increase above the line. From this analysis of all four studies, *Sophora japonica* 1 extract, Siberian ginseng extract, quercetin, ipriflavone, licorice extract, and the third-party ingredient were identified as the top BF performers as they induced BMP2 and stimulated BF in the calvarial assay.

As was done for the AR formula, the BF formula was optimized in a calvarial model by testing different combinations of the top performing botanical samples with quercetin as it was the best performing sample overall. All combinations showed increased new BF in the calvarial assay due to the inclusion of quercetin (Figure 5.8). It was determined to proceed with a formula comprised of quercetin and licorice based on cost and ingredient availability.

The final bioassays were run with varying ratios of quercetin to licorice in the calvarial assay. The data in Figure 5.9 show the results of that experiment. In the top graphs, quercetin was kept constant at 1 or 0.2 µg/mL. In the bottom graphs, licorice was kept constant at 2 or 0.2 µg/mL. Quercetin at 1 µg/mL alone was able to stimulate BF, but when licorice was added at increasing doses there appeared to be some inhibition. These combination experiments are not completely clear-cut. However, it is clear that a lower dose of licorice with increasing concentration of quercetin shows increased BF. Further testing will be required (preferably *in vivo*) to substantiate the effectiveness of this formulation.

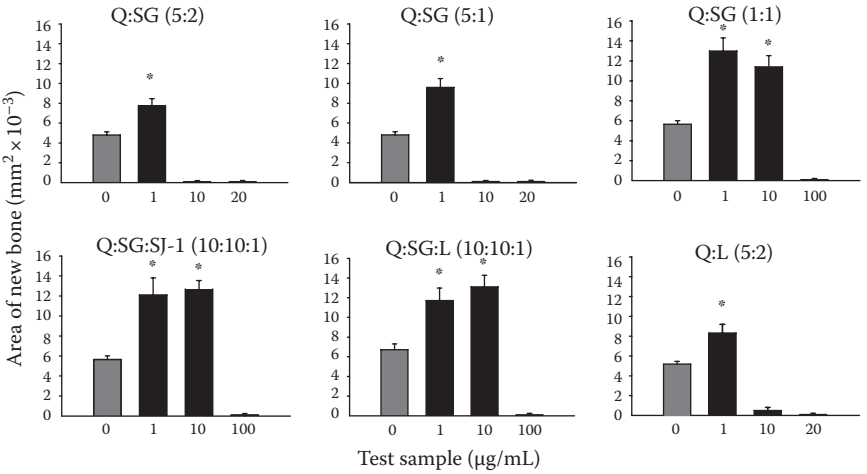

FIGURE 5.8 BF calvarial study results after treatment with botanical samples. Q, quercetin; SG, Siberian ginseng; SJ-1, *Sophora japonica* 1; L, licorice. Gray bars indicate results for the blank (untreated) samples. All data were expressed as mean ± SEM. *Indicates results that were significantly different from blank (untreated) samples ($p < 0.05$).

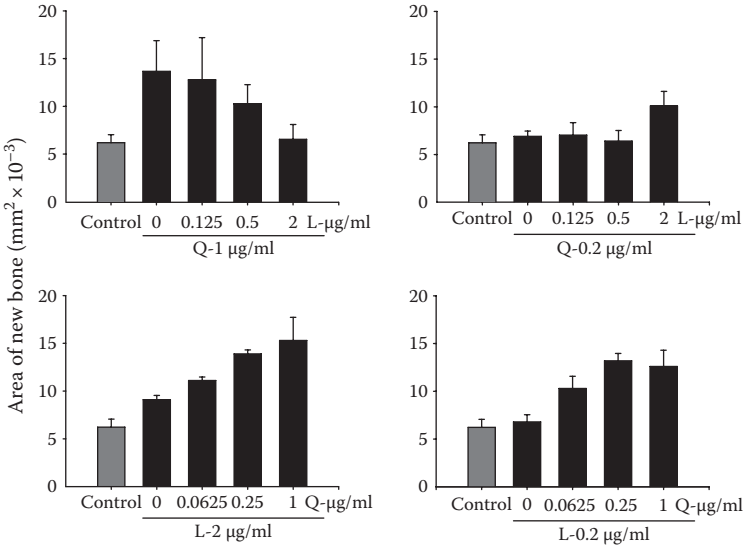

FIGURE 5.9 BF calvarial study results after treatment with a combination of quercetin and licorice. Q, Quercetin; L, licorice extract. Gray bars indicate results for the blank (untreated) samples. All data were expressed as mean ± SEM. *Indicates results that were significantly different from blank (untreated) samples ($p < 0.05$).

CHEMISTRY IDENTIFICATION

Background

Based on *in vitro* and calvarial studies, it was determined that two combinations from four botanical ingredients could be developed. Standardized extracts of pomegranate fruit and grape seeds constituted the AR formula while the BF formula contained quercetin and standardized extract of licorice root. All the botanical ingredients were in the form of hydro-ethanolic extracts standardized for specific markers or group of compounds. The pomegranate extract (*Punica granatum*) was standardized to contain 40% ellagic acid. GSE (*Vitis vinifera*) was standardized to contain 40% total polyphenols. Quercetin was sourced from a hydro-ethanolic extract of fava beans/fruit (*Dimorphandra mollis*). Licorice root extract (*Glycyrrhiza glabra*) was standardized to contain 0.05% glabridin. The processing of the botanicals was carried out by extraction procedures from commercial and in-house manufacturing facilities. The level of standardization and quality of the extracts were verified using phytochemical and analytical investigations.

Figures 5.11 through 5.14 show typical phytochemical fingerprints of the four botanical ingredients that were chosen for the AR and BF formulations. The methods and observed data will serve as key information to track and maintain the quality of these bioactive ingredients for further formulation and product development activities.

Pomegranate Fruit

Sample Information

Punica granatum fruit extract, ethanol/water extract, 35–50:1 ratio; standardization, 40% ellagic acid; analytical markers for quality, phytochemical fingerprint (qualitative), and ellagic acid (identification and quantification).

Phytochemical Fingerprint and HPLC Analysis

Raw material (200 mg) was extracted by sonicating at room temperature in methanol (100 mL). Ellagic acid was detected and quantified against a commercially available external standard. The fingerprint profile is shown in Figure 5.11.

HPLC Conditions

Mobile phase—solvent A: acetic acid 1.0% (v/v) in aqueous solution; solvent B: acetonitrile. Gradient: time (min), %B: 0 min, 3%; 9 min, 6%; 14 min, 8%; 19 min, 13%; 22 min, 22%, 24 min, 35%; 27 min, 3%; 30 min, 3%. Run time 30 min.

Flow rate: 1.0 mL/min; column temperature: ambient; sample temperature: ambient; injection volume: 10 µL; integration: peak area; detection: 260 nm; column: Phenomenex Synergi 4 µm Hydro-RP; 150 × 4.6 mm (00F-4375-EO) or equivalent.

Results and Discussion

Polyphenols constitute the major class of phytochemicals found in pomegranate fruit. The most abundant of these are ellagitannins. Ellagitannins such as punicalagins are hydrolysable tannins that contain glycosylated ellagic acid. Pomegranate fruit extract used in this study was standardized to contain 40% free ellagic acid

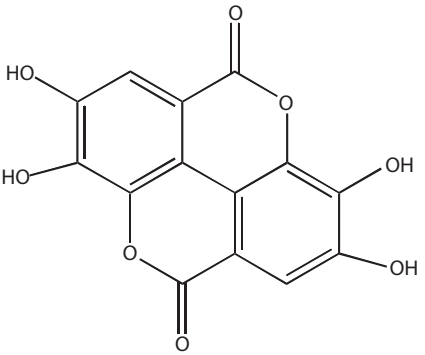

FIGURE 5.10 Chemical structure of ellagic acid from pomegranate extract.

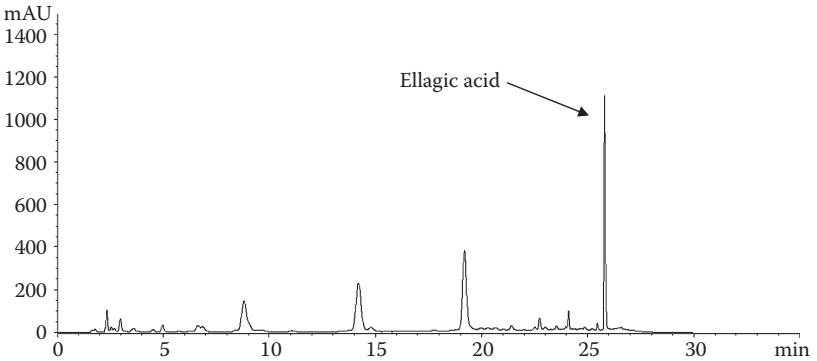

FIGURE 5.11 Phytochemical fingerprint profile of pomegranate extract.

(molecular structure in Figure 5.10). An analytical high-performance liquid chromatography (HPLC) method was developed to separate, identify, and quantify ellagic acid in the extract. The generated HPLC (Figure 5.11) also served as a benchmark phytochemical identification for the raw material.

Grape Seed Extract

Sample Information

Vitis vinifera, ethanol/water seed extract, 25:1 ratio, 50%–60% maltodextrin; standardization, 40% total polyphenols; analytical markers for quality, total polyphenols (quantification); phytochemical fingerprint (qualitative) and cyanidin (identification).

Total Polyphenols

Total polyphenols were estimated using a UV-Vis spectrophotometric determination by Folin–Ciocalteu method (Lester 1999; Vrhovsek et al. 2004). Target analytes: Polyphenols.

Method type: spectrophotometric/colorimetric. Results expressed as: Total polyphenols as gallic acid equivalents (GAE). Standard: gallic acid (as typical polyphenol). Reagent: Folin Ciocalteu Reagent (FCR) is commercially available in liquid form from Sigma-Aldrich Chemicals. It is a mixture of molybdic and tungstic acids and involves the following chemical species: 3 H2O.P2O5.13 WO3.5MoO3.10H2O and 3 H2O.P2O5.14WO3.4MoO3.10H2O.

Phytochemical Fingerprint and HPLC Analysis

A raw material (100 mg) was dissolved in methanol/hydrochloric acid (HCl), 95/5, v/v solution (25 mL). The solution was sonicated at room temperature for complete dissolution for 10 min. Ferric ammonium sulfate solution (2% in 2N HCl) was added as a catalyst (1.0 mL). The resultant solution was heated in a heat block with constant stirring at 65°C for 60 min. The resultant solution was cooled to room temperature and analyzed by HPLC. The fingerprint profile is shown in Figure 5.12.

HPLC Conditions

Mobile phase—solvent A: phosphoric acid 0.5% (v/v) in aqueous solution; solvent B: water, acetonitrile, acetic acid, phosphoric acid, 50/48.5/1.0/0.5 (v/v). Gradient: time (min),%B: 0 min, 20%; 26 min, 60%; 30 min, 20%, 35 min, 20%. Run time, 35 min.

Flow rate: 0.8 mL/min, column temperature: 30°C; sample temperature: ambient; injection volume: 10 µL; integration: peak area; detection: 520 nm; column: Hypersil ODS, 5 µm, 125 × 4.0 mm (Agilent 799260D-564) or equivalent.

Results and Discussion

Polyphenols constitute the major class of phytochemicals in GSEs. Commercially available GSE was standardized to total polyphenols. Due to this reason the sample was tested for total polyphenols using the Folin–Ciocalteu method. This procedure is based on a spectrophotometric (colorimetric) method for estimating total polyphenols in natural product matrices such as fresh herbs, feedstocks, raw material extracts, and finished product tablets. FCR is sensitive to reducing compounds. In the alkaline medium, the phenolic compounds react instantaneously with the FCR,

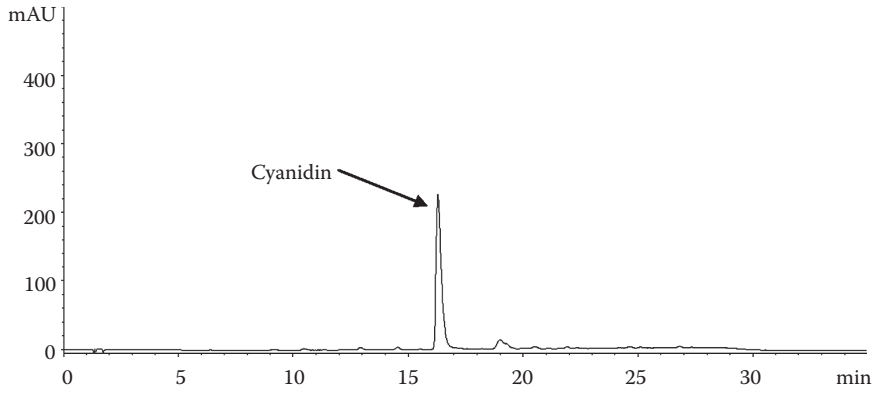

FIGURE 5.12 HPLC phytochemical fingerprint profile of hydrolyzed GSE.

resulting in blue-colored compounds that are measured at 750 nm. The blue color is a result of the oxidation of phenols in the sample that reduce the tungstates and molybdates in FCR. The reduced FCR is blue in color and is directly proportional to the total polyphenols present in the sample. The major class of polyphenols in GSE is a group of polymerized catechins called proanthocyanidins (also known as oligomeric proanthocyanidins) and anthocyanins. Oligomeric proanthocyanidins are dimers, trimers, and oligomers of catechin and epicatechin type flavanones. Materials containing these polyphenols, upon acid hydrolysis, yield cyanidin and related anthocyanidin pigments (Porter et al. 1985). The liberation of these anthocyanidins is directly proportional to total hydrolyzable polyphenols present in the original, unhydrolyzed material. Cyanidin is hydrolyzed and detected as the liberated anthocyanidin pigment from GSE as per the reaction scheme shown in Figure 5.13. An HPLC method that was developed for separation of anthocyanins in dietary

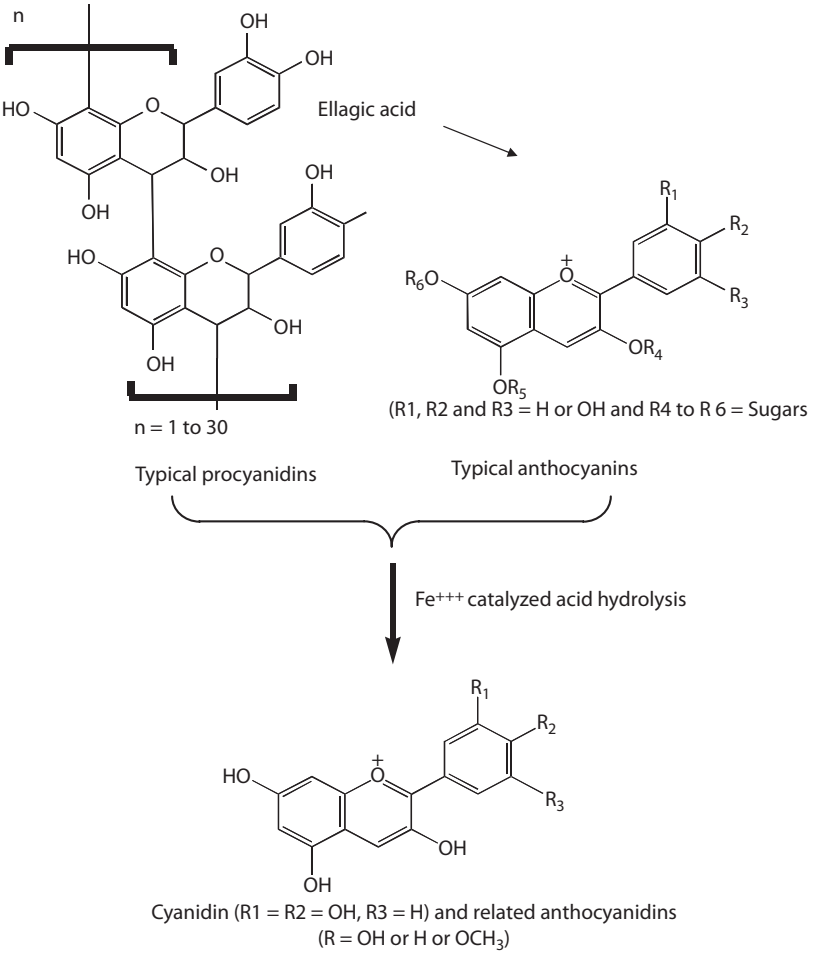

FIGURE 5.13 Liberation of anthocyanin pigments from "hydrolysable polyphenols" upon acid hydrolysis.

supplements (Chandra et al. 2001) was used to specifically separate and identify the release of cyanidin as the anthocyanin pigment from GSE and the results are shown in Figure 5.12.

Licorice Root Extract

Sample Information

Glycyrrhiza glabra, 70% ethanol/water extract, 3–6:1 ratio; standardization, 0.05% glabridin; analytical marker for quality, phytochemical fingerprint (qualitative), glabridin (identification and quantification).

Phytochemical Fingerprint and HPLC Analysis

Finely powdered licorice root extract (0.50 g) was extracted by adding 10 mL acetonitrile. The extraction was carried out by sonicating in warm water (40°C) for 30 min with occasional swirling. Samples were filtered through a 0.45 µm filter and analyzed by HPLC. Glabridin was quantified against a commercially available standard. The fingerprint profile is shown in Figure 5.14.

HPLC Conditions

Mobile phase—solvent A: phosphoric acid 0.2% (v/v) in aqueous solution; solvent B: acetonitrile; gradient: time (min), %B: 0 min, 45%; 10 min, 50%; 15 min, 60%; 19 min, 60%; 21 min, 80%; 25 min, 80%; 30 min, 45%; 35 min, 45%. Run time, 35 min.

Flow rate: 1.0 mL/min; column temperature: ambient; sample temperature: ambient; injection volume: 10 µL; integration: peak area; detection: 282 nm; column: Waters Symmetry Shield RP8; C8, 250 × 4.6 mm; 100A, 5 µm; cat # WAT200670 or equivalent.

Results and Discussion

Glabridin (Figure 5.15) belongs to the phytochemical group isoflavans that are a subclass of the group isoflavones with no double bond between C2 and C3 and no keto moiety at position C4. Glabridin is a fat soluble compound native to licorice.

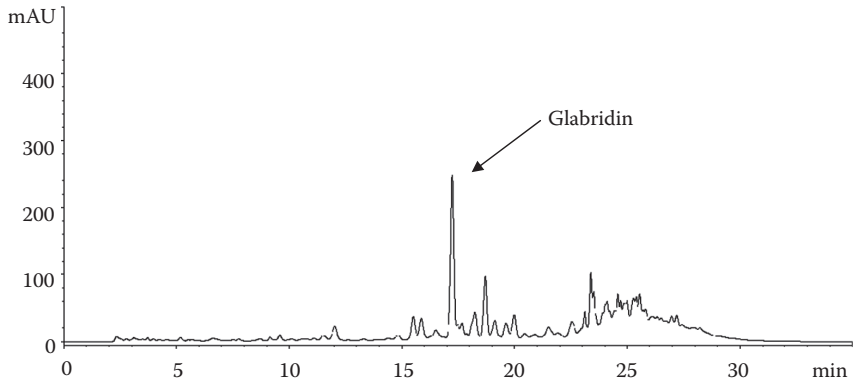

FIGURE 5.14 HPLC phytochemical fingerprint profile of licorice root extract.

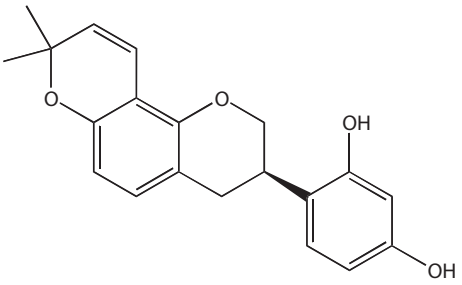

FIGURE 5.15 Chemical structure of glabridin from licorice root extract.

Its molecular structure and lipophilic nature are similar to that seen in estradiol, which make it a compound of interest for several health benefits in nutraceutical and cosmeceutical applications (Tamir et al. 2000).

Licorice root extract used in our studies has been derived from a patent pending hydro-ethanolic extraction process from licorice root feedstock that is standardized to glabridin. The HPLC method developed not only separates, identifies, and quantifies glabridin but also generates a phytochemical fingerprint identification for benchmarking the bioactive extract (Chandra et al. 2008). A typical HPLC fingerprint profile is shown in Figure 5.14.

Quercetin

Sample Information

Dimorphandra mollis fruit; ethanol/water extract, 10–15:1 ratio, no excipients; standardization: quercetin minimum 86%; analytical marker for quality: phytochemical fingerprint (qualitative), quercetin (detection, identification, and quantification). Quercetin was quantified against a commercially available standard. The fingerprint profile is shown in Figure 5.16.

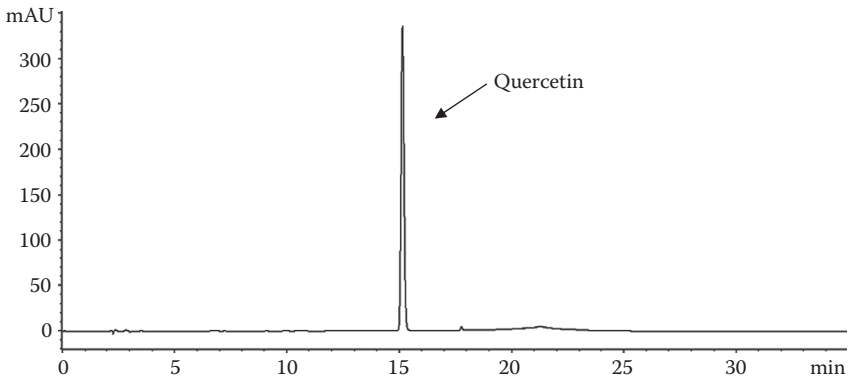

FIGURE 5.16 HPLC phytochemical fingerprint profile of quercetin.

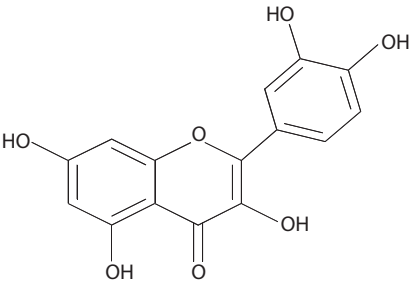

FIGURE 5.17 Chemical structure of quercetin from *Dimorphandra mollis* extract.

Phytochemical Fingerprint and HPLC Analysis

Quercetin raw material (20 mg) was dissolved in methanol/DMSO, 4/1, v/v solution (100 mL). The sample was sonicated at room temperature for 10 min with occasional shaking, mixed well, and filtered through a 0.45 µm filter prior to analysis.

HPLC Conditions

Mobile phase—solvent A: H_3PO_4 aqueous 0.2% (v/v); solvent B: methanol; solvent C: acetonitrile. Gradient: time (min), B-%, C-%: 0 min, B-19%, C-12%; 12 min, B-34%, C-15%; 18 min, B-40%, C-40%; 25 min, B-19%, C-12%; 35 min, B-19%, C-12%. Run time 35 min.

Flow rate: 1 mL/min; injection: 10 µL; analytical wavelength: 280 nm; column: Zorbax, agilent C-18, 250 × 4.6 mm, 5 µm, or equivalent; integration: peak area; column and sample temp: ambient.

Results and Discussion

Dimorphandra mollis is a tree native to Brazil. The fruit is rich in the flavone glycoside rutin from which quercetin (Figure 5.17) is derived through the removal of the sugar moiety. The concentration of quercetin in the enriched extract is more than 85% w/w.

The HPLC fingerprint method that was developed for flavonoids and related polyphenols was used for the analysis of quercetin (Chandra et al. 2004). The typical phytochemical fingerprint shown in Figure 5.16 is used for quality assurance purposes.

RESULTS AND DISCUSSION

Possible Future Testing

To further validate the efficacy of the AR and BF formulas, a human or animal study is warranted. The standard human clinical study approach is to conduct a double-blind placebo controlled study. The end-point measurement can be bone mineral density measurement using a dual-energy x-ray absorptiometry (DXA) machine. Due to the slow rate of bone turnover in humans, this type of study requires a long

intervention period and large sample size. To reduce the intervention duration and sample size, bone turnover biomarkers such as specific alkaline phosphatase (ALP), serum osteocalcin (OC), serum C-terminal cross-linked telopeptides of type I collagen (S-CTX-I), and urinary osteocalcin (U-OC) have been proposed. These markers are safe and easily performed as compared to DXA or other imaging techniques. However, the use of these markers is not without controversy due to individual differences and the complexity of the bone metabolism process (Vasikaran, Glendenning, and Morris 2006).

Gene expression analysis using microarray technology is a potentially valuable tool to study bone metabolism in humans due to its sensitivity and mechanistic target measurement approach. For example, the difference in BMP2 gene expression before and after the intervention can be compared to confirm the effect of a BF treatment. An advantage of this technology over DXA scanning is that the intervention duration and sample size can be greatly reduced. In addition, this technology has the ability to identify new molecular mechanisms of activity, which may assist future product development.

Animal models are often used to study the effect of bone health treatments. The advantages of an animal model over a conventional human model are shorter study duration as well as the ability to directly study bone histology and architecture (bone mass and strength). However, a disadvantage is that any particular species of animal model may have different nutrient metabolism than humans, especially when considering natural plant-based ingredients.

The maintenance of bone health is a long-term commitment. Safety of ingredients over this extended period of time is also an important aspect during product development. Safety validation can be performed by monitoring the change of liver enzymes and other biochemical markers during a human and/or animal study.

CONCLUSIONS

This research represents a potential new application for the use of natural product extracts in a nutraceutical formulation to prevent excessive bone loss over time. The novel selection strategy described earlier, by identifying high potential extracts for further research, has significantly reduced product development risk.

The systematic and targeted approach resulted in two different formulas. The AR formula is designed to maintain bone mass by preventing aggressive calcium loss. This formula contains pomegranate and GSE. Our results illustrate that this formula can suppress *RANKL* expression, which can, in turn, lead to suppressed osteoclast activity and reduction of bone loss. Due to this mechanism of action, the AR formula may be most suitable for peri- and postmenopausal women to effectively maintain their bone health.

The BF formula is designed to help reach and maintain maximum bone mass by stimulating BF and enhancing the calcium deposition process. This formula contains quercetin and licorice extract. Our results demonstrate that this formula can stimulate BMP2 production that can lead to increased BF. Due to the mechanism of action, the BF formula may be more suitable for young adults to reach their maximum bone mass before the onset of age-related bone loss.

ACKNOWLEDGMENTS

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6 Comprehensive Strategies for Evaluating the Adaptogenic Properties of Phytochemicals

Mary Ann Lila and Diana Cheng

CONTENTS

INTRODUCTION

This chapter focuses on experimental research methodologies to evaluate the adaptogenic benefits of phytochemicals. Adaptogen is a term used to describe a natural product that provides restorative or rejuvenating benefits to the body of animals (including humans) that ingest it. The term adaptogen was originally coined in 1947, but the functional definition was first recorded in 1968 by a Soviet scientist, Dr. I. I. Brekhman: "Adaptogens are non-toxic substances that produce nonspecific, positive responses to stress in the body, and have a normalizing influence on body metabolism and physiology (homeostasis)." In addition to use as normalizing treatments, adaptogens have been used as performance enhancers. Numerous studies have evaluated these performance-enhancing properties through measures of improved endurance, strength and recovery

time, maximum oxygen uptake, muscle mass accretion, and reduction in body fat mass (Bucci, 2000). Exercise is a form of stress on the body's mobile functions, and adaptogens are valued as a means to counter the negative ramifications of extreme physical exertion. Human and animal studies have induced stresses such as noise, high altitude, and mental stress (achieved by requiring subjects to perform complex psychomotor tests) and have monitored the ability of natural adaptogens to alleviate negative impacts on performance (Davydov and Krikorian 2000).

Adaptogenic plants have long been an ingredient in folk and traditional medicines; however, their mechanisms of action have seldom been pinpointed, nor has science, until very recently, had the capacity and the fine precision analytical instrumentation needed to elucidate their metabolism-balancing activities. Because adaptogens can have diffuse effects on the body and are assumed to interact with multiple human therapeutic targets, their pharmacodynamic activities can be challenging to quantify, and it has been difficult to attribute adaptogenic activity to discrete phytochemical groups. A range of modern *in vitro* and *in vivo* bioassays have been developed in order to gauge adaptogenic properties, and a few caveats concerning various approaches are discussed. The diversity of phenotypic effects that can be impacted by adaptogens mandates a comprehensive, multi-faceted approach to successfully gauge adaptogenic benefits *in vivo.*

ADAPTOGENS IN TRADITIONAL MEDICINE

Although the term adaptogen is relatively new and has only recently gained popularity in herbal medicine, parallel concepts of adaptogenic herbs have long been in use in ayurvedic medicine and traditional Chinese medicine (TCM). In both ayurvedic medicine and TCM, the focus is placed on individual patients, rather than on diseases, and treatment of disease symptoms takes place via the restoration of balance of the body. In TCM, the balance of the opposing energies, yin and yang, is key to maintaining homeostasis. The balance of yin and yang can be physically conceptualized into the interaction between the four bodily humors (qi, blood, moisture, and essence) and internal organ systems (Patwardhan et al., 2005; Yuan and Lin, 2000).

Similarly, in ayurvedic medicine, biological systems are governed by the interactions between the five elements of ether, air, fire, water, and earth. The interplay between them is organized into three forces, known as doshas, which are responsible for the physiological and psychological balance and therefore health of the individual (Chopra and Doiphode, 2002). The aim of ayurvedic medicine is to balance the integrated body, mind, and spirit to help prevent illness and promote wellness (nccam.nih.gov/health/ayurveda). Specifically, the *rasayana* herbs of ayurvedic medicine coincide with the adaptogenic concept. *Rasayana* plants are said to prevent aging, reestablish youth, strengthen life and brain power, and prevent disease (Rege et al., 1999). A prime example is Ashwagandha root (*Withania somnifera*), which has demonstrated anti-inflammatory, anticonvulsive, antitumor, immunosuppressive, and antioxidant properties mainly attributed to the steroidal lactones, withanolides (Bhattacharya and Muruganandam, 2003; Ganzera et al., 2003). Both ayurvedic medicine and TCM feature treatments that alleviate individual patient symptoms, emphasizing restoration and maintenance of balance.

In contrast, the Soviet concept of adaptogen was developed to distinguish a new group of chemical substances that conferred "a state of nonspecifically increased resistance" in an organism. As previously noted, the term adaptogen and its definition were originally recorded by Soviet scientists (Mamedov, 2005). Specifically, the concept was used by Lazarev to describe the effectiveness of dibazol (2-benzylbenzimedazol) on damaged regions of the nervous system and increasing nonspecific resistance to adverse influences (Brekhman and Dardymov, 1969). Brekhman further defined adaptogens as substances that must (1) be innocuous and cause minimal disorders in the physical functions of an organism; (2) be nonspecific, that is, it should increase resistance to adverse influences of a wide range of factors of physical, chemical, and biological nature; and (3) possess normalizing action irrespective of the direction of the foregoing pathological changes. Research in the former Soviet Union on adaptogenic plants focused on *Eleutherococcus senticosus* (Siberian ginseng), *Rhodiola rosea*, *Leuzea carthamoides*, *Schizandra chinensis*, and *Panax ginseng* (Brekhman and Fulder, 1980). In the former Soviet Union, adaptogens were valued for their ergogenic capacity (i.e., the ability to increase physical or mental output by eliminating fatigue) and for aiding military personnel and athletes during international competitions (Mamedov, 2005). Most of the original studies on Siberian ginseng were conducted in the former Soviet Union, and results are difficult to interpret due to a lack of published details (Bucci, 2000). In a comparison of eight subsequent investigations on Siberian ginseng and endurance performance, three were deemed to have severe methodological flaws, and the remaining five did not demonstrate any performance benefit with administration of the Siberian ginseng preparation (Goulet and Dionne, 2005).

CLASSIFICATION OF ADAPTOGENS

While a vast array of plants and plant-derived extracts have been identified and used to combat chronic disease conditions, there are comparatively few medicinal plants that can be cited in the adaptogen category. Interactions between different phytochemical groups that co-occur within a plant are expected to result in the adaptogenic benefits to the consumer, and as previously noted, the diffuse nature of the bioactive effects confound assignments of activity to certain phytochemical constituents. Adaptogenic compounds are routinely used against a plethora of diverse disorders that, by the gauges of modern medicine, seem to not have pathophysiological connections (Govindarajan et al., 2005). For these reasons, adaptogens have defied standardized classification into bioactive groups; however, a simple classification breaking adaptogens into three major groups, triterpenes, phenylpropanes, and oxylipins, has been proposed by Panossian (2003) and others as constituents common to adaptogens. The triterpenes include phytochemicals produced through the mevalonate pathway, including phytoecdysteroids, phytosterols, and saponins. The shikimate pathway produces the phenylpropane group of adaptogenic compounds, which includes lignans and flavonoids. Finally, the oxylipins are produced via the acetate pathway; the hydroxylated fatty acids fall into this group. Many adaptogens contain polysaccharides that together with the components mentioned earlier stimulate immune responses. Clearly, adaptogens include quite divergent categories of compounds, and

as stated previously, it is expected that potentiating interactions (either additive or synergistic) between them may account for the activity of the plant extract.

A few examples of recognized adaptogenic species illustrate the potentially multifaceted chemical contributions to activity. *P. ginseng* is one of the most studied and well-known adaptogenic plants from Chinese, Korean, Japanese, and Soviet origins. It has been prized as a tonic to invigorate weak bodies and help the restoration of homeostasis. The most intensively studied active phytochemicals in *P. ginseng* are ginsenosides, a unique class of steroid glycosides and triterpene saponins. Active constituents of *P. ginseng* include not only the ginsenosides but also polysaccharides, peptides, polyacetylenic alcohols, and fatty acids (Attele et al., 1999). The unique mixture of phytochemicals act through different biological mechanisms, conferring an overall adaptogenic response. Current *in vivo* and *in vitro* studies have shown ginseng's beneficial effects in a wide range of pathological conditions such as cardiovascular diseases, cancer, immune deficiency, reduction in age-related deficits, central nervous system (CNS) disorders, and neurodegenerative diseases (Radad et al., 2006). Another adaptogenic species, *E. senticosus*, has the common name Siberian ginseng although it is not botanically a true ginseng. Extracts of *E. senticosus* include phenylpropanoids, lignans, saponins, coumarins, the triterpene betulinic acid, and vitamins and provitamins (Davydov and Krikorian, 2000). Although *E. senticosus* and *P. ginseng* are both potent adaptogens and are in the Araliaceae family, they accumulate different classes of phytochemicals and promote adaptogenic effects through different physiological mechanisms. Traditionally valued adaptogens can also come from outside the plant kingdom, for example, the Tiaga (hard bracket mushroom fungus) was harvested as an immune booster by Native Americans when they observed restorative effects on injured or sick animals that sought it out and ingested it. In this case, the polysaccharides (beta-1–3-D glucans) are purported to be the most active ingredients, but fibers and other components are assumed to modify the bioactivity. Cordyceps, a fungus that grows on caterpillar larvae, is one of the most valued adaptogens in traditional Chinese medicine and considered an equal to ginseng as a restorative tonic (Huang, 1999).

Even though adaptogens are a relatively recent category, research into adaptogenic properties and the elucidation of the plant or fungal hosts that produce adaptogens are a strong current focus in the medical arena. Consumer demand in the marketplace for adaptogenic products is robust, especially those that can naturally bolster endurance or improve sports performance. In modern pharmacology, higher therapeutic value is typically assigned to the most potent and target-specific drugs, yet the nonspecific nature of adaptogens makes them ill-suited to ranking via these standard industry criteria. In contrast to the high throughput screens used to evaluate either synthetic drugs or even some natural products that combat chronic disease, evaluation methods used to gauge the bioactive potency and efficacy of adaptogens must be adjusted to accommodate nonspecific or multitarget therapeutics. Robust evaluation of adaptogens is complicated by the lack of well-accepted, reliable bioassays or clinical stress models (Rege et al., 1999). Performance evaluations require substantiated scientific criteria, and adaptogens can best be evaluated through standardization of the (multiple) bioactives involved in the effect and validation of structure–activity relationships (Chopra and Doiphode, 2002; Yuan and Lin, 2000). The pharmacodynamic actions

of adaptogens can be quite different than those of stimulant drugs or even of some phytochemicals that are recognized antagonists to specific chronic diseases such as cancer or diabetes.

Many adaptogens demonstrate antioxidant capacity *in vitro* and *in vivo*, but that is not the sole proposed mechanism of action. Since adaptogens promote optimal homeostasis (by *toning down* hyperfunctioning systems and *upregulating* hypofunctioning systems), checks and balances and more complex modus operandi are expected to be involved. To determine the mechanisms of adaptogenic plant extracts, multiple targets need to be investigated in order to produce a more holistic assessment of complex pharmacological systems (Panossian et al., 1999; Rege et al., 1999). Research is needed to fill in the gap of information from dose response to evaluation of efficacy based on the triangulation of performance, tissue, and molecular expression information.

Are adaptogens, as their classic definition implies, really "nonspecific"? Or has science just not yet identified the varied active mechanisms and therapeutic targets? These questions bear further scrutiny and allude to the need for complex, multifaceted strategies for thorough evaluation of this group of natural compounds.

SCREENING FOR ADAPTOGENIC PROPERTIES

In Vitro

Only a limited number of *in vitro* (cell culture) screens have proven routinely useful for gauging adaptogenic properties; most approaches combine *in vitro* and *in vivo* bioassays in tandem. *In vitro* animal cell cultures (muscle myotubes, microglia cell lines, etc.) have been used to measure related parameters after administration of purportedly adaptogenic natural compounds. For example, Gorelick-Feldman et al. (2008) recently used cultured C2C12 mouse skeletal muscle cell lines to detect increases in protein synthesis after an administration of 20-hydroxyecdysone (20E), a phytoecdysteroid present in spinach, as well as *Ajuga turkestanica*, an adaptogenic herb from Central Asia. Subsequently, the adaptogenic extract was found to affect a small but significant increase in the grip strength of treated mice. Similar tests can be done using primary muscle cells biopsied from animals or humans. Simple *in vitro* tests for antioxidant capacity/reduction of lipid peroxidation have been conducted on various adaptogenic herbs like *R. rosea* and *S. chinensis*. Antioxidant capacity is the single most studied mode of action and the most verified outside of the former Soviet Union, although it is well acknowledged that this cannot be the main mode of action of adaptogens (Panossian et al., 1999). Alternative assays include measurements of glucose uptake from blood into muscle cells and tissues (accounting for the "burst of energy" effect attributed to some adaptogens) and various anti-inflammation assays. Because inflammation plays a key role in arthritis and other conditions with relevance to strength and metabolic balance, Dey et al. (2008) have demonstrated anti-inflammatory activities in a range of adaptogenic herbs using gene expression assays. Murine macrophage cell lines were selected as monocytes play a central role in inflammation, and activated monocytes induce proinflammatory genes that can be monitored *in vitro*. A great advantage of these gene expression arrays (high content

screens) is that a potential mechanism of action can be evaluated before particular bioactive principles or constituents in a phytochemical mixture have been identified. Catecholamine inhibition (related to stress) and immunostimulant assays have also been applied to measurements of adaptogenic properties. *In vitro* bioassays can seek, in a simplified forum, to identify at least some of the mechanisms of action that account for the observed metabolic improvements attributed to adaptogenic compounds. Well-established screens for antioxidant capacity, CNS function, inflammation, and other bioactivities can be used in conjunction with more mechanistic *in vivo* screens. Multiple bioassays together can contribute to a clearer picture of mechanisms responsible for adaptogenic properties.

In Vivo

Animal models are invaluable tools for evaluating the potential effect that a treatment might ultimately have in human systems, but the animal model is only robust if the test subject is given the chance to respond to the *treatment—not* to possible stress (anxiety or even injury) imposed during the treatment administration. This is particularly true when adaptogenic improvements in metabolism or performance are being gauged, as stress-induced reactions can greatly obscure and confound measured results. Stress is defined as the effect produced by external events or internal factors, which induce an alteration in an animal's biological equilibrium (Institute for Laboratory Animal Research, 1992). For adaptogenic response measurements, nonspecific and chronic stress responses must be minimized, allowing only the deliberately imposed, controlled form of stress (or treatment) to impact the test animal. Both the method of adaptogen delivery and the subsequent performance measurement procedures can adversely stress test animals and skew measurements of adaptogenic response.

Treatment Delivery

In order to assess reproducible and reliable responses to adaptogen treatment, particular care must be taken to avoid stress during delivery of the adaptogen to the animal. Routine procedures such as handling, venipuncture, and orogastric gavage are acutely stressful to animals, and previous research verifies that the stress-induced responses can be quantifiable and substantial. Handling of research animals triggers stress responses such as increased concentration of corticosterone, hyperthermia, and plasma glucose (flight or fight response). Other procedures that are considered routine in animal research, including venipuncture and orogastric gavage, can lead to elevations of heart rate, blood pressure, and glucocorticoid concentrations (Balcombe et al., 2004).

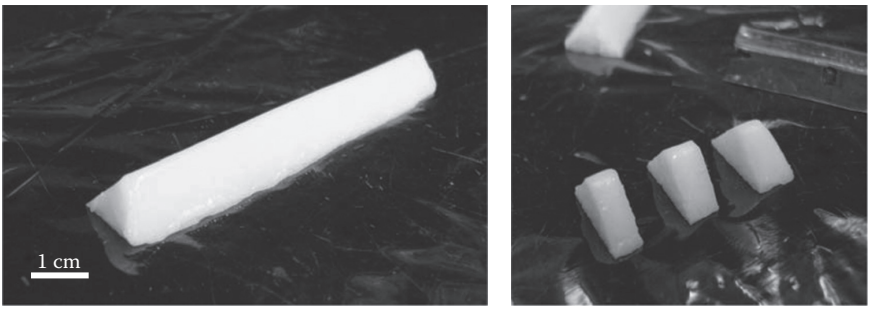
Oral Administration

To administer compounds by oral gavage, the animal must be restrained, and a rigid metal or plastic tube, with a rounded end to prevent tissue puncture, is inserted down the throat to dispense solution directly into the stomach. In addition to the stress of restraint, which must be done properly to prevent injury, the animal experiences the stress of breathing interference during the procedure and the discomfort

of stomach distension after the solution is dispensed. Other complications that may occur during gavage include inadvertent tracheal administration, reflux, aspiration pneumonia, esophageal impaction, trauma or perforation, hemothorax, and even death (Balcombe et al., 2004).

As an alternative to gavage, an orally administered compound may be incorporated into animal feed (diet) or into the water. This method of administration circumvents handling stress. However, in a feeding study, the amount of material administered is more difficult to judge as animals may not eat as much chow if the treatment adversely alters taste or palatability; water or chow may be inadvertently spilled by the animal and the treatment dosage may therefore not be accurate. In addition, feed and water intake of rodents is strongly correlated with body weight. Substantial differences in intake coincident with body weight of the test animal can further confound comparisons between animals. For example, mean adjusted water intakes can range from 5.7 ± 0.2 to 11.4 ± 0.5 mL per 30 g body weight (Bachmanov et al., 2002).

In order to gauge the effects of adaptogenic compounds, it makes sense to try to mimic the recommended methods of administration of traditional medicinal preparations, which are taken at specific doses and times, rather than throughout the day and night. One possible method, which again circumvents any handling stress, is to incorporate the adaptogenic compound into a "treat" that a laboratory animal will preferentially consume before regular chow. The treats can be periodically delivered at the times chosen in the experimental design, and the animal will willingly ingest it. For water soluble compounds, the treatment can be dissolved in a 5% sugar solution and administered to rats that have been trained to drink from a syringe (Schleimer et al., 2005). Sugar tablets or tablets with flavor additives may help mask unappealing tastes of compounds and require even less attention from the investigator. However, costs can escalate if the compound costs are high, as large quantities are needed to produce accurate doses using tablet press machines. Another method is to incorporate compounds into a gel form of the animal diet offered by Testdiet®. The compound can be added to a powder diet mix that contains a gelling agent and flavor additive. Mixing the powder with hot water activates the gelling agent, which can then be poured into molds and cut to desired quantities for administration to animals (Figure 6.1).



(a) (b)

FIGURE 6.1 Gel treat containing phytoecdysteroid treatment (20E), solidified and removed from tray mold (a). Individual gel treats are cut into triangles (b) for regulated dosage 0–50 mg/kg body weight for each animal.

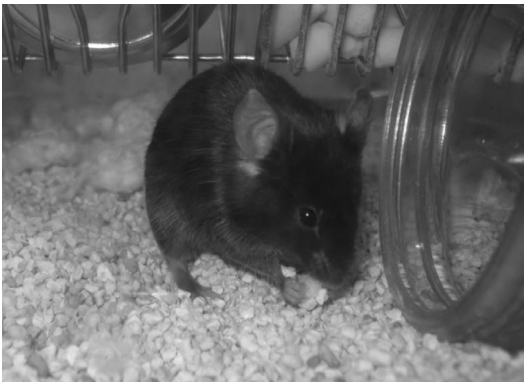


FIGURE 6.2 C57BL/6J mouse consuming a gel treat.

This method offers control of dose and timing with minimal handling by the investigator. Recently, during experiments to test the effects of the adaptogenic plant phytoecdysteroid 20E from *A. turkestanica* in a rodent model, we determined that the stress of routine gavage was not tolerated by the mice; the rodents were disadvantaged by the procedure to the extent that benefits from the administered dosage could not be gauged. As an alternative, the 20E was formulated as a gel treat, using the method described earlier. The mice preferentially consumed the full gel treat immediately at the time of introduction to the cage, ensuring that the full administered dose was consumed (Figure 6.2).

Injection or Surgical Implantation

When oral administration to the animal is not suitable (e.g., due to issues with bioavailability or if the animal model is unable to tolerate the stress of gavage), compounds can be directly delivered to the animal's system through injection or the use of implantable pumps. Small osmotic pumps, such as those produced by Alzet®, can be implanted into animals to allow for continuous infusion of compounds into circulation. However, this requires surgery and a recovery period. The invasiveness of implants may cause stress to the animal and inflammation, which can confound measurement of positive adaptogenic benefits. Multiple injections would involve the same caveats. As an alternative, injectable gel matrixes and biodegradable polymers allow for sustained release of compounds into the animal's body and eliminate the need for multiple injections (Chitkara et al., 2006; Gao et al., 1995; Kempe et al., 2008). These release agents are becoming more attractive for the controlled, time-course delivery of therapeutic agents.

Topical Administration

Another major noninvasive route of administration of therapeutic agents is by topical application through the skin. Topical administration also affords the advantage of bypassing first-pass metabolism; however, metabolic activity of the skin should not be overlooked (Calvery et al., 1946; Hadgraft and Guy, 1989; Hadgraft and Lane, 2005). Ointments, creams, gels, or patches are typically applied to the skin's

surface and the drug then diffuses through the skin. Human skin is composed of four main layers: the stratum corneum, the epidermis, the dermis, and the subcutaneous tissue that not only provide a protective barrier but also present a barrier to the absorption of topically applied drugs and eventual release into the blood stream (Foldvari, 2000). Penetration enhancers, or absorption promoters, are chemicals that reversibly disrupt the organized structure of the skin to accelerate drug permeation (Williams and Barry, 2004). After the (sometime serendipitous) discovery of some of these compounds, further research has uncovered the mechanisms of action (Hadgraft and Lane, 2005).

Dimethyl sulfoxide (DMSO) is frequently used as a vehicle for drug delivery due to the strong solvent and penetration properties afforded by its amphipathic structure (Pope and Oliver, 1966). DMSO dissolution of adaptogens (like phytoecdysteroids) and topical delivery to animal skins can be quite advantageous because no surgeries are involved. Experimental animals are marginally disadvantaged by handling stress, but this is a minor stress compared to invasive surgery or multiple injections. In addition, topical applications of a DMSO-based adaptogen can be repeated at time-course intervals, allowing a more realistic assessment of effects over time on performance and muscles/tissues.

As compounds independently diffuse through the skin in response to alterations caused by absorption promoters, other absorption promoters such as azones, alcohols, oils, glycols, surfactants, and terpenes may be more appropriate depending on the compound under investigation (Kurihara-Bergstrom et al., 1987; Williams and Barry, 2004). Although topical application of DMSO is generally well tolerated, it is important to be aware of the systemic side effects with its use and those of other penetration enhancers (Santos et al., 2003). Development of novel matrixes for noninvasive and sustained compound delivery allow for compounds with low oral bioactivity, such as resveratrol, to be therapeutically effective with minimal confounding stress factors (Hung et al., 2008).

Physiological Measures of Performance and Endurance

Ergogenic aids are any training technique, mechanical device, nutritional practice, pharmacological method, or psychological technique that can improve exercise performance capacity and/or enhance training adaptations (Kreider et al., 2004). Because adaptogens are often used as ergogenic aids, gauging their ability to enhance performance is a logical criterion for determining efficacy and possible applications. Exercise protocols should be designed to test the desired physiological adaptations without producing confounding nonspecific chronic activation of the stress response. Intensity, frequency, and duration, as well as perceived control and novelty of environment, can also have an impact on the stress response of animals (Kregel et al., 2006). Although acute activation of the stress response is normal and an attempt of the body to maintain or restore homeostasis, it is important to minimize stress that would confound measurements that attempt to gauge adaptogenic effects.

The three major modalities in gauging exercise performance capabilities in rodents are treadmill running, voluntary wheel running, and forced swimming (often weighted). Researchers attempt to reduce activation of the stress response by

repeatedly exposing the test animals to handling (and the treadmill or other apparatus) at the same time of day and by consistently assigning the same personnel to conduct the actual training sessions. These practices greatly reduce the stress response triggered by a novel environment and activity. Mice are nocturnal, so treadmill familiarization and training can be done during their dark cycles (Kregel et al., 2006). Treadmill and voluntary wheel performance assessments can also be skewed by genetic factors. Swiss Webster and FVB/NJ mice performed best and C57BL/6J mice performed the worst in treadmill running tests, whereas Swiss Webster and C57BL/6J mice had significantly longer running durations on voluntary treadmill assessments than other strains of mice (Lerman et al., 2002).

Treadmill Running

Among the three primary exercise modalities, running on a motorized treadmill allows the most control over the intensity and duration of physical performance. Some researchers consider the treadmill to be the "gold standard" for assessing the influence of adaptogens on performance in rodent models. Additionally, in combination with treadmill running, the total amount of external work can be quantified by measuring maximum endurance capacity or maximal O_2 uptake ($VO_{2\,max}$). $VO_{2\,max}$ is an indication of the capacity transport and utilization of O_2 between the lungs, cardiovascular system, and musculoskeletal system (Henderson et al., 2002). To evaluate endurance capacity, an animal is run to the point of fatigue, where $VO_{2\,max}$ is normally defined as the point at which VO_2 does not increase, even though further increases in external workload are imposed on the animal (Kregel et al., 2006). In general, $VO_{2\,max}$ correlates with exercise endurance and is a reliable method to assess fitness.

A main disadvantage of treadmill running in gauging adaptogenic effects is the stress imposed on the animal from a forced exercise modality. Often, animals need to be stimulated to exercise to their physical capacity through the use of external stimuli such as tapping their tails or hindquarters lightly with a stick, electric shock, or bursts of high-pressure air (Kregel et al., 2006). Additionally, chronic exercise training on the treadmill increases the rat's $VO_{2\,max}$ and leads to adaptations in both the cardiovascular and skeletal muscle systems that can confound effects of adaptogens (Davies et al., 1981; Kemi et al., 2002). However, motorized treadmill offers the examination of factors that contribute to exercise performance under well-defined experimental conditions.

Voluntary Wheel Running

Unlike motorized treadmill running, voluntary wheel running does not require constant surveillance by the investigator to prevent animal injury and does not impose stress responses from a forced exercise. As such, wheel running exercise studies can be performed long term with minimal intervention by the investigator. Additionally, voluntary wheel running can be used to determine the effects of adaptogenic compounds on animal physiology and behavior by monitoring changes in duration and distance run over a given time period (Dolinsky et al., 1983; Teramoto et al., 1988). Thus, exercise-promoting effects and ergogenic aid capabilities can be investigated using this exercise modality (Avraham et al., 2001). However, a major disadvantage is the difficulty in regulating exercise duration or intensity of running and

that exercise is largely determined by the animal's motivation to exercise. Another consideration with chronic use of wheel running is that substantial hypertrophy of hindlimb muscles and myocardium develops, which may confound the determination of adaptogenic effects (Henriksen et al., 1995).

Forced Swimming

Swim tests can be used to determine physiological, biochemical, and molecular changes to acute and chronic exercise (Baar et al., 2002; Jones et al., 2003). A major advantage of swimming exercise is the employment of a large volume of muscle mass and uniform exercise. However, forced swimming produces psychological stress and results in survival behavior, such as floating, climbing, diving, and bobbing, as the animal attempts to prevent drowning (Ferrandez and De la Fuente, 1999; Kregel et al., 2006). These types of behavior can confound exercise adaptations and interpretation of results due to the intermittent bouts of hypoxia. Additionally, swimming produces extensive adaptations to the cardiovascular system and is often used as a stimulus to produce adaptations rather than to determine performance (Kaplan et al., 1994). However, with training, noncontinuous swimming behavior can be reduced and the effect of adaptogens on improved performance can be measured by measuring swim duration.

Skeletal Muscle and Gene Expression Analysis

Examination of skeletal muscle can be performed to further substantiate the mechanisms by which adaptogenic compounds work *in vivo*. The analyses also uncover possible therapeutic applications for muscle diseases, through administration of natural adaptogenic extracts. Skeletal muscle is made up of heterogeneous specialized myofibers, or muscle cells, that generate force and movement of the body through contractile activity. The composition of myofibers in each muscle group determines their optimal specialized function. The myofibers are controlled by signaling pathways that respond to changes in metabolic and functional demands of the organism (Bassel-Duby and Olson, 2006).

Skeletal muscle hypertrophy in adult animals is a result of the increase in size of muscle, as opposed to hyperplasia, the increase in the number of muscle cells (Glass, 2005). Adaptogenic plants such as *L. carthamoides* and *A. turkestanica* contain compounds that have resulted in increased muscle mass after oral administration to animals (Hikino et al., 1968; Kratky et al., 1997; Koudela et al., 1995). However, increase in muscle mass alone does not necessarily benefit the functional capacity of the animal. Skeletal muscle fiber–type distribution and mitochondrial density are two major parameters of measure that determine muscle function and endurance capacity (Burkholder et al., 1994; Philippi and Sillau, 1994; Wang et al., 2004).

The ergogenic effects of certain adaptogenic compounds can be explained through investigation of fiber type composition. Skeletal muscles are generally classified as type I (red/oxidative/slow) or type II (white/glycolytic/fast) fibers. Type I fibers are rich in mitochondria and fatigue resistant due to the use of mainly oxidative metabolism, which provides a stable long-lasting supply of ATP. Type I fibers also have high levels of slow isoform contractile proteins, high capillary densities, and high levels of myoglobin, an oxygen-binding protein that give the fibers their red color. Type II

fibers have low levels of mitochondria, are susceptible to fatigue, rely mainly on glycolytic metabolism as a major energy source, and exhibit fast contractile activity (Spangenburg and Booth, 2003; Wang et al., 2004). Shifts in levels of oxidation enzymes, mitochondrial biogenesis, and fiber-type-specific contractile proteins indicate muscle fiber type switching and changes in functional capacity. Adult skeletal muscle can undergo muscle type conversion in response to exercise training, genetic manipulation, or pharmacological intervention (Booth and Thomason, 1991; Choo et al., 1992; Kadi et al., 1999; Rajab et al., 2000; Wang et al., 2004). Thus, adaptogenic compounds may increase stamina, strength, and endurance in animals through the modulation of skeletal muscle fiber–type composition.

Mitochondria are the main subcellular structures that determine the oxygen consumption and energy demand of muscle (Philippi and Sillau, 1994). One of the most important adaptations after endurance training is the increase in muscular oxidative capacity due to the increase in mitochondrial density and activities of mitochondrial enzymes (Zoll et al., 2002). Although both fiber types adjust to match functional demand, the difference in oxidative capacity between slow oxidative fibers and fast glycolytic fibers is the result of much greater mitochondrial volume density in the subsarcolemmal area of the slow oxidative fibers (Philippi and Sillau, 1994). Analysis of fiber type and mitochondrial density in skeletal tissues can identify biological changes induced by adaptogens to support their performance-enhancing capabilities.

Adaptogens, given the breadth of their influence, have potential to interface with a range of gene pathways. A valuable and efficient means of obtaining a mass screen of multiple systems is with global gene expression microarrays. With the growth of genomics and the full sequence of mice DNA and most of their gene functions, a mouse global gene expression microarray provides a robust analysis of adaptogenic effects *in vivo* (Duggan et al., 1999). Identification of pathways affected by adaptogens will uncover modes of actions to the alterations in the cellular environment responsible for the adaptogenic properties.

Global gene expression arrays provide a screen of functions and can be corroborated with studies targeting specific key pathways. Activation of gene expression appears to be instrumental in controlling the accumulation of posttranscriptional adaptations leading to structural and biochemical adaptations of the mitochondrial compartment in exercised skeletal muscle (Hoppeler and Fluck, 2003). Increase in mitochondrial volume density is supported by proportional increases in the steady-state level of a number of mRNAs encoding mitochondrial proteins (Puntschart et al., 1995). The combination of gene and protein expression, skeletal muscle composition, and physical performance capacity provides a robust illustration of the ergogenic function of adaptogens.

COMPREHENSIVE STRATEGIES TO DETERMINE THE ADAPTOGENIC ACTIVITY OF PLANTS

Natural adaptogenic phytochemicals are some of the most intriguing new targets for discovery and development for human health applications. The concept of homeostasis as a complex dynamic equilibrium persistently challenged by stress factors (Chrousos and Gold, 1992) inherently resists precise quantification by

scientific criteria. Therefore, not surprisingly, adaptogens present particular challenges for science-based evaluation. As noted earlier, the diversity of phenotypic effects that can be impacted by adaptogens mandates a comprehensive, multifaceted approach to successfully gauge adaptogenic benefits *in vivo*. Multiple mechanisms of action may be simultaneously in operation as multiple phytochemicals act together in harmonic synergy to provoke an adaptive response in the human body. *In vitro* mechanistic tests need to be combined with *in vivo* evaluations, and in addition, performance measurements should ideally be supported with physiological and gene expression data on the mechanisms of action.

Tests of adaptogen efficacy should strive to mimic, as much as possible, the methods by which an adaptogenic herb or other preparation would be administered in traditional medical practice. However, costly clinical trials are generally not warranted until many parameters (dosage, timing, composition, toxicology, etc.) have been narrowed down in a series of preclinical tests. Since distress, tension, fatigue, etc., are difficult to precisely regulate, investigators have resorted to some unusual means to simulate stress that a human subject might experience (Govindarajan et al., 2005). For example, animals injected with pathogenic bacteria (to evaluate the ability of a plant extract treatment to combat infection) have been further disadvantaged by ligating the caecum, or in other cases, animals were rendered immunosuppressed before deliberate infection (Thatte and Dahanukar, 1989). In order to evaluate the ability of an adaptogen to alleviate muscle damage, some research strategies have deliberately injected muscles with snake venom to induce muscle necrosis (Harris et al., 2000; Toth et al., 2008). The adaptogenic phytoecdysteroid compound 20E was injected into rat soleus muscle, narcotized, and treated for muscle regeneration followed by daily injections of 20E for 7 days to see if it could alleviate muscle damage/degeneration and therefore provide a potential treatment for muscle atrophy (Toth et al., 2008). Analysis of muscle fibers posttreatment showed an increase in fiber size and myonuclear number in normal and regenerating cells given 20E treatment, indicating its potential for use as a therapeutic treatment for muscle atrophy. Caveats in this case include that muscle necrosis, as induced in these experiments, is a damage that can be caused by injury (or venom injection), whereas muscle degeneration or atrophy can have multiple other causes including forced confinement (failure to exercise the muscles) or genetic factors. Snake venom, in addition to causing rapid muscle necrosis, also induces additional responses in a test animal such as inflammation and pain that would not be symptomatic of simple atrophy due to lack of muscle use (Dixon and Harris 1996; Teixeira et al., 2003). Therefore, researchers who attempt to provoke muscle damage using this mechanism must also treat to alleviate the side effects in order to evaluate muscle regeneration. According to Lee and Bianchi (1971), the lack of good experimental or clinical stress models is one major obstacle in discovery and/or development of adaptogenic formulae.

CONCLUSION

Breakthroughs in modern metabolomics, with multiple platforms for analysis, are for the first time permitting rigorous analysis of complicated adaptogenic mixtures and translating their benefits, from both well-known mainstream food crops and lesser-known endemic medicinal plant sources, to the public at large. Adaptogens are

an increasingly popular category in the industry, with much simpler routes to commercialization than traditional pharmaceuticals, and burgeoning demand from consumers interested in health maintenance and improved performance. There are multiple manifestations of adaptogenic compounds on the human metabolism. In order to thoroughly gauge the efficacy, science needs to center attention on the common mechanisms of action such as immune modulation (Rege et al. 1999). Given that multiple therapeutic targets seem to be involved, robust evaluative assays for gauging adaptogenic properties will inevitably demand evaluation of multiple complementary bioassay screens, *in vitro* and *in vivo*, and the animal screens ideally should evaluate both performance and analysis of muscle and tissue composition. By linking evaluative criteria in this way, the particularly complex and multifaceted roles of adaptogenic phytochemicals can be elucidated, and subsequently understood by consumers as proactive means for human health protection.

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7 Prevention of Obesity, Diabetes, and Cancer with Lifestyle Intervention Strategies

Jacob J. Junco and Thomas J. Slaga

CONTENTS

EVOLUTION OF OBESITY-RELATED CHRONIC DISEASE

In today's fast-paced lifestyle, technological advances have allowed for negligible levels of physical activity in daily routines, especially for people with sedentary occupations. In addition, a recent explosion of high-calorie fast food and snack food availability has hampered many people's ability to make sound dietary choices. These changes have led to a number of health-related consequences such as obesity, diabetes, and obesity-related cancers.

Before the rise of civilizations, human societies consisted almost exclusively of small hunter–gatherer tribes. Their diet at the time, now commonly referred to as the "Paleolithic diet," consisted primarily of lean meat and/or fish (depending on location), fruits, vegetables, and nuts and excluded many modern mass-produced food sources such as grains, dairy, salt, and sugar (Klonoff, 2009). This diet contained slightly higher protein intakes and similar percentages of fat and carbohydrates as modern Western diets; however, there was likely a greater consumption of vitamins and phytonutrients from natural plant sources (Eaton and Eaton, 2000; Konner and Eaton, 2010; Kuipers et al., 2010). Also, carbohydrates were obtained primarily from natural, non-refined sources like fruits, vegetables, and roots (Konner and Eaton, 2010; Kuipers et al., 2010). Many dieticians suggest today that this diet is what humans are genetically meant to eat, sparking a number of discussions among the

diet community. In addition, these early humans engaged in enormous amounts of aerobic activity, as they moved over 15 km a day to follow prey or find nuts or berries, once food sources in their region became scarce (Cordain et al., 1998).

Around 10,000 BC, the "agricultural revolution" began, bringing with it easy-to-grow, high-carbohydrate crops and domesticated meat sources. This lifestyle shift was the primary factor in the formation of large modern societies and civilizations by providing a consistent source of food in one area. High-glycemic-index crops like rice, potato, and corn provided the backbone of these mass-produced diets and still do today in the form of basic staples as well as fast food and snack food ingredients. In addition, with mass production of food, fewer people had to spend their days searching for food, enabling them to focus on the earliest tenets of civilization, including art, literature, and government. However, these early "white collar" jobs had one of the same consequences they do today: a sedentary lifestyle. Physical inactivity was only further exacerbated with the rise of automation during the past few centuries. Plentiful high-carbohydrate crops as well as fatty meat from domesticated animals and increasingly sedentary lifestyles were a large contrast to the routines to which humans had physiologically adapted for hundreds of thousands of years. In essence, human lifestyle shifted many times faster than could human evolution, allowing for rapid increases in chronic diseases related to obesity, which are largely absent under the healthy diet and exercise-heavy lifestyle of hunter–gatherers (Eaton et al., 1988; O'Dea, 1991). While these diseases were likely introduced in large part due to the formation of civilization thousands of years ago, the sharp rise in obesity, diabetes, and obesity-related cancers in industrialized and industrializing countries over the past few decades can be attributed to a modern dietary shift as well as an increasingly sedentary lifestyle.

MODERN LIFESTYLE SHIFTS AND THE "FRENCH PARADOX"

Over the past few decades in many countries, increased consumption of fatty "fast foods" as well as less physically active work environments has driven obesity epidemics leading to sharply heightened incidences of diabetes, heart disease, and many cancer types. In long-industrialized countries like the United States and England (Lusignan et al., 2005; Howel, 2011), and even in recently wealthy countries like Qatar and Saudi Arabia (Al-Nozha et al., 2007; Ng et al., 2011), high-calorie, low-nutrition foods are readily available while technological conveniences have removed daily exercise from many routines, leading to alarmingly high rates of obesity and associated chronic diseases. In the United States, incidence of type II diabetes among adults has more than doubled since the late 1970s (Fox et al., 2006; Zhang et al., 2009a), with a peak at 11.3% according to recent statistics (Centers for Disease Control and Prevention, 2011). The United States also recently saw a strong increase in hypertensive adults, from about 20% of the population in 1990 (Belfiglio, 2005) to nearly 30% in 2000 (Egan et al., 2010). In addition, many epidemiological studies in the United States and other countries have suggested high-grade prostate cancer (Freedland and Platz, 2007; Rodriguez et al., 2007; Strom et al., 2008), postmenopausal breast cancer (Magnusson et al., 1998; Rosato et al., 2011), and colorectal cancer (Campbell et al., 2010; Siddiqui, 2011) risks are directly linked to obesity. Studies have also shown

links between diabetes or insulin resistance, a precursor to developing type II diabetes, and incidence of breast cancer (Goodwin et al., 2002; Lipscombe et al., 2006) and colorectal cancers (Colangelo et al., 2002; Ren et al., 2009; Flood et al., 2010). The role of diabetes in prostate cancer is more complex and may depend on serum levels of insulin at different stages in diabetic progression (Giovannucci et al., 1998; Rodriguez et al., 2005; Kasper and Giovannucci, 2006). Overall, incidences of prostate cancer nearly doubled between the 1970s and early 2000s (Penson and Chan, 2007), while breast cancer incidence rose about 75% in the same time frame (Lacey et al., 2002; Jatoi et al., 2005). Breast cancer incidences appear to have stabilized more recently, but it has been suggested that the rapid rise in obesity has prevented a decrease in breast cancer incidence (Polednak, 2008). Colorectal cancer incidence in the United States, however, decreased by about 25% during that time span, likely due to better detection combined with improved treatment methods (Cheng et al., 2011).

The recent explosion of obesity, diabetes, hypertension, and some cancers in the United States can be largely attributed to two key well-known factors: lack of proper diet and physical activity. The past few decades have seen a surge in readily available high-fat, high-calorie, low-nutritive meals in fast food restaurants. The number of fast food restaurants in the United States increased from around 30,000 to over 200,000 between the early 1970s and mid-2000s (Rosenheck, 2008), while the population only increased by 50%. Between 1997 and 2006, the portion of restaurants categorized as fast food increased from 17% to approximately 30% (Powell et al., 2007). The consumption of fast food meals (Guthrie et al., 2002) and calories obtained from soda drinks (Nielsen and Popkin, 2004) nearly tripled between the 1970s and 1990s in adults, with a five times increase in children's fast food consumption (Guthrie et al., 2002). In other words, dining out choices for Americans are much more composed of high-calorie unhealthy foods than they ever were before. Epidemiological studies in different areas have also shown a correlation between eating fast-food-type meals and increased incidences of diabetes (Krishnan et al., 2010), colorectal cancer (Slattery et al., 2003), and cancers of the mouth (Wang et al., 2006; Amtha et al., 2009). In addition, despite the increasing availability of 24-hour gyms and fitness programs, Americans get less physical activity today than they did in past decades. A decrease in exercise may be due to the fact that less Americans are performing physical labor and are spending more time in a cubicle or an office, for reasons as varied as recent technological advancements (machines performing the work of many humans) and the 1900s' prominent rural to urban population transformation (Brownson et al., 2005). Epidemiological studies have strongly linked decreased levels of physical activity to higher risks of breast (Eliassen et al., 2010) and colorectal cancers (Howard et al., 2008; Friedenreich et al., 2010) in the United States, with some evidence for exercise protecting against prostate cancers (Antonelli et al., 2009). This shift to a further sedentary lifestyle, along with an increasingly poor diet, seems a strong contributor to the recent pandemic of obesity and obesity-related chronic diseases in industrialized countries like the United States.

Rapidly industrializing countries, including China (Popkin and Du, 2003; Van de Poel et al., 2009; Zhao et al., 2010), India (Bhardwaj et al., 2008; Misra and Khurana, 2009), and Mexico (Rivera et al., 2002), have also demonstrated a sharp recent increase in obesity-related chronic diseases. In Mexico, the percentage of overweight

or obese adults rose by 12% between 2000 and 2006; now almost a third of Mexican adults are obese (Barquera et al., 2006; Ford and Mokdad, 2008). A more striking obesity increase has been observed in Mexican children (5–11 years of age); among this group, obesity increased from 5.5% to 8.9% between 1999 and 2006 (Bonvecchio et al., 2009). The percentage of Mexican adults with diabetes surged from 6.7% in 1993 to 14.4% in 2006 (Villalpando et al., 2010), and coronary-heart-disease-related death rates in Mexico increased by over 90% between 1970 and 2000 (Rodríguez et al., 2006). Also, the proportion of deaths related to diabetes or hypertension in Mexico rose by over 50% between 1980 and 1998 (Rivera et al., 2002). With regard to obesity-related cancers, long-term epidemiological cancer incidence studies on a large scale are limited in Mexico, and in many cases, cancer incidences are undetected and/or underreported (Knaul et al., 2008; Villarreal-Garza et al., 2010). However, the years between the early 1970s and late 1990s saw an increase in mortality from prostate cancer (124%) (Malvezzi et al., 2004), breast cancer (85%) (Malvezzi et al., 2004; Villarreal-Garza et al., 2010), and colorectal cancer in men (45%) (Malvezzi et al., 2004) in Mexico. Death rates of prostate cancer and breast cancer stopped increasing and slightly reversed over the past decade, likely due to better diagnosis and treatment, while colorectal cancer rates continued to rise (Villarreal-Garza et al., 2010; Bosetti et al., 2011). As mentioned earlier, these cancers are associated with obesity in a large number of epidemiological studies.

Parallel to the increase in chronic diseases, Mexico saw a concomitant shift in food consumption patterns during the 1990s and early 2000s. The percentage calories from fat in the diet of Mexican women from four major regions of Mexico (North, Central, South, and Mexico City) increased from an average of 23.5% to 30.3%, with a concomitant rise in obesity incidence from 9.4% to 24.4% between 1988 and 1999 (Rivera et al., 2002). Between 1984 and 1998, purchase of fruits and vegetables per capita in Mexico decreased by almost 30% (Rivera et al., 2002). Also, between 1999 and 2006, intake of calories from high-sugar soda and sweetened drinks more than doubled in 5–11 year old Mexican children (Barquera et al., 2010). Even larger intake increases in high-energy drinks were seen in Mexican adolescents and adult women between 1999 and 2006 (Barquera et al., 2008). Other studies of different communities in Mexico showed recent heightened rates of obesity, heart disease, hypertension, and/or diabetes along with higher intakes of total calories and saturated fat (Rodríguez-Morán et al., 2008, 2009).

The recent poor dietary trends in Mexico may be linked to increasing urbanization in the country. In Mexico, studies have shown urban and higher socioeconomic groups generally have higher intakes of total and saturated fat and lower intakes of fiber than rural and lower socioeconomic groups (Aguilar-Salinas et al., 2001; Barquera et al., 2003; Barquera et al., 2009; Flores et al., 2009). Also, an escalation in availability of fast-food-type restaurants occurred concomitantly with urbanization in Mexico (Ortiz-Hernández et al., 2006). Intake of high-calorie soft drinks was also demonstrated to be higher in urban than in rural areas (Barquera et al., 2008, 2010). A number of epidemiological studies showed that obesity and related disorders like diabetes are more prominent in urban than rural adults in Mexico (Ramírez-Vargas et al., 2007; Flores et al., 2009; Rojas et al., 2010). Other studies found that Mexican urban adolescents spend more time in sedentary activities, such

as watching television (Lajous et al., 2009). Similar physical activity trends were observed for Mexican adults living in cities versus rural areas (Ortiz-Hernández and Ramos-Ibáñez, 2010). Finally, urban Mexican populations have higher rates of mortality from obesity-related cancers, such as those of the breast (Tovar-Guzmán et al., 2000; Palacio-Mejía et al., 2009) and colorectum (Tovar-Guzmán et. al., 1998). As mentioned before, both cancer types are linked to obesity, diabetes, and a lack of physical activity. The rising rates of obesity associated with poor diets and lack of physical activity, especially among youths, reveal an alarming trend that will likely result in increases in obesity-related diseases and deaths in Mexico.

The prevalence of chronic diseases like diabetes, hypertension, and related cancers remains high in industrialized countries and is rapidly increasing in modernizing countries, especially those with recent fast food booms and more sedentary lifestyles. However, certain industrialized countries like France still maintain a relatively low incidence of obesity and related disease. French incidence of obesity is much less than that in the United States (9% vs. over 30%); however, obesity incidence in both countries is increasing (Sassi et al., 2009; Schneider et al., 2010; Austin et al., 2011). Also, French incidence of diabetes is roughly half that of the United States (Kusnik-Joinville et al., 2008; Bringer et al., 2009; Zhang et al., 2009a; Centers for Disease Control and Prevention, 2011). With regard to obesity-related cancers, epidemiological data from the mid-2000s indicated France has a lower incidence of colon cancer than the United States (Chauvenet et al., 2011; Cheng et al., 2011; Jooste et al., 2011; Murphy et al., 2011b). Also, while France and the United States had approximately equal breast cancer incidences over the past two decades among the total population (Héry et al., 2008b; Curado, 2011), France was shown to have a lower breast cancer incidence among those 70+ years of age (Héry et al., 2008a,b). In addition, the incidence of prostate cancer in France has been consistently less than half of that found in the United States, according to studies conducted in the mid-1970s, late 1980s/early 1990s, and early 2000s, although incidences of prostate cancer in both countries are increasing (Hsing et al., 2000; Baade et al., 2009). Despite these differing health statistics, studies have demonstrated that the French consume a diet equal to or slightly higher in total and saturated fat (Volatier and Verger, 1999; Perrin et al., 2002; Wright and Wang, 2010) and total calories (Perrin et al., 2002; Dubuisson et al., 2010; Wright and Wang, 2010) compared to Americans; however, the French consume approximately 20% more fruits and vegetables (Tamers et al., 2009). In addition, epidemiological studies have suggested that among adults (Sisson et al., 2009; Charreire et al., 2011) and adolescents (Imperatore et al., 2006; Kahn et al., 2008; Thibault et al., 2010) the French are approximately as sedentary as their American counterparts. These odd statistics form the basis for the "French paradox," named so because of the perplexingly lower incidence of obesity and related diseases despite similar lifestyle factors. The French diet, which still consists of high levels of saturated fat and sodium, also contains more antioxidant-rich foods like fruits, vegetables, wine (average consumption in France is approximately six times higher than that of the United States) (International Organisation of Wine and Vine (OIV), 2011), and olive oil (slightly more than twice the average U.S. consumption) (Anania and D'Andrea, 2008). Both of these foods have protective effects on obesity-related diseases. Olive oil consumption is inversely correlated with incidences of coronary heart disease (Bendinelli et al., 2011) and breast and colorectal cancers, among other types, in adults in Europe (Bessaoud et al., 2008; Pelucchi et al., 2009). Red wine consumption is associated

with a reduction of diabetic symptoms (Napoli et al., 2005) and coronary heart disease (Grønbaek and Sørensen, 1996). Finally, moderate (one to two drinks per day) red wine drinking has been shown to be inversely proportional to rates of cardiovascular and cancer-related deaths (Grønbaek et al., 2000; Renaud et al., 2004).

This modern day dietary paradox has much in common with the Paleolithic diet, which has higher protein and roughly equal fat and carbohydrate contents compared to modern Western diets but decreased levels of processed carbohydrates and higher levels of phytonutrient-rich fruits and vegetables. The Paleolithic diet was also found to improve metabolic parameters and reduce blood pressure in diabetics when compared to a standard "diabetes diet" (Jönsson et al., 2009) and in healthy individuals compared to a standard diet (Frassetto et al., 2009). A large number of studies have detailed the effects modern poor dietary and exercise habits can have on obesity-related diseases, such as diabetes, breast cancer, colon cancer, and prostate cancer. These diseases, which are the by-products of increased food availability and a more sedentary lifestyle throughout the building of civilizations, have spiked remarkably in the past few decades especially in established industrialized and in industrializing nations. The common etiology of these diseases, obesity-related inflammation, provides a relatively simplistic prevention strategy used by less and less societies over time. A lifestyle implementing physical activity, limited calorie intake, and a diet high in phytonutrient-rich foods including red wine and olive oil can help prevent obesity-related diseases like diabetes and certain cancer types.

MECHANISTIC LINKS BETWEEN OBESITY, DIABETES, AND CANCER

Type II diabetes, hypertension, and heart disease are components of the "metabolic syndrome," a generalized term for maladies typically developed as a consequence of being overweight/obese and physically inactive. In healthy humans, increased blood sugar and free fatty acid levels induce the β-cells in the pancreas to secrete insulin, which causes insulin-sensing cells like those in muscle and fat to increase glucose uptake and causes the liver to shut down gluconeogenesis (Pessin and Saltiel, 2000; Zeyda and Stulnig, 2009). In obese humans, chronic exposure to high blood glucose and free fatty acids leads to high levels of insulin production. In addition, obesity-related inflammation mediates insulin resistance in insulin target cells, leading to higher insulin production in order to reduce blood glucose and fatty acid levels. The increased demand for insulin production leads to the eventual breakdown of insulin-producing β-cells in the pancreas and full type II diabetes (Klöppel et al., 1985; Butler et al., 2003).

Obese humans have high levels of inflammation in different tissues, stemming from increased activation of the inflammatory transcription factor nuclear factor kappa B (NFκB) (Ajuwon and Spurlock, 2005; Lappas et al., 2005; Maury et al., 2009) likely via increased macrophage accumulation in fat tissue (Weisberg et al., 2003; Cinti et al., 2005). Adipose tissue of obese humans secretes high amounts of tumor necrosis factor α (TNFα) (Kern et al., 1995; Winkler et al., 1998) and other inflammatory factors (Visser et al., 1999; Bastard et al., 2000; Vozarova et al., 2001). These cytokines can activate various signaling pathways including those of NFκB and mammalian target of rapamycin (mTOR), a kinase that regulates many processes including protein translation. In addition, plasma insulin and glucose levels

are raised in insulin resistance and diabetes, respectively, and both have been shown to activate the NFκB (Pieper and Riaz-ul-Haq, 1997; Iwasaki et al., 2009) and mTOR (Xu et al., 1998; Yeshao et al., 2005; Vander Haar et al., 2007) pathways. Components of the NFκB (Gao et al., 2002, 2003; de Alvaro et al., 2004) and mTOR (Khamzina et al., 2005; Ueno et al., 2005) signaling pathways induce insulin resistance by phosphorylating insulin receptor substrate 1 (IRS-1) on serine residues, which abrogates its ability to signal downstream in response to insulin. mTOR is a normal component of insulin signaling; its serine phosphorylation of IRS-1 is a negative feedback mechanism that can be exacerbated with chronic mTOR activation (Gual et al., 2003; Manning, 2004). Mice genetically deficient in mTOR (Um et al., 2004) and NFκB (Kim et al., 2001) pathway activation have been found to be refractory to formation of insulin resistance. In addition, a number of antidiabetic drugs likely function via NFκB inhibition (Ruan et al., 2003; Cameron and Cotter, 2008), and blockade of mTOR was shown to stimulate insulin-mediated glucose uptake in healthy humans (Krebs et al., 2007). Finally, besides their ability to downregulate insulin signaling, the NFκB and mTOR pathways have direct effects on β-cell function and maintenance. NFκB inhibition prevented β-cell death in different studies (Friberg et al., 2010; Yuan and Chung, 2010), while the role of mTOR is more complex. mTOR signaling initially resulted in increased β-cell mass and insulin output (Rachdi et al., 2008), but this eventually led to early β-cell breakdown in these animals (Shigeyama et al., 2008), much the same as in normal diabetes etiology. Overall, these data implicate mTOR and NFκB as potential therapeutic or preventative targets for insulin resistance and diabetes.

As mentioned earlier, incidences of cancers associated with the metabolic syndrome are increasing in industrialized and industrializing nations. Cancer forms through a few distinct stages: initiation, where mutated DNA leads to activation of oncogenes and inactivation of tumor suppressor genes; promotion, where high levels of proliferation, angiogenesis, inflammation, and apoptotic resistance allow for tumors to grow; and progression, where the tumor can become malignant (Walaszek et al., 2004). In initiation, DNA is damaged via various insults, including exposure to ultraviolet (UV) light, reactive oxygen species (ROS), and chemicals like those found in cigarette smoke (Minamoto et al., 1999). However, mutations predisposing to cancer types can also be germ-line mutations that are strongly associated within families, like in the case of breast cancer susceptibility (BRCA) mutations in breast cancer (Nicoletto et al., 2001) and adenomatous polyposis coli (APC) mutations in colorectal cancer (Half et al., 2009; Kwong and Dove, 2009). In tumor promotion, activation of oncogenic signals and downstream transcription factors like NFκB leads to production or activation of proliferative, inflammatory, anti-apoptotic, angiogenic, and invasive factors (Kundu and Surh, 2008; Prasad et al., 2010). The kinase mTOR also contributes to tumor promotion via protein synthesis of factors important for cell proliferation and angiogenesis (Azim et al., 2010; Zoncu et al., 2011). Finally, tumor progression occurs when the tumor microenvironment created during promotion as well as additional genetic alterations allow cells to invade the surrounding tissues and enter the bloodstream, leading to cancer metastasis to distant organ sites (Walaszek et al., 2004; Gialeli et al., 2011).

A number of cancer types are encouraged by an obese or diabetic state, as demonstrated via the aforementioned human epidemiological studies as well as

animal models. Mice with diet-induced obesity have shown increased tumorigenesis in models of breast cancer (Nuñez et al., 2008), colorectal cancer (Yakar et al., 2006), pancreatic cancer (White et al., 2010), and skin cancer (Dinkova-Kostova et al., 2008), among others. In addition, transgenic diabetic, fatless mice developed more tumors and larger tumors in models of both skin and breast cancer, indicating that metabolic disorders may promote tumor formation independent of obesity or body fat (Nuñez et al., 2006). The link between obesity, metabolic disorders, and certain cancers may be explained by the activation of common signal transduction factors, especially NFκB and mTOR. Studies in mice have shown that NFκB activity is crucial for the formation of a number of cancer types. In one model, inducible overexpression of a stable variant of NFκB inhibitor IκBα decreased tumor incidence and increased tumor latency when activated in a spontaneous mouse model of breast cancer (Liu et al., 2010). Another model of breast cancer was treated with a chemical NFκB inhibitor to reduce mammary tumor size (Connelly et al., 2011). Also, stable IκBα expression reduced tumor size, invasiveness, and NFκB activity of tumors created by injecting metastatic human prostate cancer cells into the prostate of nude mice (Huang et al., 2001). Other *in vivo* results implicate NFκB in the pathogenesis and proliferation of colorectal cancer (Umar et al., 2008; Yang et al., 2010). In humans, inflammatory diseases with increased NFκB activation such as inflammatory bowel disease are risk factors for intestinal and colorectal cancers (Neurath et al., 1998; Canavan et al., 2006; Pedersen et al., 2010). NFκB activity was found to be upregulated in a number of cancer types in humans, including breast cancer (Sovak et al., 1997; Cogswell et al., 2000), prostate cancer (Suh and Rabson, 2004), and colorectal cancer (Lind et al., 2001), among others (McNulty et al., 2004; Karin, 2009). Similar results have also been shown with increases in mTOR, making it a potential target for cancer treatment (Zhou et al., 2004; Seeliger et al., 2007; Dai et al., 2009). In mice, mTOR inhibition reduced tumorigenesis in models of colorectal cancer (Gulhati et al., 2009), prostate cancer (Zhang et al., 2009b), and breast cancer (Namba et al., 2006).

Chronic diseases such as cancer and diabetes include some of the same risk factors, including obesity and related inflammation, and many epidemiological studies also show a strong correlation between these diseases. In addition to the common etiology of obesity and inflammation, these maladies likely also contribute to the formation of each other via similar pathophysiological signal transduction pathways, such as those involving NFκB and mTOR. The association between the metabolic syndrome and increased risk of certain cancers, in the context of an ever-increasing obese and diabetic population, only further demonstrates the need for lifestyle interventions to prevent obesity-related illnesses.

IMPORTANCE OF DIET, EXERCISE, AND PHYTONUTRIENT CONSUMPTION

Certain lifestyle strategies, including reducing calorie and fat intake, increasing physical activity, and increasing consumption of certain antioxidant-rich foods, are linked to reduced incidence of metabolic disorders and related cancers. As obesity, metabolic disorders, and cancer types like breast, prostate, and colorectal cancers

have similar risk factors, certain lifestyles may provide an efficient preventative or even treatment mechanism for these chronic diseases.

Many studies show that reduced calorie intake, or "calorie restriction," inhibits a vast array of degenerative and chronic diseases, including of the metabolic syndrome and cancer. Short-term (10 day) administration of a hypocalorie diet (1200–1400 calories/day) improved performance in the oral glucose tolerance test in non-obese humans with impaired glucose tolerance (Molfino et al., 2010). Another study showed 14 day administration of a calorie-reduced diet (about 800 calories/day) improved fasting insulin and blood pressure in overweight hypertensive individuals, despite no difference in salt intake (Nakano et al., 2001). Similar antidiabetic effects were also seen with short-term calorie restriction, even before substantial weight loss, in obese subjects (Kelley et al., 1993). Calorie restriction also has anticancer effects in humans and animals. Decreasing dietary fat intake, with reduction in body weight, resulted in a decrease of relapses in breast cancer patients (Chlebowski et al., 2006). Bariatric surgery and resulting weight loss also decreased incidence of a number of cancers, especially breast cancer (Christou et al., 2008). Calorie restriction also prevented tumor formation in various mouse models of cancer, including those of the brain (Shelton et al., 2010), colon (Mai et al., 2003), breast (Fernandes et al., 1995), prostate (Blando et al., 2011), and skin (Birt et al., 1991). Calorie restriction likely exerts these various beneficial effects through a number of different mechanisms, providing many different targets for which calorie restriction mimetic drugs can be designed.

Calorie restriction may induce beneficial antidiabetic and anticancer effects via Sirt1, a mammalian homologue of the NAD+-dependent energy-sensing deacetylase Sir2. Studies have shown calorie restriction can increase the expression of Sirt1 in humans (Civitarese et al., 2007; Pedersen et al., 2008), and lean women have higher Sirt1 expression than obese women (Pedersen et al., 2008). Downstream, Sirt1 has beneficial calorie-restriction-related effects by inhibiting pathways implicated in the metabolic syndrome and tumor formation, like the NFκB and mTOR pathways. Sirt1 overexpression in rat pancreatic β-cells protected the cells from inflammatory cytokine-induced cell death, likely via deacetylation of NFκB resulting in lowered NFκB activation and decreased inflammatory response (Lee et al., 2009b). Overexpression of Sirt1 in the liver of diabetic mice and obese mice improved insulin sensitivity, likely via inhibition of mTOR signaling and a reversal of IRS-1 serine phosphorylation (Li et al., 2011). Also, Sirt1 expression was found to be associated with insulin sensitivity in nondiabetic individuals with a family history of diabetes (Rutanen et al., 2010). Finally, a number of small molecule Sirt1 activators improved insulin sensitivity and lowered resting glucose in obese mice and rats (Milne et al., 2007). With regard to cancer, Sirt1 overexpression in the intestinal epithelium inhibited colon cancer formation in a mouse model (Firestein et al., 2008). While Sirt1 activation seems to protect from colon cancer, its implications in breast and prostate cancer are unclear. Sirt1 inhibition induced apoptosis in BRCA-intact breast cancer cell lines (Kalle et al., 2010; Peck et al., 2010), but Sirt1 overexpression contributed to apoptosis of BRCA-null breast cancer cells and inhibited their ability to form tumors in mice (Wang et al., 2008). Also, Sirt1 inhibitor melatonin induced apoptosis in prostate cancer cell lines and inhibited prostate cancer progression in a mouse model, along with a reduction of Sirt1 levels in the prostate (Jung-Hynes et al., 2011). These results

suggest that implication of Sirt1 in the antitumor effects of calorie restriction may be different among different tumor types. Also, in addition to ameliorating aging-associated diseases like diabetes and some cancer types, studies have shown Sirt1 or homologue Sir2 can mediate calorie restriction-induced increases in the lifespan of different animals, including worms, flies, and mice (Boily et al., 2008), giving Sirt1 the controversial distinction as a potential "fountain of youth" molecule. Finally, as will be discussed later in this chapter, natural compounds with calorie restriction-like effects are currently researched as preventers or treatments for diabetes and cancer.

Exercise also functions as both a preventative and treatment strategy for the metabolic syndrome and related cancers. Exercise has long been verified to promote improvement of diabetic symptoms, and even does so before significant weight loss (Kishimoto et al., 2002; Kirwan et al., 2009). Also, epidemiological studies have suggested exercise can help prevent prostate cancer progression (Richman et al., 2011), colon cancer recurrence (Meyerhardt et al., 2006), and death of breast cancer patients (Irwin et al., 2008). Exercise studies using animal models of cancer have corroborated these findings. One study using prostate-cancer-predisposed mice showed that mice who ran at least 5 km/day had a greatly reduced incidence of prostate neoplasia compared to mice who ran less (Esser et al., 2009). Also, immunodeficient mice injected with human prostate cancer cells or pancreas cancer cells had lower levels of tumor cell proliferation and higher levels of tumor cell apoptosis, resulting in overall decreased tumor sizes, if the mice had access to a running wheel. These differences in tumor activity were seen despite no difference in mouse weight but approximately a 30% decrease in fat pad mass (Zheng et al., 2008), indicating that exercise likely exerts antitumor effects by a mechanism other than or in addition to weight loss, such as overall fat loss combined with muscle gain. Similar effects of exercise were also seen on incidence of chemically induced colon cancers in rats (Andrianopoulos et al., 1987), even without weight loss (Reddy et al., 1988). The antitumor and antidiabetic effects of exercise may be manifest by activation of the energy-sensing kinase AMP-activated protein kinase (AMPK). AMPK is activated by increases in the AMP/ATP ratio and functions to restore ATP levels by activating catabolic processes like fat oxidation and glucose uptake while inhibiting anabolic processes like fatty acid synthesis and gluconeogenesis (Viollet et al., 2009). Tumor incidence in a chemically induced rat model of breast cancer was significantly reduced in rats with running wheel access, along with a similar decrease in weight (Zhu et al., 2008). This study also showed exercise stimulated activation of AMPK and suppresses mTOR activity. In addition, activation of AMPK inhibited tumorigenesis, while knockdown of an upstream kinase of AMPK enhanced tumorigenesis, in tumor-susceptible mice (Huang et al., 2008). Also, AMPK has been found to suppress mTOR (Inoki et al., 2003; Green et al., 2010) and NFκB (Hattori et al., 2006) activities, and AMPK can reverse IRS-1 serine phosphorylation at mTOR target sites (Ju et al., 2007). Studies in high-fat diet-fed rats also show that exercise reversed mTOR activation and insulin resistance in the muscle, likely via increased AMPK signaling (Rivas et al., 2009). Finally, exercise has been found to activate AMPK signaling in human muscle (Birk and Wojtaszewski, 2006; Dreyer et al., 2006; Koopman et al., 2006), and common antidiabetic drugs may function via AMPK (Zhou et al., 2001; Musi et al., 2002; Fediuc et al., 2008, Xiao et al., 2010). This suggests that exercise may prevent or

treat insulin resistance, diabetes, and obesity-related cancers by inhibiting mTOR and NFκB activities via AMPK.

Although NFκB and mTOR inhibition reduce metabolic syndrome and cancer formation, in a number of studies in humans exercise acutely increased mTOR (Mascher et al., 2008; Dreyer et al., 2010; Mascher et al., 2011) and NFκB (Cuevas et al., 2005; Kim et al., 2009) signaling in muscle, suggesting the protective effects of exercise on the metabolic syndrome and cancer may also be mediated by a somewhat different mechanism than that of calorie restriction. One hypothesis is that intermittent acute (as opposed to chronic) activation of NFκB and mTOR after exercise is a response to exercise-induced increases in ROS, and this acute activation of NFκB and mTOR can lead to prevention of chronic ROS (Kramer and Goodyear, 2007; Gomez-Cabrera et al., 2008; Ji, 2008). Indeed, levels of antioxidant enzymes like superoxide dismutase (Ji et al., 2007) and catalase (Vasilaki et al., 2006) were upregulated by exercise, likely via increased ROS and resulting NFκB activation, and these enzymes can inhibit NFκB (Manna et al., 1998; Lüpertz et al., 2008) and mTOR (Carpenter et al., 2011) activities. This suggests that exercise helps induce a protective or adaptive response via ROS insults. Exercise may acutely increase ROS and activate NFκB and mTOR, resulting in an adaptive response to upregulate antioxidant enzymes, inhibit chronic ROS, and inhibit chronic NFκB and mTOR activation and related diseases like diabetes and cancer. One study found that diabetic patients have significantly higher basal NFκB activity (chronic) than nondiabetic lean controls, and a nonstatistically significant increase over obese nondiabetic patients. Following 210 min of moderate exercise, muscle NFκB activity was significantly increased (acute) in both lean and overweight nondiabetic patients but not diabetic patients (Tantiwong et al., 2010). These acute increases in NFκB activity in nondiabetic patients may protect them from chronic NFκB activation seen in the diabetic patients, likely via production of antioxidant enzymes and inhibition of chronic ROS. Chronic ROS has been shown to suppress insulin sensitivity (Yang et al., 2011a), while exercise-induced acute ROS may increase insulin sensitivity. Supplementation of humans with antioxidant vitamins C and E prevented exercise-mediated production of antioxidant enzymes and increases in insulin sensitivity in healthy humans, indicating exercise-induced ROS may be necessary for these beneficial effects (Ristow et al., 2009). Also, AMPK activation has been shown to increase antioxidant enzyme production (Park et al., 2010; Zrelli et al., 2011), and AMPK was activated by oxidative stress in various systems (Wang et al., 2011; Sarre et al., 2012). This evidence suggests exercise can acutely increase oxidative stress with a concomitant rise in mTOR, NFκB, and AMPK activities. Activation of these signaling pathways can lead to increased production of antioxidant enzymes, while AMPK can also directly interfere with activation of NFκB and mTOR. This results in decreased chronic oxidative stress; decreased chronic NFκB and mTOR activation; and perhaps a reduction in associated insulin resistance, diabetes, and cancers.

Finally, AMPK and Sirt1 are strongly intertwined and likely promote the activities of each other (Fulco and Sartorelli, 2008; Lin et al., 2010; Ruderman et al., 2010), as do mTOR and upstream components of the NFκB activation pathway (Lee et al., 2007; Dan et al., 2008). Overall, the mechanisms of action of calorie restriction and exercise can contribute to each other (Cox et al., 1996; Larson-Meyer et al., 2010),

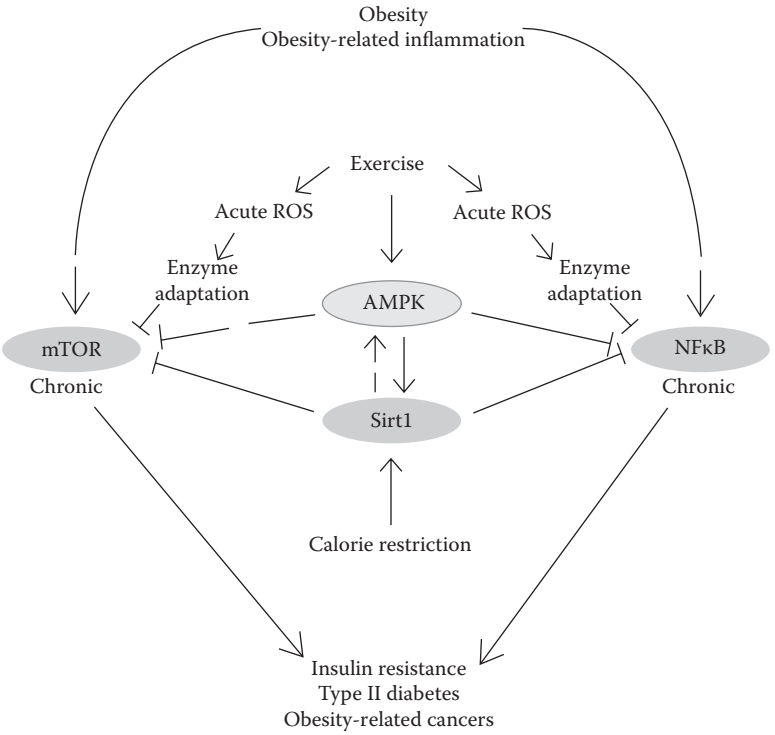

FIGURE 7.1 Exercise and calorie restriction inhibit chronic mTOR, chronic NFκB, and related metabolic diseases and cancers.

revealing a potential synergistic effect of calorie restriction plus exercise as a preventer of the metabolic syndrome and certain cancer types as shown in Figure 7.1.

Calorie restriction and exercise are important preventers of the metabolic syndrome and cancer. In addition, certain natural phytonutrients can activate Sirt1 or AMPK and/or inhibit mTOR or NFκB, leading downstream to similar effects of calorie restriction or exercise. These compounds may provide similar benefits as calorie restriction or exercise without the high failure rate these profound "self-administered" lifestyle changes typically have. A large number of recent studies suggest that smaller lifestyle changes, such as the incorporation of phytonutrient-rich fruits and vegetables into the diet, may combat metabolic diseases and cancer. Some of the most beneficial effects of increased fruit and vegetable intake come from the large variety of phytonutrients found in these plant products. Phytonutrients include classes of anthocyanidins, flavonoids, isoflavonoids, carotenoids, organosulfides, polyphenols, and terpenoids, among others. Components of phytonutrient-rich plants have been used for millennia in Eastern medicine as cures for such maladies as inflammation, arthritis, fevers, hypertension, and cancer (Meng et al., 2009; Li-Weber, 2010). More modern studies have shown that consumption of a number of phytonutrient-rich foods, including green tea (Yang et al., 2011b; Zheng et al., 2011), apples (Jedrychowski et al., 2010), red wine (Grønbaek et al., 2000), and olive oil (Bessaoud et al., 2008; Pelucchi et al., 2009),

is inversely correlated with risk of different cancers in humans. Much of current phytonutrient research focuses on extracts or individual components of these foods, in order to understand the various molecular mechanisms by which these natural compounds exert their benefits. A number of phytonutrients, their main sources, and their effects on previously described molecular targets are listed in Table 7.1.

TABLE 7.1
Phytonutrients Which Mimic the Downstream Molecular Effects of Calorie Restriction or Exercise

Compound	Main Sources	Structure Class	Target(s) of Action
Resveratrol	Grapes	Polyphenol	↑Sirt1, ↑AMPK, ↓NFκB, ↓mTOR
Ursolic acid	Apples, rosemary	Pentacyclic triterpene	↑AMPK, ↓NFκB, ↓mTOR
Oleanolic acid	Olives, rosemary	Pentacyclic triterpene	↑AMPK, ↓NFκB (Shyu et al., 2010), ↓mTOR
6-Shogaol	Ginger	Phenolic ketone	↓NFκB (Ling et al., 2010) ↓mTOR (Hung et al., 2009)
Avicins	*Acacia victoriae* (Australian desert tree)	Triterpenoid saponin	↑AMPK (Xu et al., 2007) ↓NFκB (Haridas et al., 2001) ↓mTOR (Xu et al., 2007)
Curcumin	Turmeric	Polyphenol	↑AMPK (Lee et al., 2009a) ↓NFκB (Han et al., 2002) ↓mTOR (Clark et al., 2010)
Delphinidin	Pomegranate, berries, black rice	Anthocyanidin	↓NFκB (Yun et al., 2009) ↓mTOR (Syed et al., 2008)
Epigallocatechin gallate (EGCG)	Green tea	Catechin	↓Sirt1 (Feng et al., 2009) ↑AMPK (Hwang et al., 2005) ↓NFκB (Kundu and Surh, 2007) ↓mTOR (Van Aller et al., 2011)
Genistein	Soybeans	Isoflavonoid	↓Sirt1 (Kikuno et al., 2008) ↑AMPK (Hwang et al., 2005) ↓NFκB (Li and Sarkar, 2002)
Lycopene	Tomatoes, red carrots	Carotenoid	↓NFκB (Kim et al., 2004) ↓mTOR (Tang et al., 2009)
Quercetin	Teas	Flavonoid polyphenol	↑ Sirt1 (Davis et al., 2009) ↑AMPK (Suchankova et al., 2009) ↓NFκB (Puangpraphant and de Mejia, 2009) ↓mTOR (Olson et al., 2010)

A large variety of phytonutrients inhibit the metabolic syndrome and related cancers. Studies of mice fed a high-fat diet showed that coadministration of the juice from purple carrots, which primarily contained high levels of anthocyanins, reduced increases in blood pressure, left ventricle inflammation, and disturbances in liver function (Poudyal et al., 2010). In rats, a high-fructose diet decreased insulin sensitivity and raised free fatty acids, triglycerides, and oxidative stress, all of which were strongly prevented or reversed when rats received 5 g/kg of an anthocyanin-rich extract of black rice in their diet throughout the study or after the establishment of insulin resistance (Guo et al., 2007). In addition, it has also been observed that certain phytonutrients can directly inhibit the inflammation associated with obesity and the metabolic syndrome. In one study, macrophage migration and inflammatory cytokine production were induced when cells were grown in media that previously contained adipocytes from high-fat diet-fed mice. These effects were strongly suppressed when the macrophages were coincubated with various phytonutrients including curcumin (a polyphenol found in turmeric) and zingerone (a flavonol found in ginger) (Woo et al., 2007). Dietary curcumin also inhibited diseases associated with inflammation, including arthritis as well as intestinal inflammation and intestinal tumorigenesis in mouse models (Murphy et al., 2011a). Finally, 30 day oral curcumin treatment significantly reduced the number of aberrant crypt foci in the rectum of smokers, indicating a potential of curcumin to inhibit colorectal cancer in humans (Carroll et al., 2011). Human studies with other phytonutrients will hopefully yield similar positive results in the coming years.

Some of the most promising phytonutrients currently under study are resveratrol, ursolic acid, and ursolic acid isomer oleanolic acid (Figure 7.2). Resveratrol, a polyphenol found primarily in grapes and red wine, is a strong candidate for explanation of the "French paradox." As described before, the French have lower incidences of metabolic disorders and related cancers despite similar saturated fat intake and physical activity relative to other Western countries, potentially due to increased intake of phytonutrients including resveratrol in foods like red wine. Resveratrol has been a component of Eastern medicine for centuries and also exerts similar effects of calorie restriction and exercise, likely via the same signal transduction pathways. Resveratrol suppressed weight gain rate in lemurs during their seasonal body weight gain, by both decreasing food intake and increasing metabolism with no change in locomotor activity (Dal-Pan et al., 2010). Resveratrol also inhibited weight gain in rats fed an atherogenic diet (1% cholesterol), with an accompanying decrease in liver inflammation (Ahn et al., 2008). In addition, dietary resveratrol

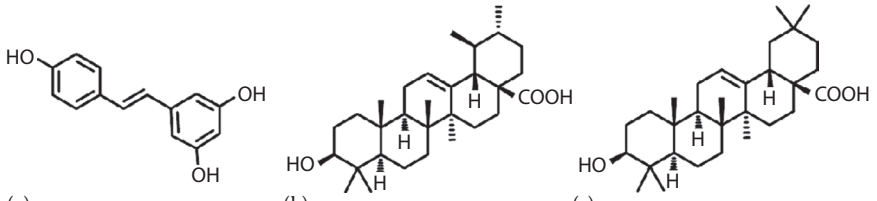

FIGURE 7.2 Structures of (a) resveratrol, (b) ursolic acid, and (c) oleanolic acid.

improved insulin sensitivity in mice fed a high-calorie diet, reversed mTOR and NFκB pathway activation in the liver, and induced liver AMPK activity (Baur et al., 2006). Dietary resveratrol increased insulin sensitivity and glucose tolerance in normal mice, but not AMPK deficient mice, challenged with a high-fat diet, indicating AMPK is crucial for resveratrol's antidiabetic effects (Um et al., 2010). Another study found that resveratrol improved insulin sensitivity and induced glycogen synthesis in muscle cells in a Sirt1-dependent manner, highlighting the importance of Sirt1 in mediating resveratrol's antidiabetic effects (Sun et al., 2007). In another study, NFκB activation and IRS-1 serine phosphorylation were induced in adipocytes cultured with pro-inflammatory media previously incubated with activated macrophages. These changes were prevented if macrophages were cocultured with resveratrol (Kang et al., 2010). Finally, in type II diabetic humans, insulin sensitivity was improved by the fourth week following 10 mg daily oral resveratrol, relative to placebo (Brasnyó et al., 2011).

In line with its effects involving Sirt1 and AMPK, resveratrol also functions as a potent preventer of cancer. Resveratrol administered in the diet reduced incidence and delayed formation of chemically induced mammary tumors in rats. Chemically induced NFκB activation was also strongly suppressed in the mammary tissue of most rats that are also treated with resveratrol (Banerjee et al., 2002). Also, i.p.-administered resveratrol inhibited increases in tumor size in tumors established by injection of a breast cancer cell line into nude mice. In the same cell line, resveratrol was found to inhibit mTOR in an AMPK- and Sirt-dependent manner, with AMPK activation dependent on Sirt1. This shows a potential antitumor mechanism of resveratrol via Sirt1 activation, leading to AMPK activation and mTOR downregulation (Lin et al., 2010). Oral resveratrol also suppressed tumorigenesis in rodent models of prostate cancer (Harper et al., 2007) and chemically induced colon cancer (Cui et al., 2010). Finally, clinical trials with high oral doses showed that resveratrol may slow colorectal cancer growth (Patel et al., 2010) and induce apoptosis in liver tumors (Howells et al., 2011).

Results indicate that resveratrol inhibits the metabolic syndrome and cancer via similar signaling pathways as calorie restriction and exercise. In addition, the oral resveratrol dose used by the antidiabetic human study is easily achieved with moderate red wine consumption. For the animal antitumor studies mentioned, oral resveratrol doses used correspond to roughly 60–600 mg daily oral dose for humans, depending on animal used and amount of food ingested (Reagan-Shaw et al., 2008). Also, the human tumor studies used anywhere from 0.5 to 5.0 g resveratrol. However, these studies did not necessarily assess the lowest possible effective resveratrol dose, so lower doses of resveratrol corresponding to moderate red wine drinking may also be effective. The 10 mg dose of resveratrol used to improve metabolic syndrome symptoms in human studies is achievable with as low as two glasses of red wine, depending on the type and source of grapes used. For example, the average resveratrol content of red wines from France is more than double that of the United States (Stervbo et al., 2007), providing another potential explanation for the French paradox.

Ursolic acid and oleanolic acid are isomeric pentacyclic triterpenes found in many plants including rosemary, apples, and olives (Allouche et al., 2009;

Jäger et al., 2009). These compounds have antidiabetic and anticancer effects similar to those of resveratrol. Dietary ursolic acid (0.05% w/w) in high-fat-fed streptozotocin-induced diabetic rats decreased resting glucose levels and improved glucose tolerance and insulin sensitivity. This may be due to the inhibitory effects of ursolic acid on streptozotocin-mediated β-cell toxicity (Jang et al., 2009). Oleanolic acid had similar effects on blood glucose levels in streptozotocin-induced diabetic rats (Musabayane et al., 2010). Also, in a rat model of hypertension, 60 mg/kg i.p.-injected ursolic acid or oleanolic acid lowered resting glucose, LDL cholesterol, and triglycerides to near control levels, and raised antioxidant enzymes and HDL cholesterol after 6 weeks of treatment (Somova et al., 2003). In addition, oleanolic acid (Wang et al., 2009) and ursolic acid (Ha et al., 2009) have been shown to activate AMPK in cell culture, indicating these triterpenes may function via a similar mechanism as exercise. Ursolic acid and oleanolic acid also induced cell death in human colon cancer cell lines (Li et al., 2002; Shan et al., 2009) and inhibited the formation of aberrant crypt foci (the precursors of colorectal polyps) when given orally (25 mg/kg/day) in a chemically induced rat model of colon cancer (Furtado et al., 2008). Both of these pentacyclic triterpenes have been found to induce apoptosis of breast cancer cell lines (Kassi et al., 2009; Allouche et al., 2011), with an inhibition of NFκB (shown for ursolic acid) and mTOR signaling (shown for both ursolic and oleanolic acids) (Chu et al., 2010; Yeh et al., 2010). Oral ursolic acid also inhibited tumor numbers and tumor growth, as well as mTOR signaling in a mouse model of postmenopausal breast cancer (De Angel et al., 2010). A number of oleanolic acid derivatives, like CDDO-Me, as well as fresh apple extracts in the diet inhibited mammary tumorigenesis when applied in different rodent models (Liu et al., 2005; Ling et al., 2007; Liby et al., 2008). Ursolic acid also impaired viability of different prostate cancer cell lines (Kassi et al., 2007), and CDDO-Me demonstrated an anticancer effect in a rat model of prostate cancer (Hyer et al., 2008). Finally, the oral doses of ursolic acid and oleanolic acid used in these studies are achievable in humans with moderate consumption of triterpene-rich fruits and vegetables. The rat model of colon cancer study used the human equivalent of 200–300 mg ursolic acid or oleanolic acid (Reagan-Shaw et al., 2008; Furtado et al., 2008), while the rat model of diabetes used 0.05% w/w ursolic acid (Jang et al., 2009). These oral doses can be obtained with just ~25 to 100 g dried apple for ursolic acid and ~100 g dried apple or ~100 g dried olives for oleanolic acid (Reagan-Shaw et al., 2008; Jäger et al., 2009).

Resveratrol, ursolic acid, and ursolic acid isomer oleanolic acid have been shown to inhibit metabolic disorders and obesity-related cancers in a number of different systems. Molecular targets for these compounds include those implicated in the benefits of calorie restriction and exercise, such as Sirt1 (for resveratrol), AMPK, NFκB, and mTOR. Via these pathways, resveratrol, ursolic acid, and oleanolic acid essentially mimic the positive effects of calorie restriction and exercise on insulin resistance, diabetes, and obesity-related cancers. These phytonutrients, found in natural products like red wine, rosemary, apples, and olives, may offer protection from these chronic diseases and likely contribute to the oddly lower incidences of metabolic syndrome and obesity-related cancers seen in the French paradox.

SKIN CARCINOGENESIS AND PHYTONUTRIENTS

One popular avenue of phytonutrient research involves using topical treatments as preventers of skin damage and skin cancer. Skin cancer incidence in the United States has been increasing over the past decades (Karagas et al., 1999, Geller et al., 2007) and is now present in about 1% of the adult population, with over one million new cases diagnosed each year (American cancer society, 2007; Rogers et al., 2010). In addition, skin cancer is associated with a 15%–30% higher risk of other forms of cancer such as those of the colon, breast, and prostate, making it crucial to determine potential mechanisms to prevent or treat skin carcinogenesis (Chen et al., 2008; Krueger et al., 2010). Skin cancer consists of two main types: melanoma skin cancer and non-melanoma skin cancer. Both have similar etiologies in humans as determined by epidemiological studies, including higher risks with increased sun exposure (Boscoe and Schymura, 2006), especially early in life (Lea et al., 2007). In addition, melanoma risk has been found to be increased with obesity (Gallus et al., 2006; Samanic et al., 2006) while non-melanoma skin cancer risk is higher in certain proliferative (Frentz and Olsen, 1999) and inflammatory disorders (Long et al., 2010).

Like other cancer types, skin cancer proceeds via three phases: initiation, promotion, and progression. Initiation leading to skin cancer is normally mediated via UV light, which reaches the earth's surface in two main wavelength groupings: UVB (290–320 nm) and UVA (320–400 nm). UVB and UVA can induce DNA damage via ROS production (Bickers and Athar, 2006) and pyrimidine dimer photoproducts (Liardet et al., 2001; Narbutt et al., 2007). Other skin tumor initiators normally encountered include polycyclic aromatic hydrocarbons, which are highly present in smoke and can form DNA adducts when metabolized (Boffetta et al., 1997; Baird et al., 2005). In addition to initiating tumorigenesis, UV light functions as a potent tumor promoter. Promotion of skin cancer is characterized by increases in inflammation and production of genes associated with proliferation, angiogenesis, and a resistance to apoptosis (Katiyar et al., 1999; Loercher et al., 2004). In this stage, the activities of NFκB and mTOR in skin cells are increased. Studies found that UVB treatment of human skin *in vivo* activated NFκB (Pfundt et al., 2001) and UVB treatment of *in vitro* human skin cells activated NFκB (Afaq et al., 2003) and mTOR (Brenneisen et al., 2000; Olson et al., 2010). In line with inflammation associated with skin tumor promotion, application of different inflammation-inducing compounds promoted tumorigenesis in initiated mouse skin (Slaga, 1983). As mentioned before, inflammation-associated disorders in the body can also promote skin tumor formation.

Recently, a number of studies have focused on the use of either topical or oral application of various phytonutrients to treat or prevent both chemically and UVB-mediated skin cancer formation in mouse models (Park et al., 1998; Sancheti et al., 2005; Katiyar, 2011). One of the primary mouse models of skin cancer formation is the two-stage chemical carcinogenesis model, in which initiator and promoter are applied to the shaved backs of number of rodent types (Slaga, 1986; Hennings et al., 1993). This model involves initiating tumorigenesis with a single topical application of 7, 12-dimethylbenz(a)anthracene (DMBA), a polycyclic aromatic hydrocarbon that induces mutations in tumor suppressor genes like p53 and proto-oncogenes like ras, which contribute to tumorigenicity in skin and other organs (Wang et al., 1998;

Ise et al., 2000; Park et al., 2004). One week after initiation, skin tumorigenesis is promoted with twice weekly topical treatments of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an inflammatory compound that activates mTOR and NFκB in the epidermis, resulting in tumor formation in less than 10 weeks depending on mouse strain used (Affara et al., 2006; Moore et al., 2008; Cichocki et al., 2010). This paradigm is especially useful because it separates the initiation and promotion phases of cancer formation, which allows for studies to examine specific preventers of these phases. A number of phytonutrients have been shown to have inhibitory effects on a variety of processes involved with skin tumor initiation, promotion, and progression (Figure 7.3).

Topical resveratrol treatment prior to each TPA dose has been found to inhibit DMBA/TPA-mediated tumor incidence and tumor volume per mouse and increase tumor latency, as confirmed by a number of studies (Jang et al., 1997; Kapadia et al., 2002).

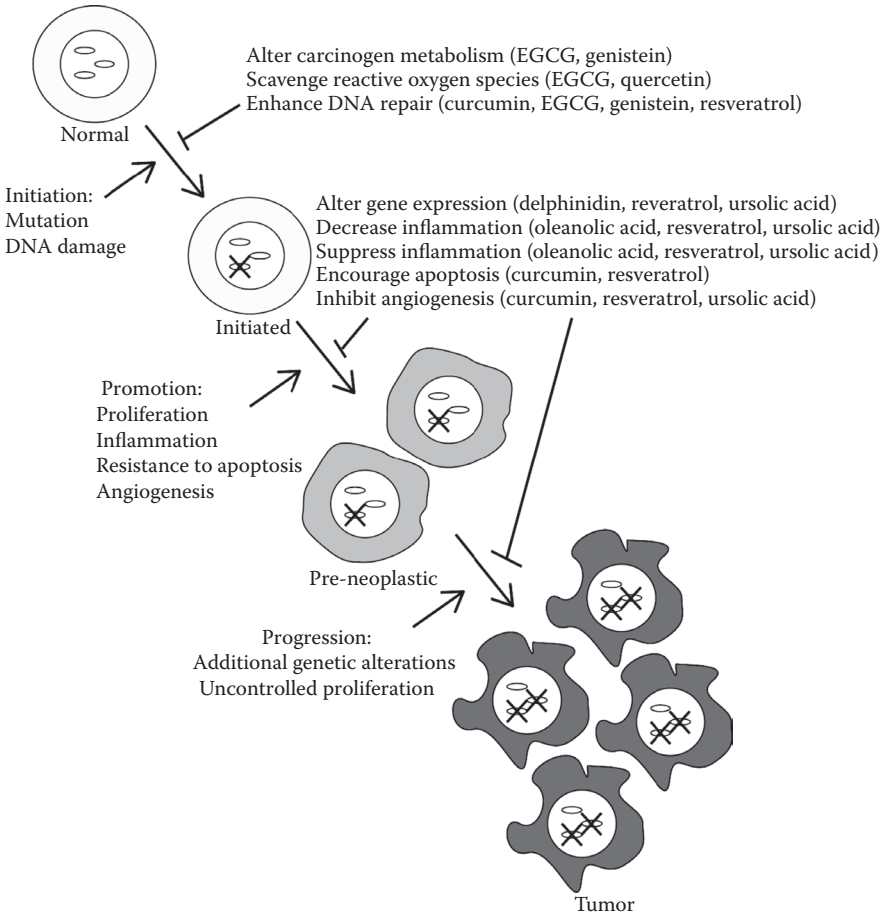

FIGURE 7.3 Phytonutrients inhibit tumor initiation, promotion, and progression stages in skin.

In addition, studies with Sirt1 knockout mice treated with the DMBA/TPA protocol showed that these mice had no significant difference in skin tumor incidence compared to control mice. Topical resveratrol treatment still reduced tumor incidence, multiplicity, and volume in the Sirt1 knockout mice but not to the same level as that of control mice, indicating that the protective effect of resveratrol on skin tumorigenesis is at least partially mediated by Sirt1 (Boily et al., 2009). Resveratrol also inhibited NFκB activation in TPA-treated mouse skin, providing another potential mechanism for its antitumor promotion activities (Kundu et al., 2006). Ursolic acid and oleanolic acid have also demonstrated antitumor promotion activity when topically applied during the TPA stage in the DMBA/TPA model of skin carcinogenesis in mice (Tokuda et al., 1986; Huang et al., 1994). Both compounds also inhibited TPA-induced inflammation in mice (Banno et al., 2004). As mentioned before, ursolic acid and oleanolic acid have demonstrated anti-NFκB and anti-mTOR activities in a number of models, suggesting a potential downstream mechanism for the anti-skin tumor promotion effects of ursolic acid and oleanolic acid. Finally, ursolic acid inhibited UVA-induced (Soo Lee et al., 2003) and UVB-induced (Ramachandran and Prasad, 2008) changes in human cells, while resveratrol (Aziz et al., 2005) suppressed UVB-mediated tumorigenesis when topically applied in a mouse model. This indicates these compounds may be useful as preventers of UV-mediated human skin cancers.

CONCLUSIONS

The common etiology of certain chronic diseases, including metabolic syndrome-related disorders and related cancers, allows for similar preventative strategies. Signal transduction pathways activated in obesity and metabolic diseases promote formation of these cancers, leading to a higher risk of breast, prostate, and colorectal cancers among the obese and diabetic population. In addition, the percentage of those with metabolic syndrome, and at increased risk for these cancer types, has spiked in recent years in both industrialized and industrializing nations. This is a by-product of an increasingly poor diet and sedentary lifestyle, a lifestyle that is far from the hunter–gatherer lifestyle humans had for hundreds of thousands of years. Certain intervention strategies today try to mimic some traits of the healthier hunter–gatherer lifestyle, such as favorable energy balance, exercise, and increased intake of phytonutrient-rich fruits and vegetable products. Certain natural food products and their beneficial phytonutrients, such as red wine, rich in resveratrol, and apples and olives, rich in ursolic acid and oleanolic acid, may be used to prevent or even treat metabolic disorders and related cancers by inhibiting mutual signal transduction pathways.

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8 Alterations in the Adiposity and Dyslipidemia of Obesity by Berries and Berry Phytochemicals

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CONTENTS

INTRODUCTION

Anthocyanins have important functions in plant physiology and may have health effects in the prevention of chronic diseases. Berries are particularly rich sources of anthocyanins (Wu et al. 2006a). Different types of berries provide unique patterns of anthocyanins to study due to both differences in anthocyanin concentrations and differences in individual anthocyanins. (Wu et al. 2002, 2004, 2005, 2006b). For example, total anthocyanin concentrations in freeze-dried strawberries, blueberries, and black raspberries were found to be 2.9, 27.2, and 43.7 mg/g dry weight, respectively. Aside from the considerable differences in anthocyanin concentration, the anthocyanin composition among the berries can also vary. Black raspberries are rich in cyanidin-based anthocyanins; blueberries contain a complex mixture of delphinidin-, cyanidin-, peonidin-, petunidin-, and malvidin-glycosides; and

strawberries (Wu et al. 2006a) are one of only a few berries in which pelargonidin is the primary anthocyanidin. Pelargonidin is much more stable and/or more readily absorbed *in vivo* than other anthocyanins. Even though the concentration of pelargonidin-based anthocyanins in strawberries is low relative to anthocyanins in other berries, its apparent absorption may be as much as 10 times higher (Wu et al. 2004; Carkeet et al. 2008). Specific health effects of anthocyanins *in vivo* are still under investigation, although there are several possibilities related to obesity, cardiovascular disease, and cancer (Hou 2003; Martin et al. 2003; Prior 2004; Prior and Wu 2006; Alarcon-Aguilar et al. 2007; Kwon et al. 2007; Sasaki et al. 2007; Singletary et al. 2007; Zafra-Stone et al. 2007; Tsuda 2008).

This review will focus on what is known about the effects of anthocyanins on obesity. All of the early published data related to obesity were derived using purified anthocyanin preparations from berries and other foods (Tsuda et al. 2003; Jayaprakasam et al. 2006) or purified anthocyanins *in vitro* (Tsuda et al. 2004, 2005, 2006). However, from a nutritional standpoint, the relevant question is whether the anthocyanins would be effective when consumed as part of the whole food. Our initial studies focused on effects of consuming the whole berry.

ANTHOCYANINS IN WHOLE BERRIES/FOODS AND OBESITY

Early *in vivo* studies from our laboratory demonstrated the potential benefits of whole berry consumption on body composition. Rats were placed on a low-fat diet, from weaning to post-natal day 34, consisting of 0%, 2.5%, 5%, or 10% as freeze-dried whole blueberry powder. Abdominal fat pad weight, as a percentage of body weight, was measured and found to be significantly lower with 10% blueberry powder in the diet in both male and female rats (Prior and Wu, unpublished data). A similar trend was observed in gonadal fat weight, although the effects were not statistically significant (Prior and Wu, unpublished data). These data suggested that whole blueberries in the diet might impact adipose tissue formation.

C57BL/6J mice were fed a high-fat (HF) diet (either 45% or 60% kcal from fat) and were supplemented with whole blueberry or strawberry powders, or anthocyanin-rich extracts from these two berries (Prior et al. 2008). These differing dietary regimens were compared for their effects in preventing obesity in these HF-fed mice. The diet containing whole strawberry powder (114 g/kg; 24.7 mg/kcal) demonstrated some impact on slowing the development of obesity. However, mice fed an HF diet containing whole blueberry powder had increased body weight gain and increased adiposity (% body fat ↑ 15%) relative to mice fed a matching HF diet without whole blueberry powder (Prior et al. 2008). Weight gain and adipose tissue weights did not change in C57BL/6 mice fed low-fat diets (10% kcal from fat) supplemented with the same whole berry powders. Furthermore, freeze-dried powders of black raspberry and Concord grape as dietary treatments did not alter the development of obesity in mice fed an HF diet (Prior et al. 2009). These two sources represent extremely high (black raspberry) or relatively low (Concord grape) anthocyanin content.

Thus, feeding of the whole berry in the context of an HF diet has no effect on weight gain and, in some cases, may actually increase adiposity. However, as indicated in the next section, feeding purified forms of anthocyanins may decrease obesity.

ISOLATED ANTHOCYANINS *IN VIVO* AND OBESITY

Eight studies are summarized in Table 8.1 that relate to effects of purified anthocyanins on development of obesity. Sources of the anthocyanins include the Cornelian cherry, purple corn, blueberry, strawberry, black soybean, and black raspberry. The content of individual anthocyanins from these different foods varies, with cyanidin-3-glucoside being present in all sources, and is the predominant anthocyanin in Cornelian cherry, purple corn, and black soybean. Pelargonidin predominates in strawberry, cyanidin-3-rutinoside in black raspberry, and malvidin-3-glucoside in grape. Total anthocyanins consumed in the different experiments with rodents, as presented in Table 8.1, ranged from ~0.2 to ~6 mg/day (Table 8.1). Purified grape anthocyanins (Lefevre et al. 2008) and black raspberry (Prior et al. 2010a) anthocyanins were ineffective in altering the development of obesity in the C57BL/6 mouse model. The lack of response with grape may relate to the particular type of anthocyanins found or to the amount of anthocyanins consumed. Malvidin is a predominant anthocyanin found in grape, and grape has the lowest anthocyanin content (~0.2 mg/day) of any of the treatments studied (Table 8.1). In this study (Lefevre et al. 2008), C57BL/6 mice were divided into two groups and were fed a proatherogenic diet containing 39.9% kcal from fat with or without a semipurified grape anthocyanin extract (70% anthocyanins) incorporated at a level of 0.1 mg/mL into the drinking water. After 6 weeks, mice supplemented with anthocyanins tended to gain more weight and had increased adipose tissue mass, although these effects did not achieve statistical significance compared to control mice (Lefevre et al. 2008). The lack of effect of black raspberry anthocyanins on obesity (Prior et al. 2010a) is not clear but may be related to the predominance of di- and tri-glycosides in this berry.

Other purified anthocyanin preparations were effective in slowing the development of obesity (Tsuda et al. 2003; Jayaprakasam et al. 2006; Kwon et al. 2007; Prior et al. 2008, 2009, 2010b). Purified anthocyanins supplemented in an HF diet resulted in decreased liver lipids in two studies (Tsuda et al. 2003; Jayaprakasam et al. 2006), but no changes in three other experiments (Prior et al. 2008, 2009, 2010b). Body weight gains were consistently decreased except in studies where purified anthocyanins from grape or black raspberry were fed (Lefevre et al. 2008; Prior et al. 2010a). Responses in various biomarkers of obesity measured were not totally consistent across experiments. Insulin levels tended to decrease with anthocyanin treatment, but this decline was not consistent in all studies. Some of these responses will be discussed later.

ANTHOCYANINS AND LIPID METABOLISM

Feeding purified anthocyanins prevented the development of postprandial dyslipidemia and obesity in mice (Prior et al. 2009). Serum triglycerides were increased in mice fed an HF diet (45% kcal from fat) but were increased further when whole blueberry powder was included in the regimen (Prior et al. 2008). Liver total lipids and triglycerides increased in mice fed an HF diet relative to those fed a low-fat diet; yet these results were not significantly different from the control HF diet when 10% of the diet was supplemented with the whole freeze-dried powder of strawberry, blueberry, black raspberry, or concord grape (Prior et al. 2009).

TABLE 8.1
Influence of Anthocyanins (ACNs) from Various Food Sources on Development of Obesity in Rodent Models Fed an HF Diet

Food Source of Anthocyanins	Primary ACNs	HF Diet	ACNs Fed in	Dose	Responses Relative to HF Control	Reference
Cornelian cherry (Male C57BL/6 mice)	Cy-3-gal Pg-3-gal Del-3-gal	60% kcal fat	Diet	1 mg/g diet (2.2 mg/day)	16% ↓BW gain (d 56) NC blood glucose (Fasting) 27% ↓liver lipid 16% ↓liver triglycerides 1200 X ↑serum insulin	Jayaprakasam et al. 2006
Purple corn (Male C57BL/6J mice)	Cy-3-glu	57% kcal fat	Diet	2 mg/g diet (~6 mg/day)	22% ↓BW gain (d 84) 55% ↓body fat (d 84) 30% ↓blood glucose 35% ↓liver lipids 44% ↓serum insulin 83% ↓serum leptin	Tsuda et al. 2008
Blueberry (Male C57BL/6J mice)	Mixture of 19 ACNs	60% kcal fat	Water	1 mg/mL (2.8 mg/day)	11% ↓BW gain (d 63) 39% ↓body fat (d 63) NC heart wt NC liver wt 8% ↓blood glucose NC liver lipids 8% ↓serum triglycerides 41% ↓serum insulin (NS) 48% ↓serum leptin	Prior et al. 2008, 2009

TABLE 8.1 (continued)
Influence of Anthocyanins (ACNs) from Various Food Sources on Development of Obesity in Rodent Models Fed an HF Diet

Food Source of Anthocyanins	Primary ACNs	HF Diet	ACNs Fed in	Dose	Responses Relative to HF Control	Reference
Strawberry (Male C57BL/6J mice)	Pg-3-glu Cy-3-Sop Cy-3-glu	60% kcal fat	Water	1 mg/mL (1.6 mg/day)	16% ↓BW gain (d 63) 14% ↓body fat (d 63) NC heart wt NC liver wt 15% ↓Blood glucose NC liver lipids 50% ↓serum triglycerides 34% ↓serum insulin (NS) 32% ↓Serum leptin	Prior et al. 2008, 2009
Blueberry (Male C57BL/6J mice)	Mixture of 19 ACNs	45% kcal fat	Water	1.8 mg/mL (5.27 mg/day)	16% ↓BW gain (d 72)(NS) 26% ↓body fat (d 72) 12% ↑heart wt NC liver wt 12% ↑kidney wt 45% ↓blood glucose NC liver lipids NC serum triglycerides NC serum insulin 48% ↓serum leptin	Prior et al. 2010b
Blueberry (Male C57BL/6J mice)	Mixture of 19 ACNs	45% kcal fat	BB juice in place of water	0.2 mg/mL (0.49 mg/day)	17% ↓BW gain (d 72) 8% ↓body fat (d72) 17% ↑heart wt NC liver wt 15% ↑kidney wt NC blood glucose	Prior et al. 2010b

(*continued*)

TABLE 8.1 (continued)
Influence of Anthocyanins (ACNs) from Various Food Sources on Development of Obesity in Rodent Models Fed an HF Diet

Food Source of Anthocyanins	Primary ACNs	HF Diet	ACNs Fed in	Dose	Responses Relative to HF Control	Reference
					NC liver lipids NC serum TG NC serum Insulin 55% ↓serum Leptin	
Grape (Male C57BL/6 mice)	Mixture Mal-glu Peon-Glu Del-glu Pet-Glu	39.9 kcal fat	Water	0.1 mg/mL	20% ↑ BW gain (NS) 10% ↓heart wt 6% ↓liver wt NC serum insulin 44% ↑serum leptin (NS)	Lefevre et al. 2008
Black soybean (Male SD rats)	Cy-3-glu Del-3-glu Pet-3-glu	37% kcal fat	Diet (6.6 mg/day)	0.4 mg/g diet	29% ↓BW gain (d 28) NC body fat (d 28) 17% ↑heart wt 11% ↓liver wt (NS) NC kidney wt 45% ↓serum triglycerides	Kwon et al. 2010
Black raspberry (Male C57BL/6J mice)	Cy-3-rut Cy-3-xyl-rt Cy-3-glu Pg-3-rt	60% kcal fat	Water	1.25 mg/mL (2.66 mg/day)	NC BW gain (d 70) NC body fat (d 70) NC heart wt NC liver wt NC kidney wt NC serum triglycerides NC serum insulin NC serum leptin	Prior et al. 2010a

NC/NS, no change or differences not statistically significant; BW, body weight; Cy, cyanidin; Del, delphinidin; Peon, peonidin; rt, rutinoside; xyl, xylosyl; SD, Sprague Dawley.

Anthocyanins from purple corn (primarily cyanidin-3-glucoside), when fed in an HF diet to mice, were effective in reducing liver triglycerides and total lipids compared to mice fed only the HF diet (Tsuda et al. 2003). mRNA expression of lipogenic enzymes (fatty acid synthetase, acyl-CoA synthase, glycerol-3-phosphate acyltransferase) in liver and white adipose tissue was suppressed in mice fed anthocyanins from purple corn (Tsuda et al. 2003). Mice fed an HF diet (60% kcal from fat) plus purified anthocyanins from blueberry in the drinking water had lower body weight gains and body fat than the control mice not fed anthocyanins. Postprandial serum cholesterol and triglyceride levels were elevated in mice fed an HF diet, but when purified anthocyanins from either strawberries or blueberries were included in the drinking water, these levels were not different from that seen in control mice fed a low-fat diet (Prior et al. 2010). Rats fed black soybean anthocyanins in an HF diet also had reduced serum triglyceride (↓45%) and cholesterol (↓26%) levels relative to those fed an HF diet without anthocyanins. Supplementation of a black rice pigment fraction improved the lipid profile in addition to modulating atherosclerotic lesions in rabbits (Ling et al. 2002) and apolipoprotein E–deficient mice (Xia et al. 2003). An anthocyanin-rich extract (43.2%) from black rice enhanced atherosclerotic plaque stabilization in apolipoprotein E–deficient mice (Xia et al. 2006) and lowered fasting plasma free fatty acids, triglycerides, and HDL-C in fructose-fed rats (Guo et al. 2007).

Rat adipocytes treated *in vitro* with anthocyanins showed up-regulated lipid metabolism and signal transduction-related genes; however, the specific altered genes were different between the cyanidin-3-glucoside- and cyanidin (aglycone)-treated groups. Based on the gene expression profile, the up-regulation of hormone-sensitive lipase and enhancement of the lipolytic activity by the treatment of adipocytes with cyanidin-3-glucoside or cyanidin (Tsuda et al. 2005) was demonstrated.

ANTHOCYANINS AND HORMONE/CYTOKINE PRODUCTION

Leptin

A consistent observation in the *in vivo* obesity studies has been that when the anthocyanins were effective in slowing the development of obesity, circulating levels of leptin were decreased (Tsuda et al. 2003; Prior et al. 2008, 2009, 2010a), and when there was not an effect of anthocyanins, the serum leptin did not change (Prior et al. 2010a) or was increased (Lefevre et al. 2008). Leptin is a product of the *ob* gene, and its production increases proportionally with adiposity; leptin levels are high in rodent models of diet-induced obesity. The development of HF-diet-induced obesity in C57BL/6J mice over a 19 week period has been shown (Lin et al. 2000) to be divided into three stages: (1) an early stage in response to an HF diet that mice were sensitive to exogenous leptin; (2) a reduced food intake stage when mice had an increase in leptin production and still retained central leptin sensitivity; and (3) an increased food intake stage, accompanied by a reduction of central leptin sensitivity (Lin et al. 2000). Most likely in all the studies reported here, the obesity had not developed to the 3rd stage in which there was a reduction in central leptin sensitivity.

The amount of leptin in circulation is related to adipose tissue mass; however, with anthocyanin treatment, it is not clear whether the levels just reflect adipose tissue mass or whether other metabolic changes occur that increase leptin production or secretion. *In vitro* treatment of adipocytes with cyanidin significantly enhanced leptin secretion into the media (1.4 fold). However, treatment with cyanidin-3-glucoside did not enhance leptin secretion compared to control (Tsuda et al. 2004). The results with cyanidin may not have relevance to *in vivo* findings because the aglycone form is very unstable and rapidly degrades to protocatechuic acid *in vitro* (Kern et al. 2007). Further, the aglycone form is not found in the circulation *in vivo*. Glycosylated and acylated anthocyanins are rapidly degraded by intestinal microflora, and the major stable products of anthocyanin degradation are the corresponding phenolic acids derived from the B-ring of the anthocyanin skeleton. (Fleschhut et al. 2006).

Insulin

Anthocyanins from Cornelian cherry (*Cornus mas*) were also observed to ameliorate obesity and insulin resistance in C57BL6 mice fed an HF diet (~57% kcal from fat) containing anthocyanins at 1 g/kg (Jayaprakasam et al. 2006). Mice receiving anthocyanins exhibited elevated insulin levels, and anthocyanin consumption preserved pancreatic architecture and insulin staining, relative to mice receiving no anthocyanins (Jayaprakasam et al. 2006). In this study, the mice were fed the HF diet for a period of 4 weeks before switching to the anthocyanin-containing diet. The large elevation of plasma insulin in mice fed the HF diet plus anthocyanins (Table 8.1) was due to the maintenance of pancreatic islet function in the anthocyanin-treated mice (Jayaprakasam et al. 2006). Anthocyanins from purple corn fed to C57BL/6J mice on an HF diet also reduced serum insulin levels compared to the control HF diet (Tsuda et al. 2003). Dietary cyanidin-3-glucoside significantly reduced serum glucose concentration and increased insulin sensitivity in type 2 diabetic mice (Sasaki et al. 2007). KK-A(y) mice fed a diet containing 0.2% of cyanidin-3-glucoside for 5 weeks had significantly reduced blood glucose concentrations and enhanced insulin sensitivity compared to mice fed a control diet.

Inflammatory Cytokines

Adipocyte dysfunction is associated with the development of obesity and insulin resistance. The regulation of adipocytokine expression may be an important target for the prevention of obesity and improvement of insulin sensitivity. The inflammatory molecules, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α), and/or the generation of reactive oxygen species may contribute to the development of insulin resistance in obesity and diabetes. An increase in the mRNA levels of TNF-α was observed in mice fed an HF diet, and these levels returned to normal upon consumption of a diet containing anthocyanins from purple corn (Tsuda et al. 2003). However, serum TNF-α levels were not increased with HF feeding, nor were changes observed in serum TNF-α levels in mice fed an HF diet plus purified anthocyanins from blueberry or strawberry (Prior et al. 2009).

Sasaki and coworkers (Sasaki et al. 2007) suggested that a down-regulation of MCP-1 and TNF-α in white adipose tissue by cyanidin-3-glucoside could lead to an up-regulation of glucose transporter 4 (Glut4) and down-regulation of retinol binding protein 4 (RBP4) expression, leading to a suppression of liver gluconeogenesis, decreased serum glucose levels, and increased insulin sensitivity.

Gene expression of adiponectin was also up-regulated in white adipose tissue in mice fed an anthocyanin supplemented diet. As one of the possible mechanisms, AMP-activated protein kinase activation would be associated with these changes; however, the AMP:ATP ratio was significantly decreased by the administration of anthocyanins (Tsuda et al. 2004).

ANTHOCYANINS STRUCTURE CONSIDERATIONS

Of the anthocyanin sources that have demonstrated bioactivity in slowing the development of obesity in animal models, most have cyanidin-3-glucoside/galactoside as the primary anthocyanin or a mixture of anthocyanin monoglycosides (Blueberry). Pelargonidin-3-glucoside from strawberry also seems to be effective in the mouse obesity model (Prior et al. 2008, 2009). Anthocyanins from black raspberry (Prior et al. 2010a) are not effective in slowing the development of obesity in the mouse model. Cyanidin in black raspberry is present primarily as the rutinoside with smaller amounts of a triglycoside and some glucoside. If the intact anthocyanin molecule is the active component, then the size and perhaps conformation of a diglycoside or triglycoside may prevent the molecule from accessing a receptor or other binding sites to signal the changes that result in decreased adipose tissue deposition.

The competitive inhibition of cyclic nucleotide phosphodiesterase (PDE), an elevation in cAMP level, and subsequent activation of protein kinase A (cAMP-dependent protein kinase) by flavonoids have been proposed (Peluso 2006) as mechanisms to induce neutral lipid hydrolysis from lipid stores in adipose tissue and liver. Indeed, the three-dimensional structure of many flavonoids is sterically and electrostatically compatible with the catalytic site of cAMP PDE3 and PDE4. Flavonoid-mediated PDE inhibition is dependent on the ability of the flavonoid to sterically fit in the cyclic nucleotide binding pocket (Ferrell et al. 1979). Findings from molecular docking investigations support the contention that many of the biological effects of plant flavonoids are attributable to competitive inhibition of specific cyclic nucleotide PDE isoforms (Peluso 2006). Indeed, flavonoids such as apigenin, genistein, daidzein, and quercetin fit very well in the catalytic site of x-ray crystallographic models of human PDE3B, PDE4B, and PDE4D (Peluso 2006). What has not been demonstrated is whether the glycoside forms of flavonoids, and in particular anthocyanidin glycosides, would be able to dock in the catalytic site of PDE. Increased steric hindrance with diglycosides of cyanidin could well prevent inhibitory effects of black raspberry anthocyanins in the process.

ANTHOCYANINS AND HYPERGLYCEMIA

Anthocyanins have been found to have an anti-hyperglycemic effect due to an inhibition of the digestion of carbohydrates via α-glucosidase (Matsui et al. 2001a,b). *Aronia melanocarpa* fruit juice, which contains a high concentration of anthocyanins,

when fed to diabetic rats (10 or 20 mL/kg) significantly reduced plasma glucose by 44% and 42% and TG by 35% and 39%, respectively. These reduced levels did not significantly differ from those of the normal control rats (Valcheva-Kuzmanova et al. 2007). Dietary supplementation with *Aronia melanocarpa* fruit extract (0.2%) decreased the activity of maltase and sucrase with a distinct hypoglycemic response in rats (Jurgonski et al. 2008). The mechanism of the glucose reduction is likely to be multifactorial, but likely related to the decreased activity of mucosal disaccharidases in the gastrointestinal tract.

CONCLUSIONS

Obesity is a multi-faceted disease process involving life style, nutrition, genetics, and other factors. Understanding the role of components in different foods in altering the development of obesity is another factor important in understanding and preventing obesity. The positive effect of purified anthocyanins in slowing the development of obesity in animal models, and the opposite effect observed with whole berries and other foods, presents an interesting dilemma, the solution of which may provide important insights into preventing this disease process. Anthocyanins have been shown to down-regulate fatty acid synthase and acyl-CoA synthase enzymes associated with fatty acid biosynthesis. Up-regulation of hormone-sensitive lipase may enhance lipolytic activity in adipocytes. Changes in both of these processes would have the effect of decreasing the amount of adipose tissue deposited. Understanding the role of anthocyanins as well as other flavonoids that have been shown to impact the development of obesity provides one more approach to consider in preventing the near-epidemic proportions of obesity that we face in the United States.

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9 Literature Review on the Ergogenic Effects of Quercetin

Alyson Mitchell, Jihyun Lee, and Valeria Acquarone

CONTENTS

INTRODUCTION

Flavonols (3-hydroxyflavone) are the most abundant subclass of plant-derived polyphenolics. Flavonols are of tremendous scientific interest because of their potential beneficial effects on human health including antioxidant, anti-inflammatory, and antiviral activities. Moreover, epidemiological studies demonstrate an association of improved cardiovascular health with increased fruit and vegetable intake. Fruits and vegetables are the main source of flavonols in the diet and are largely thought to be responsible for many of the health improvements observed in populations consuming high levels of fruits and vegetables in synergy with vitamins, minerals, and fiber. Quercetin is the most abundant and studied of the flavonols and has biological properties consistent with improved cardiovascular health (as reviewed by Formica and Regelson [1995]). Quercetin demonstrates anti-inflammatory activity and antioxidant activity, prevents platelet aggregation, and promotes relaxation of cardiovascular smooth muscle. In addition, quercetin has been shown to have antiviral (Kaul et al., 1985) and anticarcinogenic properties (Verma et al., 1988).

There is an increasing interest in the use of quercetin in sports science as an adjunct to improve athletic performance as its antioxidant, anti-inflammatory, and psycho-stimulatory activity may improve mental and physical performance. Emerging research suggests that quercetin may improve athletic performance in humans, and

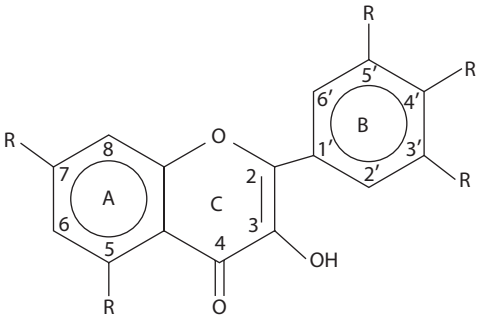

FIGURE 9.1 General structure of flavonoids.

reduce post-exercise infection risk. In addition, quercetin has been shown to stimulate mitochondrial biogenesis *in vivo* (mice). To date, exercise training has been the only practical way to increase the amount of mitochondria in cells and, in turn, increase aerobic capacity and endurance. Cell culture and animal data support this association, whereas human trials have demonstrated only modest benefits at best. However, drawing conclusions on the biological activity of flavonoids based upon available data presents many difficulties due to complexity of comparing (1) a wide range of chemical structures with tremendous variability in glycosidic bond structure, (2) *in vitro* data with animal and human data, (3) types of intervention studies (acute, subacute, chronic, etc.), (4) study design (double blind, crossover, placebo controlled, etc.), (5) dose discrepancies (i.e., a wide range of doses/dosages/exposures routes were used), (6) delivery vehicle differences, (7) different levels of athletic training, (8) different types of exercise (e.g., cycling and endurance running), and (9) different endpoints and biomarkers. In addition, many of the available studies do not account for habitual dietary habits or flavonoid–food matrix interactions and important to athletic performance, pretrial caffeine use, and hydration status. To date, little is known regarding the basic chemical reactions (e.g., oxidation, reduction, and polymerization) and changes in chemical composition that occur in fruits, vegetables, and grains as a result of food processing. These factors need to be explored in order to better understand relationships between flavonoids and health. In this chapter, the scientific evidence for quercetin to improved athletic performance is reviewed (Figure 9.1).

Pharmacokinetics of Quercetin

The chemical structures of flavonols (i.e., glycoside and hydroxylation patterns) influence food matrix interactions and importantly their bioavailability (i.e., the amount absorbed into the blood stream). The flavonols most frequently found in plants are those with B-ring hydroxylation in the 3′,4′-positions (quercetin), 4′-position (kaempferol), and 3′,4′,5′-positions (myricetin). The preferred site of gycoslylation is in the 3-position. In the case of diglycosides, 3-*O*-glycosides and 3,7-diglycosides occur most frequently. D-glucose is the most frequent sugar residue, but D-galactose, L-rhamnose, L-arabinose, D-xylose, D-apiose, and D-glucuronic acid are found as well. Rutin is a diglycoside of quercetin and rutinose (rhamnose and glucose).

Flavonoid glycoside profiles are species and cultivar specific, and differences in the predominant glycosides in foods are often sighted as possible reasons for discrepancies noted between feeding trial outcomes.

Glycosidic linkages also have the potential to influence metabolism and the range of metabolites circulating free in plasma. This has important consequences as the bioavailability of flavonols from different sources will vary widely due to differences in chemical forms of the flavonoid in these sources. For example, quercetin when consumed from onions as the 4′-*O*-glucoside reaches a peak plasma concentration (0.74 μM) at ~45 min, whereas when it is consumed from apples as a mixture of 3-*O*-galactoside, 3-*O*-glucoside, 3-*O*-rhamnoside, and 3-*O*-rutinoside, the maximum plasma level (0.30 μM) is reached at 2.5 h (Hollman et al., 1997). To date, only the aglycone form of quercetin (i.e., quercetin without a sugar attached) is available to manufactures for supplementation. However, Hollman et al. (1995) demonstrated that the absorption of the glycosides surpasses that of the aglycone (52% of the glycosides is absorbed versus 24% of the aglycone) in healthy ileostomy volunteers. This has been confirmed in other studies as well (Hollman et al., 1997; Erlund et al., 2000). Flavonoid glycosides are substrates for enterobacterial β-glucosidases and, especially, lactase phlorizin hydrolase (LPH) present in enteric membranes. The affinity of LPH for various forms of quercetin glycosides is likely to affect their absorption and thus bioavailability. Carrier-mediated transport of quercetin glycosides via the sodium-dependent glucose transporter-1 has also been suggested (Gee et al., 1998; Arts et al., 2002). Most human feeding trials relating quercetin (or other flavonols) intake with biomarkers of health, inflammation, or athletic performance have either not included quantitative information on the composition of quercetin glycosides used in the test diets or used the quercetin agylcone. This may, in part, help explain discrepancies in outcomes found among human feeding trials that compare the effect of quercetin but utilize different foods or supplements as a source of the quercetin. On average, the oral absorption of the quercetin aglycone ranges between 36%–53% and the $t_{1/2}$ 20 and 72 h. Quercetin undergoes extensive first-pass metabolism in the small intestine, colon, liver, and kidney. Metabolites formed are mainly the result of phase II metabolism and therefore include the methylated, sulfated, and glucuronidated forms of quercetin. Dr. Mitchell's laboratory has identified 21 different quercetin-related metabolites after the ingestion of quercetin glycosides from onion (Hong and Mitchell, 2004). The main route of elimination is an exhalation of CO_2 (21%–81%). Normally, human quercetin plasma concentrations are in the low nanomolar range, but upon quercetin supplementation they may increase to the high nanomolar or low micromolar range. Typical supplement doses for quercetin range between 500 and 1000 mg/day. The most common dose for supplementation is subchronic (<30 days) administration of oral quercetin aglycone incorporated into a chew, sports bar, or capsule at 1000 mg/day.

IMPROVEMENT IN ATHLETIC PERFORMANCE

Proposed Mechanisms of Flavonoid Action in Athletic Performance

Our review showed that the most vastly studied flavonoid in relation to athletic performance endpoints is quercetin.

There are numerous reasons to consider a role of quercetin in athletic performance improvement. For example, quercetin is a potent antioxidant and may help to minimize oxidative damage to contractile and structural proteins in skeletal muscle. Exercise results in the increased production of reactive oxygen species (ROS) that can lead to fatigue, inflammation, and muscle injury (Powers et al., 2004; Reid and Hawley, 2008). Quercetin has also been shown to have anti-inflammatory and antiviral properties and may reduce postexercise susceptibility to infection (Davis et al., 2008). Quercetin is also an adenosine A_1-receptor antagonist and may reduce perception of effort and pain and improve endurance (Alexander et al., 2006). More recently, animal studies in mice suggest that quercetin can increase mitochondrial biogenesis (Davis et al., 2009). This is an exciting finding, as increased mitochondrial biogenesis would potentially increase the aerobic capacity of skeletal muscle and improve endurance (Davis et al., 2009). Currently, this effect has only been observed in animals, and there is no evidence for this result in humans.

Several studies have investigated the direct effect of quercetin on athletic performance enhancement in humans. For example, Davis et al. (2010) tested the effects of dietary quercetin on increasing maximal O_2 intake (VO_{2max}) and endurance capacity (Table 9.1). This randomized, double-blind, placebo-controlled, crossover clinical study investigated the impact of consuming 500 mg of quercetin twice daily in a vitamin-enriched Tang or placebo (vitamin-enriched Tang) for 1 week. Twelve healthy fit but untrained male and female college students consumed this beverage. Test measures included VO_{2max} and ride time to fatigue on a bicycle ergometer. After 7 days of quercetin treatment, the data indicated a modest increase of VO_{2max} (3.9%) compared with placebo ($p < 0.05$) and a 13.2% increase in ride time to fatigue as compared with placebo ($p < 0.05$).

In a study by Nieman et al. (2010), the effects of 2 weeks of quercetin supplementation compared with placebo was investigated on exercise performance and skeletal muscle mitochondrial biogenesis in untrained young adult males ($n = 26$). This study was randomized, double blind, and crossover with a preceding 2 week washout period. The study participants were given a standardized beverage containing 500 mg quercetin aglycone twice in a day. Twelve minute time trials on 15% graded treadmills were performed after 60 min of moderate exercise preloads at 60% VO_{2max}. Blood and muscle biopsy samples were obtained before and after supplementation periods. Mean plasma quercetin levels were significantly higher (910 ± 70 nmol/L) after the 2 week quercetin supplementation compared with placebo (331 nmol/L) ($p < 0.001$). The quercetin group went 2.9% farther than the control group during their second time trial. Quercetin supplementation in untrained men was associated with a small but significant improvement in 12 min treadmill time trial performance compared with placebo ($p = 0.038$).

In another randomized, double-blind, and crossover design clinical study, MacRae and Mefferd (2006) tested the effects of an antioxidant cocktail containing 300 mg of quercetin, taken twice daily for 6 weeks, against a placebo (antioxidant cocktail with no quercetin) on cycling time trial performance in 11 highly trained elite male cyclists. Subjects performed the time trial at baseline and at 3 and 6 week intervals. The antioxidant cocktail contained the following: vitamins C (150 mg), E (50 mg), B_{12} (0.008 mg), B_6 (2.5 mg) and B_1 (1.9 mg), caffeine (45 mg), niacin (25 mg), taurine

TABLE 9.1
Summary of Studies Investigating the Effects of Quercetin on Athletic Performance

Study Design	No. of Subjects	Dose (mg/Day)	Duration	Plasma Total Quercetin (nmol/L)	General Results	References
Randomized, double blind, placebo controlled; no crossover	n = 12	1000	Subacute: 7 days	NA	Modest increase of VO_{2max} (3.9% vs. placebo) and 13.2% increase in ride time to fatigue after 7 days	Davis et al. (2010)
Randomized, double blind, crossover with a 2 week washout period; blood and muscle biopsy samples pre- and post-supplementation periods	n = 26	1000	Subacute: 2 weeks	910 ± 70	Quercetin supplementation in untrained men was associated with a small and insignificant improvement in 12 min treadmill time trial performance and modest but insignificant increases in the relative copy number of mtDNA and mRNA levels of four genes related to mitochondrial biogenesis	Nieman et al. (2010)
Randomized, double blind, crossover, placebo controlled (*)	n = 11	600	Chronic: 6 weeks	NA	The cyclists taking the quercetin-containing formula improved their time to complete the 30 km time trial by 3.1% over baseline time ($p < 0.01$); their time during the critical last 5 km was improved by 2% ($p < 0.05$); cyclists consuming the quercetin-containing formula generated greater power during their time trials, which resulted in faster speed; no change in $V0_{2max}$ or heart rate was observed	MacRae and Mefferd (2006)
Randomized, placebo controlled (*)	n = 39	500	Subacute: 2 weeks before trials and 1 week after	~1900 ± 400 (quercetin) and ~2400 ± 500 (quercetin-EGCG)	Two week supplementation with Q-EGCG increased GOBA and decreased inflammation after 3 days of heavy exertion in trained cyclists but not increased athletic performance measures	Nieman et al. (2009)

(*continued*)

TABLE 9.1 (continued)
Summary of Studies Investigating the Effects of Quercetin on Athletic Performance

Study Design	No. of Subjects	Dose (mg/Day)	Duration	Plasma Total Quercetin (nmol/L)	General Results	References
Randomized, double blind, no crossover placebo controlled	n = 15 in each group	1000	Subacute: 7–16 days	~560 ± 260	Quercetin levels were elevated in the quercetin group; no significant changes in muscle oxidative capacity, measures of substrate utilization, cycling economy, or perception of effort	Cureton et al. (2009)
Randomized, double blind, crossover and placebo controlled; groups included caffeine (9 mg/kg), quercetin (2000 mg), or placebo	n = 10	2000	Acute	~4800 ± 2600	No physiological or perceptual effects seen; nutritional adenosine receptor antagonists (caffeine and quercetin) did not enhance endurance exercise performance under compensable heat stress; quercetin did not change RPE, RP, RTC, or RM	Cheuvront et al. (2009)
Randomized, double blind, and placebo controlled; no crossover	n = 20 in each group (quercetin and placebo group)	1000	Subchronic: 3 weeks before and during the 3 day period of intensified exercise	1000 ± 230	Quercetin supplementation did not affect gross efficiency or fuel utilization	Dumke et al. (2009)
					Quercetin supplementation increased plasma quercetin levels but did not affect markers of oxidative stress, inflammation, or plasma antioxidant capacity and significantly reduced URTI incidence in cyclists during the 2 week period after intensified exercise	McAnulty et al. (2008)

Randomized, double blind, and placebo controlled (*)	n = 1002	500 or 1000	Chronic: 12 weeks	~500 (placebo), 1400 (500 mg quercetin) and 2000 (1000 mg quercetin)	A dose-dependent increase in plasma quercetin; however, these increases did not affect F2-isoprostanes, oxidized LDL, reduced glutathione, FRAP, or ORAC values	Shanely et al. (2010)
Randomized, single blind, no crossover, placebo controlled	n = 8 in each group	500	Chronic: 6 weeks	0.2 (quercetin) and 0.009 (isorhamnetin)	Six weeks of supplementation increased plasma quercetin, isorhamnetin, and kaempferol levels but had no effect on plasma antioxidant capacity, lymphocyte DNA damage, or urinary malondialdehyde levels	Boyle et al. (2000)
Randomized, double blind, and placebo controlled (*)	Placebo n = 21; quercetin n = 18	1000	Subacute: 3 weeks	2100 ± 400	No effect on illness rates, perturbations in leukocyte subset counts, salivary IgA, or decreases in granulocyte burst activity	Henson et al. (2008)
					Increased plasma quercetin level but did not attenuate markers of muscle damage inflammation or in leukocyte cytokine mRNA	Nieman et al. (2007)
					No effect on attenuation of aqueous-phase antioxidant capacity (FRAP); No changes in TEAC, F2-isoprostains, blood plasma lipid oxidation, oxidative damage, or protein carbonyls	Quindry et al. (2008)
					No effect on rating of RPE in athletes and on race times	Utter et al. (2009)

VO_{2max}, maximal O_2 intake; Q-EGCG, quercetin with docosahexaenoic acid; GOBA, granulocyte oxidative burst activity; RPE, perceived exertion; RP, pain; RTC, ratings of motivation; RM, thermal comfort; URTI, upper-respiratory tract infection; FRAP, ferric-reducing ability of plasma; ORAC, oxygen radical absorbance capacity; TEAC, Trolox-equivalent antioxidant capacity.
*no quercetin-only control group.

(9 mg), and green tea extract (300 mg). Unfortunately, in this study, there was no true "quercetin-only" control group because green tea extract contains quercetin (2.69 mg/100 mL green tea) and caffeine (USDA, 2007). The cyclists taking the quercetin improved their time to complete the 30 km time trial by 3.1% over baseline time ($p < 0.01$). Their time during the critical last 5 km was improved by 2% ($p < 0.05$). Cyclists consuming the quercetin-containing formula generated greater power (303 W) compared with placebo (293 W) during their time trials, which resulted in faster speed. No changes in VO_{2max} or heart rate were observed. The investigators in this study suggested that the improvement in time trial performance could be attributable to the ability of quercetin to inhibit catechol-*O*-methyltransferase (COMT), an enzyme that degrades norepinephrine (MacRae et al., 2006). However, both the cocktail with and without quercetin resulted in changes from baseline, making it difficult to ascribe effects to quercetin alone.

Nieman et al. (2009) determined the effects of quercetin on athletic performance in 39 trained cyclists (32 men, 7 women). Study groups included: a placebo chew (brown rice syrup, evaporated cane juice, vitamin C, soy lecithin, carnauba wax, natural flavors, gelatin, palm oil, citric acid, and FD&C yellow #5 and blue #1), quercetin (Q: placebo + 250 mg vitamin C, 10 mg niacinamide, 200 μg folic acid, and 250 mg quercetin), and quercetin-EGCG (Q-EGCG: Q + 30 mg epigallocatechin 3-gallate [EGCG] from green tea extract, 100 mg quercetin 3-glucoside, and 100 mg n3-polyunsaturated fatty acid [PUFA] [55 mg eicosapentaenoic acid + 45 mg docosahexaenoic acid]). This study is difficult to interpret as the study groups were not controlled for green tea extract, PUFAs, or quercetin glucoside. Each cyclist was given two (supplemented or placebo) chews to take each morning and two chews to take each afternoon for 2 weeks before, during the 3 days of exercise, and 7 days after exercise. Supplementation had no effect on markers of mitochondrial biogenesis as compared with the placebo group. Two weeks of supplementation with Q-EGCG effectively augmented granulocyte oxidative burst activity (GOBA) and countered inflammation after 3 days of heavy exertion in trained cyclists. However, it is unclear if this was an effect of quercetin, quercetin glucoside, or a synergistic interaction between EGCG and quercetin. There were no significant effects of Q and Q-EGCG on athletic performance measures (power, heart rate, VO_{2max}, and total time trial duration).

Conversely, numerous studies indicate no or minimal effects of quercetin on athletic performance in humans. For example, in a recent randomized, double-blind, placebo-controlled study by Cureton et al. (2009), the effects of quercetin on muscle oxidative capacity, VO_{2max}, and metabolic and perceptual responses on a 10 min maximal-effort cycling test following submaximal cycling, and voluntary and electrically evoked strength loss following cycling were measured before and after treatment with either quercetin (1000 mg/day) or a placebo provided in a beverage. The quercetin supplementation had no significant effect on athletic performance measures compared with placebo.

Cheuvront et al. (2009) investigated the effects of quercetin on endurance exercise performance using 30 min cycle ergometry under heat stress at 50% VO_{2max}, followed by a 15 min time trial in 10 healthy men. In this randomized, double-blind, placebo-controlled, crossover study, groups included caffeine (9 mg/kg), quercetin

(2000 mg quercetin aglycone), or placebo. Quercetin was provided in an energy bar. Plasma quercetin levels reached 4788 nmol/L. No physiological or perceptual effects were shown. Nutritional adenosine receptor antagonists (caffeine and quercetin) did not enhance endurance exercise performance under compensable heat stress. Quercetin did not change perceived exertion pain, ratings of motivation, or thermal comfort.

In another study, Dumke et al. (2009) evaluated whether quercetin ingestion would influence efficiency and fuel utilization in 40 trained male cyclists. After 3 weeks of normal training, the cyclists rode intensely for 3 h/day at 57% work maximum for 3 days in a row. This study was randomized, double blind, and placebo controlled. This study was not of crossover design; therefore, each group represented an n = 20. Subjects consumed energy drinks (Tang) with 500 mg quercetin aglycone with morning and evening meals for 3 weeks before and during a 3 day period of intensified exercise and 2 weeks afterward. Placebo was Tang without an additive. Blood levels of quercetin reached 1006 nmol/L in the quercetin group and 156 nmol/L in the placebo group. Quercetin supplementation did not affect gross efficiency (power output:energy expenditure) or fuel utilization.

REDUCTION IN BIOMARKERS OF OXIDATIVE STRESS

Exercise results in the increased production of ROS that can lead to inflammation, fatigue, and muscle injury (Powers et al., 2004; Reid and Hawley, 2008). Various antioxidants such as vitamins C and E have been studied for their potential to counteract the effects of intense exercise on inflammation and muscle damage (Nieman and Bishop, 2006). Quercetin is a potent *in vitro* antioxidant that strongly chelates certain transition metals, acts as a potent electron donor, and scavenges free radicals. The radical-scavenging ability of quercetin inhibits lipid oxidation *in vitro* (Cheng and Breen, 2000; Lemanska et al., 2004), and it is theoretically possible for dietary flavonoids to help prevent atherosclerosis via the same mechanism *in vivo*. However, outcomes in clinical studies are inconsistent as it relates to the ability of quercetin to modify typical markers of oxidative stress (e.g., antioxidant capacity of the plasma [e.g., ferric reducing antioxidant power (FRAP)], oxygen radical absorbance capacity (ORAC), uric acid, GSH, malondialdehyde, 4-HNE, 8-Hydroxy-2-deoxyguanosine, F2-isoprostains, etc.). An ideal biomarker for oxidative stress is not yet identified, and it likely the balance between antioxidants and by-products of oxidative stress in the organism that present the best approach for the evaluation of oxidative stress.

Nieman et al. (2007) reported the effects of quercetin on markers of muscle damage in 39 ultra-marathon runners (quercetin n = 18 and placebo n = 21) running the 2006 Western States Endurance Run (WSER). The subjects ran under severe conditions that year due to fires in the area of the racecourse. This study was randomized, double blind, and placebo controlled (although vitamin C and niacin were not included in the placebo). Quercetin in the amount of 250 mg was provided in a chew with 250 mg vitamin C, 20 mg niacin, or placebo; two chews were given twice daily with morning and evening meals for 3 weeks prior to the race. Plasma quercetin reached 2151 nmol/L. Increased plasma quercetin levels did not

attenuate markers of muscle damage (C-reactive protein, creatine kinase, cortisol, and glucose). The authors noted that the plasma levels of quercetin dropped to pre-race placebo group levels when measured just after the 30 h race and theorized that quercetin levels were at this level during a good part of the race as well and that this is why no effect was seen. The half-life of quercetin has been reported to range 15–72 h in human (Erlund et al., 2000; Walle et al., 2001), and Nieman et al. (2010) noted that quercetin might have dropped faster during the race than expected in their study.

In a study conducted by Utter et al. (2009), based on data from the same participants in the 2006 WSER ultra-marathon as described earlier, quercetin had no effect on rating of Rated Perceived Exertion (RPE) in athletes, and race times were not significantly different between athlete and placebo groups. The authors had theorized that quercetin would attenuate muscle fatigue by minimizing oxidative damage and through A_1 adenosine receptor antagonist activity (like caffeine), producing an analgesic effect and improving RPE.

The data of Henson et al. (2008) were also based on that from the same participants in the 2006 WSER ultra-marathon described earlier. Quercetin supplementation 3 weeks before and 2 weeks after the WSER did not affect illness rates, perturbations in leukocyte subset counts, salivary IgA, or granulocyte burst activity. The authors believed that quercetin would attenuate the immune dysfunction that follows prolonged and intense exercise. This assumption was based on animal studies or *in vitro* models that used pharmacological levels of quercetin aglycone. The lack of effect may have resulted from the overwhelming amount of oxidative stress and inflammation promoted by the severe conditions in 2006 or from the large drop in quercetin levels during the race event. Based on the data from the same participants of the 2006 WSER ultra-marathon described earlier, Quindry et al. (2008) found that quercetin did not attenuate aqueous-phase antioxidant capacity (ferric-reducing ability of plasma [FRAP]). Exercise did not change Trolox-equivalent antioxidant capacity, F2-isoprostains, or protein carbonyls. Quercetin supplementation did not alter blood plasma lipid oxidation, aqueous-phase antioxidant capacity, or oxidative damage. Although the authors theorized that quercetin would attenuate the oxidative damage that occurs during prolonged and intense exercise, no significant protective effect was found.

In another study, McAnulty et al. (2008) determined the effects of subchronic consumption of quercetin on multiple measures of inflammation, oxidative stress, and upper-respiratory tract infection after 3 weeks of normal training in 40 trained male cyclists. This study data came from the participants in the study of Dumke et al. (2009) as described earlier. Quercetin supplementation increased plasma quercetin levels but did not affect markers of oxidative stress, inflammation, or plasma antioxidant capacity.

A study evaluated the influence of ingesting quercetin on plasma measures of oxidative stress and antioxidant capacity in untrained male and female subjects (n = 1002, 18–85 years) (Shanely et al., 2010). This study was randomized, double blind, and placebo controlled. The study was not controlled for vitamin C or niacin exposure as they were not included in the placebo. The test chew contained 125 or 250 mg quercetin with either 125 or 250 mg vitamin C, 5 or 10 mg niacin,

or the placebo. Quercetin supplementation was given twice daily with meals over 12 weeks in doses of 500 or 1000 mg quercetin/day. Plasma quercetin levels reached ~400 nmol/L with placebo, ~1350 nmol/L with 500 mg quercetin, and ~2000 nmol/L with 1000 mg quercetin. Quercetin supplementation resulted in a significant dose-dependent increase in plasma quercetin concentrations ($p < 0.05$). However, this increase did not significantly affect F2-isoprostanes, oxidized LDL, reduced glutathione, FRAP, or ORAC values.

In another randomized, single-blind, placebo-controlled study (n = 8 in each group), Boyle et al. (2000) evaluated the influence of rutin (quercetin-*O*-rutinoside) supplementation on the antioxidant activity of plasma. Six weeks of quercetin supplementation or placebo were given daily as a tablet containing 500 mg quercetin or a placebo *VENICAP* containing 300 mg glucose, respectively. Blood levels reached ~50 ng/mL quercetin and ~30 ng/mL isorhamnetin for the quercetin group. Six weeks of supplementation also increased plasma isorhamnetin and kaempferol levels but had no effect on plasma antioxidant capacity, lymphocyte DNA damage, or urinary malondialdehyde levels.

In a study conducted by Cureton et al. (2009), the effects of short-duration chronic quercetin supplementation (7–16 days supplementation of 100 mg quercetin given in a sports hydration drink) on muscle oxidative capacity, cycling economy, and perception of effort were evaluated in 30 non-endurance trained men. Quercetin levels in plasma were elevated in the quercetin group (170 ng/mL); however, no significant changes were observed in muscle oxidative capacity, measures of substrate utilization, cycling economy, or perception of effort. Conversely, animal studies showed that 7 days of quercetin supplementation increases run time to exhaustion by 36%–37% in mice (Davis et al., 2010). The authors (Cureton et al., 2009) cautioned that the metabolic and physical performances observed with quercetin supplementation in mice should not be generalized to humans.

EFFECTS OF QUERCETIN ON IMMUNITY AND INFECTION

Quercetin reportedly protects against a wide variety of pathogenic viruses and bacteria. Although the mechanism of quercetin protection is not clear, *in vitro* studies suggested that quercetin may decrease the infectivity and replication of various respiratory viruses at an early stage (Chiang et al., 2003; Chen et al., 2006). The evidence for a role of quercetin supplementation in reducing postexercise upper respiratory tract infection (URTI) is limited but compelling. For example, a study of trained cyclists given 3 weeks of quercetin at 1000 mg/day indicated that quercetin supplementation did not alter exercise-induced changes in several measures of immune function, but it significantly reduced URTI incidence in cyclists during the 2 week period after intensified exercise (McAnulty et al., 2008). Additionally, a more recent randomized community clinical trial (n = 1002) demonstrated that although quercetin supplementation (1000 mg quercetin/day, twice daily dosing for 12 weeks) had no significant influence on URTI rates or symptomatology compared with placebo, a reduction in URTI total sick days and severity was noted in middle-aged and older subjects who rated themselves as physically fit (Heinz et al., 2010).

MITOCHONDRIAL BIOGENESIS

Mitochondrial biogenesis plays an important role in aging-associated diseases such as cancer and diabetes and in the adapted response to exercise training (Tarnopolsky and Raha, 2005; Wallace, 2005). Mitochondria contain enzymes that are responsible for energy production in cells. Muscle cells adapt to exercise training by increasing mitochondrial content (Booth and Baldwin, 1997). This increases aerobic capacity, as measured by maximal oxygen update (or VO_2max), which is considered a key measure of cardiorespiratory fitness. Mitochondrial increase also contributes to slower muscle glycogen and glucose utilization, a greater reliance on fat oxidation for energy, and a decrease in lactate production in the muscles (Holloszy and Coyle, 1984). All of these effects enhance the ability to perform prolonged exercise.

Recently, polyphenolics, including quercetin and resveratrol, have been of great interest due to their effects on mitochondrial biogenesis in animal studies. These compounds are thought to increase mitochondrial biogenesis via intracellular signaling pathways involving peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) and sirtuin (SIRT1). These pathways have been linked to improved endurance and health in mice and rabbits (Lagouge et al., 2006; Rasbach and Schnellmann, 2008). Davis et al. (2009) demonstrated that 1 week of quercetin aglycone supplementation significantly increased the gene expression of the mitochondrial biogenesis coactivators SIRT1 and PGC-1α and mitochondrial DNA in brain and skeletal muscle as well as the run time to exhaustion by 36%–37% in sedentary mice.

To date, human studies have resulted in mixed outcomes with respect to mitochondrial biogenesis. For example, in a study by Nieman et al. (2010), quercetin supplementation in untrained men (n = 26) was associated with modest but insignificant increases in the relative copy number of mitochondrial DNA and messenger RNA levels of four genes related to mitochondrial biogenesis. Muscle mitochondrial DNA relative copy number increased 4.1% with quercetin and a 6.0% decrease with placebo ($p = 0.098$) compared with pre-supplementation. Skeletal muscle messenger RNA expression increased (range = 16%–25%) for SIRT1 (interaction effect, $p = 0.152$), PGC-1α ($p = 0.192$), cytochrome *c* oxidase ($p = 0.081$), and citrate synthase ($p = 0.166$) after quercetin supplementation compared with pre-supplementation.

IMPROVED PERIPHERAL VASODILATION

Peripheral vasodilators, most importantly nitric oxide (NO), dilate blood vessels and theoretically enhance blood flow for performance improvement in sport activities. Quercetin supplementation (10 mg/kg/day by gavage for 3 weeks) increased NO status in rats (plasma S-nitrosothiols, nitrite, and urinary nitrate concentrations) in a study by Loke et al. (2008). Quercetin inhibited a specific vasoconstrictor—endothelin-1 (ET-1)—by activating NADPH oxidase and uncoupled nitric oxide synthase in the rat aorta (Loomis et al., 2005). However, there was no direct evidence that quercetin supplements acted as peripheral vasodilators or enhanced blood flow during athletic performance. For example, plasma volume did not change after 3 days of exercise post–2 weeks of 100 mg/day quercetin supplementation in 39 trained cyclists (Cureton et al., 2009).

CONCLUSIONS

Clinical studies of the influence of quercetin on athletic performance have produced a range of results that require careful interpretation. Studies to date have been conducted using a variety of methodologies, and, as a result, it is difficult to draw generalized learning due to differences in test subject fitness, training, and age; chemical structures and formulation of quercetin supplements; dosage regimes and dose levels (acute, subacute, chronic, etc.); the type of exercise evaluated (e.g., cycling and endurance running); and endpoints and biomarkers measured. Moreover, environmental conditions such as temperature, humidity, and wind may also be result in study discrepancies. Additionally, the parameters measured in different studies may not always be sensitive enough to detect changes (e.g., increases in mitochondrial biogenesis). And lastly, prestudy habitual diets and meals consumed prior to endurance tests may affect study results. Individuals with higher levels of fruits and vegetables in their diets would be expected to have higher body loads of bioactives (e.g., carotenoids, flavonoids, and vitamins E and C), and these may attenuate, or act in synergy with, test flavonoids to influence trial outcomes.

Nonetheless, findings to date suggest a role for quercetin in improving human health and warrant further investigation. Future studies will benefit from increased harmonization and standardization. Studies should employ a randomized, double-blind, placebo-controlled crossover design to help eliminate variability when comparing treatment effects and reduce the sample size needed to detect significant effects. Moreover, the vehicle for delivery should be carefully considered and defined in terms of its glycoside composition and matrix interactions. Better knowledge of the basic chemical reactions (e.g., oxidation, reduction, and polymerization) and changes in chemical composition that occur in fruits, vegetables, and grains during food processing (e.g., extrusion, thermal processing, and edible films) are critical for understanding relationships between flavonoids and health and harmonizing data.

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10 Berry Fruit and Nuts
Their Role in Reducing Oxidative Stress and Inflammation in the Aging Brain

*Marshall G. Miller, Barbara Shukitt-Hale, and James A. Joseph**

CONTENTS

INTRODUCTION

Advances in healthcare, nutrition, and sanitation during the last century have almost doubled human life spans. As a result, the percent of the population that is over the age of 65 years has and continues to increase. This increase is made even more salient in the United States by the impending retirement of the "baby boom" generation. Due to the anticipated population increase among older adults, age-related pathology is a growing concern. The risk of many diseases, such as arthritis, cardiovascular disease, cancer, cataract, dementia, hypertension, metabolic disorder, and osteoporosis increases with age; however, dementia is one of the most costly. While other diseases may be more likely fatal (Kochanek, 2011), dementia robs older

* In memory of James A. Joseph, our valued colleague and friend, who passed away during the writing of this paper.

adults of their independence, control, and identity. Even in the absence of neurodegenerative disease, age-related cognitive decline is observed during "normal" aging. The progressive disability associated with cognitive impairment among older adults necessitates increasingly constant care and, on a global scale, the economic impact of dementias is currently estimated at US$604 billion (ADI, 2010). New strategies to protect brain function during aging are required to meet the increased incidence of age-related neuropathology.

In the last half-century, nutrition has been identified as an important factor in age-related disease. General dietary patterns such as the Mediterranean diet were linked with enhanced health beginning in the 1960s, and, more recently, individual classes of food have been explored (e.g., links between nut consumption and improved cardiac health in the 1990s). Over the last decade, a growing body of research has investigated the protective effect of various fruits and nuts on the brain during aging. The present chapter summarizes much of the evidence related to the consumption of berry fruits and nuts and improved brain function during aging.

Aging, Oxidation, and Inflammation

All living organisms, following an initial developmental period, undergo a process of aging. Aging is a condition wherein an organism's protective functions fail to counteract internal and external stressors, leading to developmental dysregulation over time (Ashford et al., 2005). Within an organism, the aging process affects all biological systems, if somewhat unevenly. The central nervous system, however, is uniquely affected by aging due to the presence of the neurons in its cellular makeup. Neurons are an electrically and chemically sensitive cell type, found only in the central nervous system, which regulate both biological and behavioral processes throughout the body. Unlike other cell types, neurons are nondividing and, once differentiated during the fetal period, are retained throughout the life span, although recent research suggests limited adult neurogenesis occurring in the lateral ventricles and dentate gyrus (Eriksson et al., 1998; Sanai et al., 2004). Therefore, any failure to combat stressors within the brain may result in neuronal loss or alteration, causing permanent modification of biological and behavioral processes.

Excess oxidation is one source of stress on the brain. Despite its small size (≈2% by weight), the brain uses a disproportionate amount of the total inspired oxygen (≈20%), relative to the rest of the body, due to its high level of metabolic activity (Sokoloff, 1960; Halliwell, 1992; Maiese, 2002). During cellular metabolism, reactive oxygen species (ROS) are produced as by-products of mitochondrial electron transport (Beckman and Ames, 1998; Balaban et al., 2005). ROS feature a single unpaired electron in the atom's outermost electron shell. This unpaired electron makes the oxygen species highly reactive. Excess ROS induce deleterious chemical changes within cells throughout life. This cumulative oxidation within an organism is often referred to as oxidative stress, wherein the accumulation of oxidative damage threatens the normal functions of cells and/or the organism (Blomhoff, 2005). Oxidative stress has been implicated as a factor in a variety of neurodegenerative diseases (Subbarao et al., 1990; Smith et al., 1991).

ROS are capable of damaging all components of a cell; however, lipids, nucleic acids, and proteins are particularly vulnerable. When ROS interact with other molecules and gain an electron, therefore stabilizing their electron configuration, the affected molecule is often transformed into a free radical (Gemma et al., 2007). Polyunsaturated fatty acid chains, such as those found in cell membranes, are particularly vulnerable to this type of chain reaction, and neurons' branching processes and elongated morphology necessitate an unusually high membrane to cytoplasm ratio, making neurons especially vulnerable to peroxidation. Because cellular metabolism, centralized in the mitochondria of cells, is a significant source of ROS, mitochondrial deoxyribonucleic acid (DNA) is also particularly prone to oxidation, and accumulating oxidative damage eventually leads to metabolic dysregulation and cell death (Cadenas and Davies, 2000). Oxidative damage is implicated as a primary factor in many age-related neurodegenerative diseases, as well as "normal" aging.

Despite their potential for cellular damage, the production of free radicals (e.g., ROS) is a necessary biological function. Therefore, ROS are normally kept in balance by antioxidants—chemicals capable of donating or sharing an electron without becoming a free radical themselves and thus neutralizing the ROS. However, the oxidative damage that accumulates in the brain during aging is exacerbated by a decline in endogenous antioxidant defense mechanisms (Harman, 1981; Olanow, 1993; Carney et al., 1994; Yu, 1994; Bokov et al., 2004).

In conjunction with oxidative stressors, chronic immune response and inflammation are also central processes of aging and age-related neurodegeneration. Far less numerous than neurons (Pelvig et al., 2008; Azevedo et al., 2009), microglia are a type of glial cell that act as the brain's immune effector within the blood–brain barrier. In adults, microglia exist in two forms, ramified microglia and reactive microglia. Ramified microglia maintain contact with, and are thought to monitor, nearby cells (Fetler and Amigorena, 2005). In the presence of distressed cells or cellular debris, as can result from oxidative damage, ramified microglia become reactive—transforming to phagocytose the offending biological materials (Stence et al., 2001). Microglial activation has been shown to increase with age (Rozovsky et al., 1998) and contributes to chronic inflammation.

Activated microglia are a primary source of pro-inflammatory signaling molecules within the brain, such as complement proteins and cytokines. Increased levels of inflammatory mediators such as C-reactive protein, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) are observed during aging (Dobbs et al., 1999; Bruunsgaard et al., 2001; Kushner, 2001; Volpato et al., 2001; Forsey et al., 2003; Steinert et al., 2010) and in neurodegenerative disease (Luterman et al., 2000; Tarkowski et al., 2003). After binding with receptors in adjacent cells, these inflammatory mediators induce inflammation and apoptosis in damaged cells and play a key role in chronic inflammation. Once activated, microglia are also a significant source of free radicals, particularly superoxide and nitric oxide (NO), in the brain (Dringen, 2005), thus creating a positive feedback loop between oxidative stress and immune response.

The presence of extracellular pro-inflammatory signals and ROS initiate a variety of intracellular signaling cascades, such as mitogen-activated protein kinases (MAPK). MAPK are a large family of serine/threonine protein kinases, including

extracellular signal-regulated kinases (ERK), c-Jun amino (N)-terminal kinases (JNK), and p38 isoforms (among others) that, in turn, trigger a variety of cellular responses, including transcription factors such as nuclear factor-kappa B (NF-κB) and p53. NF-κB, once activated in neurons, translocates from the cytoplasm to the nucleus where it induces expression of genes related to inflammation, cell survival, and proliferation. In the brain, NF-κB has also been implicated in modulating synaptic plasticity (Albensi and Mattson, 2000). P53, under normal conditions, induces the expression of antioxidant genes; however, when cells become stressed, p53 can have the opposite effect, inducing increased ROS production and p53-mediated apoptosis (Sablina et al., 2005).

On a broader scale, the complex and deleterious interplay between oxidative and inflammatory processes are central to the changes observed in the brain during aging. It is not surprising, then, that significant cerebral atrophy and alteration of neurotransmitter levels are observed in the brain during aging (Seidler et al., 2010), which ultimately result in functional declines in motor and cognitive behavior. Though some cognitive domains appear to be maintained in aging, for example, knowledge, domains that show decline in aging include attentional flexibility, executive function, memory, processing speed, reasoning, spatial ability, and working memory (Moffat and Resnick, 2002; Salthouse, 2004; Driscoll et al., 2005; Inzitari et al., 2007; Yogev et al., 2008; Montero-Odasso et al., 2009). Additionally, complex motor behaviors such as balance and gait rely on a variety of neuronal subsystems (e.g., vision, proprioception, motor control and planning, etc.) which suffer functional declines during aging (Joseph, 1992; Bickford, 1993; Horak, 2006; Kang and Dingwell, 2008). Even mild motor and cognitive impairments, observed in aging, require an average of 8.5 h of additional care each week (Langa et al., 2001).

Antioxidants and Phytochemicals

Given the links between free radicals, inflammation, and neurodegeneration during aging, it follows that increased intake of antioxidants should reduce the effects of aging. Epidemiological studies have largely found that the consumption of antioxidant-rich diets, such as the Mediterranean diet, decreases the risk of cognitive impairment (Commenges et al., 2000; Engelhart et al., 2002; Morris et al., 2002a,b; Morris et al., 2005); although, some have not (Luchsinger et al., 2003). However, clinical trials with individual antioxidants, specifically vitamin E or C, on the progression of dementia during aging have provided conflicting evidence for even a marginal beneficial effect from supplementation (see Sano et al., 1997; Boothby and Deoring, 2005; Fillenbaum et al., 2005; Petersen et al., 2005; Praticò, 2008). If the reduced risk for dementia, observed in epidemiological studies, is not directly attributable to the antioxidant effects of these nutrients alone, then it is possible that the beneficial effects of supplementation are only realized using a combination of antioxidants or that beneficial effects occur outside the scope of most dietary intervention trials (Kesse-Guyot et al., 2011). Nonnutrient phytochemicals may also account for the beneficial effects resulting from the consumption of an otherwise antioxidant-rich diet. Recent research has, therefore, focused on both the whole

foods found in these fruit- and vegetable-rich diets and the wide variety of phytochemicals found within them, particularly phenolics.

Phenols are a class of organic substances containing a benzene ring and one or more hydroxyl substituents (Harborne, 1989) that are found throughout the plant kingdom. Polyphenols are comprised of one or more phenol groups, and the thousands of unique structures can be categorized into a variety of polyphenolic families (Cheynier, 2005; Liu, 2004), for example, tannins, flavonoids, stilbenes, coumarins, and phenolic acids. Polyphenols are often referred to as "secondary" compounds or metabolites because they are not directly involved in metabolism; however, phenols are essential to plant growth, reproduction, signaling, pigment (notably, blue, orange, red, and yellow), resistance to pathogens, and defense from UV radiation. Total phenolic intake has been estimated at ≈1 g/day in Western populations (Manach et al., 2003). However, the phenolic content of most whole foods is poorly characterized and highly dependent on cultivar, growing conditions, storage, ripeness, and preparation. Furthermore, individual intake of polyphenols is highly variable due to individual food preferences and seasonal availability. Unfortunately, these sources of variability conspire to make dietary intake estimates general at best.

Among plant foods (but excluding herbs and spices), berries and nuts represent particularly rich sources of polyphenols and other antioxidants (e.g., vitamins E and C). Açai fruit, raspberry, chokeberry, elderberry, blueberry, cranberry, and blackberry display particularly high total oxygen radical absorption capacity (ORAC) as do pecans, pistachios, and walnuts (USDA, 2010). The remainder of this chapter describes both the preclinical and clinical studies on the neuroprotective effects of berry fruit and nuts during aging.

BERRY FRUIT

The common English usage of the term "berry" defies botanical classifications in that any bite-sized fruit is often referred to as a "berry." Berry fruits are commonly consumed throughout the world. Berry fruits commonly consumed in North America include blackberries (*Rubus* spp.), blueberries (*Vaccinium corymbosum*), cranberries (*Vaccinium macrocarpon*), raspberries (*Rubus idaeus* or *occidentalis*), and strawberries (*Fragaria ananassa*; Seeram, 2008). Berries are nutrient dense and contain high levels of polyphenols, such as flavonols and anthocyanins, as well as fiber. Phenolics are generally concentrated in the berries' skin; however, the meat of some berries, such as cherries and strawberries, contain higher levels of anthocyanin (Basu et al., 2010). In the past decade, a growing literature has developed detailing *in vitro*, *in vivo*, and clinical research into the effects of berry fruit on the brain during aging.

In Vitro Evidence

Studies from our laboratory have shown a variety of beneficial effects of berries on neurons and glia, the primary cell types found in the brain. In one study (Joseph et al., 2007), cultured primary hippocampal cells from neonate rats were challenged with the neurotransmitter dopamine, amyloid-$\beta_{(42)}$, $A\beta_{(40)}$, or $A\beta_{(25-35)}$. Increased levels of

Aβ peptides are found in aging (Braak and Braak, 1991), and Aβ is the major component of the neuritic plaques found in the brain in Alzheimer's disease (Perl, 2010). The neurons were then treated with KCl to induce depolarization. During depolarization, neurons experience an influx of calcium; however, efficient clearance of calcium, following depolarization, is necessary for maintaining calcium homeostasis and is required for subsequent depolarization. Dopamine and $A\beta_{(42)}$ significantly reduced the percent of primary neurons, which recovered from depolarization (50%–70% calcium clearance); however, pretreatment with blueberry (0.5 mg/mL) significantly mitigated these effects, increasing the percent of primary neurons that recovered following depolarization. amyloid-$\beta_{(40)}$ and $A\beta_{(25–35)}$ only minimally compromised calcium recovery, and therefore, pretreatment with blueberry was ineffective at preventing these declines. Furthermore, dopamine significantly increased levels of protein kinase C-gamma (PKCγ) and phosphorylated cyclic adenosine monophosphate response element-binding (pCREB) protein, another MAPK-activated transcription factor that is involved in learning and memory. Pretreatment with blueberry prevented these alterations in signaling. Meanwhile, $A\beta_{(42)}$ significantly increased levels of phosphorylated MAPK, PKCγ, and pCREB while lowering levels of PKCα; as with dopamine, pretreatment with blueberry prevented these alterations.

In a subsequent study (Joseph et al., 2010), cultured primary hippocampal cells from neonate rats were treated with the dopamine, $A\beta_{(42)}$, or lipopolysaccharide (LPS), a potent inducer of inflammation. Cells were pretreated with whole blueberry extract (500 μg/mL), concentrated wild blueberry juice (250 μg/mL), a fraction without sugars and organic acids (17.5 μg/mL), an anthocyanin fraction (15 μg/mL), total proanthocyanidins (14 μg/mL), high and low molecular weight proanthocyanidins' fractions (11.5 and 5.5 μg/mL, respectively), or chlorogenic acid (15 μg/mL). When calcium clearance was assessed, dopamine, $A\beta_{(42)}$, and LPS each significantly reduced the percent of primary neurons that recovered following depolarization, with dopamine causing the greatest reduction in calcium recovery. In each case, pretreatment with whole blueberry extract prevented the declines in recovery caused by the stressors. Notably, chlorogenic acid failed to provide protection against any stressor. The remaining fractions provided variable degrees of protection, depending on the stressor, but none were quite as good alone as the whole blueberry extract. Additionally, each of the stressors was shown to significantly reduce cell viability and increase ROS; however, whole blueberry extract provided partial protection in each case while the various fractions' degree of protection varied according to the stressor. Interestingly, whole blueberry extract, the anthocyanin fraction, and concentrated blueberry juice increased levels of ERK 1/2. When primary neurons were exposed to dopamine, increases in the activation of JNK and P38 MAPKs as well as NF-κB were observed; however, whole blueberry extract provided superior protection against activation of these stress signals, relative to other fractions. These findings indicate that the protective effects of whole blueberry extract are more effective than any of its constituent fractions, perhaps indicating a synergistic effect of the combined polyphenols.

Lau et al. (2007) examined the effects of blueberry on LPS-activated BV2 murine microglia. BV2 cells were treated with media alone or media containing LPS in

addition to 0, 50, 100, 250, or 500 μg/mL blueberry extract. LPS-treated microglia showed significantly increased levels of both COX-2 and iNOS mRNA and protein and released increased levels of nitrites and the cytokines IL-1β and TNF-α into cell-conditioned media, relative to untreated cells. Meanwhile, blueberry significantly inhibited these increases in both early (IL-1β and TNF-α) and late (COX-2 and iNOS) inflammatory mediators in a dose-dependent manner. Furthermore, blueberry reduced intracellular ROS in a dose-dependent manner. These findings highlight blueberry's ability to stifle microglial activation by suppressing ROS and inflammatory mediators at multiple points along the inflammatory response cascade.

In Vivo Evidence

A variety of studies have shown that consumption of berries can protect behavioral function from age-induced decrements *in vivo*. Initial studies focused on blueberries due to their common consumption and high antioxidant activity; however, a variety of berries are now being investigated. In an early study in our laboratory (Joseph et al., 1999), 19 month old Fischer 344 rats whose diet contained 1.86% lyophilized blueberry or 1.48% lyophilized strawberry (matched according to ORAC) displayed a variety of behavioral improvements after 2 months, relative to age-matched controls consuming a modified AIN-93 rodent diet. Specifically, blueberry-fed animals showed enhanced balance when standing on a narrow rod or walking on a slowly accelerating, rotating rod (rotarod), relative to controls. Furthermore, both strawberry- and blueberry-fed animals showed improved performance in a working memory version of the Morris water maze (MWM), relative to controls. During this task, rats must locate a submerged platform in a pool of opaque water, which is relocated after each pair of trials. Berry-fed rats located the submerged platform faster on the second trial of each pair. Following behavioral testing, analysis of the animals' striatum, an area of the brain involved in motor control and procedural learning, revealed lower levels of 2′,7′-dichlorofluorescin diacetate (DCF), a marker of oxidative stress, in the berry-fed animals.

Goyarzu et al. (2004) compared young (4 months) and aged (15 months) Fischer 344 rats that had consumed either a 2% blueberry or a modified NIH-31 control diet. After 4 months on the diet, animals were administered a novel object test. During this test, animals were first allowed to explore two identical objects, placed within an arena, until they had spent a total of 30 s closely exploring each object and were then returned to their home cages. After a 30 s or 60 min delay, the animals were returned to the arena in which an object identical to those used during the prior phase was paired with a novel object and the amount of time rats spent investigating the familiar and novel object was measured. No differences were observed between groups in the 30 s delay condition. However, in the 60 min delay condition, blueberry-fed animals spent a significantly higher percent of time investigating the novel object, relative to age-matched controls, indicating better memory for the familiar object. Furthermore, the aged, blueberry-fed rats' exploration of the novel object was similar in duration to that of the young rats, indicating spared object memory. Following behavioral testing, NF-κB protein was measured in various brain regions.

Aged rats consuming the control diet showed significantly higher NF-κB levels than young rats in all brain regions; however, aged, blueberry-fed rats showed attenuated NF-κB levels in the frontal cortex, hippocampus, striatum, and cerebellum, which were similar to those of the young rats. Interestingly, among the aged animals, a significant negative correlation existed between the percent of time spent investigating the novel object and the level of NF-κB proteins, averaged across the brain regions.

A further study (Malin et al., 2011) explored the time course of dietary blueberry's effects. Aged Fischer 344 rats were administered behavioral testing at 19 months, following 1 or 2 months consuming a modified NIH-31 diet or a diet containing 2% blueberry. Rats completed a novel object test, similar to the one used in the previous study, using a 60 min delay between initial exposure and subsequent testing. Rats that consumed the control diet spent equal amounts of time exploring the familiar and novel object, indicating no memory for the object they had previously explored. Rats that consumed blueberry for 1 or 2 months, however, spent significantly more time (≈70%) investigating the novel object, indicating that they recognized the previously explored item. Berry-fed rats were then returned to the control diet and behavior was retested at 2 and 4 weeks thereafter. Rats that had previously consumed the blueberry diet for 2 months showed no decline in performance after 2 or 4 weeks. However, rats that had previously consumed the blueberry diet for only 1 month showed a decline in performance after 2 weeks and were not significantly different from the age-matched controls after 4 weeks on the control diet. To follow up on these findings, Malin et al. (2011) conducted a second experiment in which aged Fischer 344 rats were tested on novel object recognition at 19 months and subsequently fed either a modified NIH-31 diet or a diet containing 2% blueberry. After a month on these diets, the rats were tested again. Rats that consumed the control diet showed no change in performance; however, blueberry-fed rats showed a significant increase in performance relative to baseline. These findings show that cognition is improved (reversed?) in aged rats by as little as 1 month of dietary supplementation with blueberry; however, at least 2 months of supplementation are required to maintain the benefit long term.

Andres-Lacueva et al. (2005) maintained 19 month old Fischer rats on a 2% lyophilized blueberry diet or a control diet for 2 months. While blueberry-fed animals performed no better than controls in a nonworking memory version of the MWM, a variety of anthocyanins were found in the cerebellum, cortex, hippocampus, and striatum but not in the brains of age-matched controls. Furthermore, the level of total anthocyanins found in the cortex and hippocampus correlated significantly with MWM behavior. Animals with higher cortical anthocyanin levels required less time to locate a hidden platform during a relearning trial in which the platform was relocated, indicating faster learning. Meanwhile, animals with higher hippocampal anthocyanin levels crossed the previous location of the platform more times, indicating better memory for the platform's location. These findings show that, once consumed, dietary polyphenols from berries are both neuroavailable and related to cognitive performance.

Other berries are also being investigated for their effect on the brain during aging. After 2 months, 19 month old Fischer 344 rats whose diet contained 2% lyophilized blackberry showed improved balance on a narrow plank and accelerating rotarod,

relative to age-matched rats consuming a control diet (Shukitt-Hale et al., 2009). Blackberry-fed rats also showed improved performance in a working memory version of the MWM (see Joseph et al., 1999), finding a hidden platform more quickly than age-matched controls.

In another study (Shukitt-Hale et al., 2005), 19 month old Fischer 344 rats were maintained on an NIH-31 control diet or a diet containing 2% blueberry, 2% cranberry, 2% black currant, or 2% boysenberry for 2 months. When rats were tested on an inclined screen, blueberry- and cranberry-fed rats were able to cling to the screen significantly longer than control-fed rats, suggesting increased muscle tone and control. Interestingly, performance on the inclined screen correlated positively with the extent to which heat shock protein 70 (HSP70), a protein which protects proteins from aggregation and misfolding, increased when hippocampal sections from these animals were challenged with LPS *in vitro*. When striatal dopamine activity was assessed, rats that consumed black currant or cranberry showed enhanced oxotremorine-enhanced dopamine release.

In a further study (Shukitt-Hale et al., 2006), aged Fischer 344 rats (19 months) were maintained on Concord grape juice or an isocaloric placebo for a period of 2 months. Rats consumed 50% grape juice, 10% grape juice plus 40% placebo, or 50% placebo instead of water in their home cages. Rats that consumed the higher concentration of grape juice exhibited enhanced balance on a narrow rod and a narrow plank, as well as increased forelimb strength when suspended from a horizontal wire, relative to rats that consumed placebo. Dietary supplementation with grape juice also enhanced performance in a working memory version of the MWM in which rats attempted to locate a hidden platform whose location changed after each pair of trials. Rats that consumed the lower concentration of grape juice were significantly faster at locating the hidden platform on trial two of each pair, relative to rats that consumed placebo. When oxotremorine enhancement of dopamine release was examined in striatal sections from the brains of these animals, rats that consumed the lower dose of grape juice also showed enhanced dopamine release relative to rats consuming placebo. Given current concerns that high-calorie beverages such as fruit juice and soda contribute to obesity, it is noteworthy that the beneficial effects of grape juice are obtained by consuming only a modest dose each day, that is, 10% grape juice.

Exposure to high-energy particles has also been shown to produce a pattern of behavioral decrements similar to those seen in aging (Denisova et al., 2002; Shukitt-Hale et al., 2003; Casadesus et al., 2004a) in which motor behavior and dopaminergic function are compromised. Using this model of aging, Rabin et al. (2005) maintained young Sprague-Dawley rats (2 months) on either a modified NIH-31 control diet, a diet containing 2% blueberry, or a diet containing 2% strawberry for 2 months. Rats were then irradiated with 1.5 Gy of 1 GeV/n high-energy ^{56}Fe particles. After recovery, rats were mildly food deprived and trained in an operant chamber to bar press to obtain food. After 6 and 12 months, rats were again mildly food deprived and tested on an ascending fixed ratio schedule of reinforcement wherein each day more bar presses are required to obtain a food reward. While no differences were observed 6 months following irradiation, at 12 months, irradiated rats failed to increase bar-press behavior in response to the ascending ratio of reinforcement, relative to

nonirradiated rats. Strawberry-fed, irradiated rats, however, showed no impairment in bar-press behavior, performing similarly to nonirradiated controls. This behavior has been shown to be sensitive to dopaminergic dysregulation, particularly in aging (Salamone et al., 1993; Lindner et al., 1999, 1997) wherein animals with compromised dopaminergic systems fail to exhibit increased bar-press behavior.

Subsequently, Shukitt-Hale et al. (2007) maintained young Sprague-Dawley rats (2 months) on either a modified NIH-31 control diet, a diet containing 2% blueberry, or a diet containing 2% strawberry. After 2 months, rats were irradiated with 1.5 Gy of 1 GeV/n high-energy ^{56}Fe particles and then allowed to recover for an additional month. Rats were then tested in the MWM for 4 days, during which irradiated rats exhibited greater latencies to locate the platform during training trials. Rats received a probe trial on days two through four, in which the platform was removed. Irradiated controls passed through the location where the platform had been significantly fewer times, during the first probe trial, than did nonirradiated controls; however, the irradiated rats that consumed the strawberry diet spent more time in the quadrant containing the former location of the platform, passed through the former location of the platform significantly more times, and spent significantly more time in the exact former location of the platform than did irradiated rats consuming the control diet. Improved performance during the initial probe trial indicates that the strawberry diet protected spatial ability, allowing for earlier learning of the task. Using spatial strategies to accurately locate the platform is dependent on intact hippocampal function. On the fourth day, the platform was relocated to the opposite side of the pool, and rats had to learn the new platform location. Interestingly, there was a trend toward decreased latency to locate the new platform's location among blueberry-fed but not strawberry-fed rats, relative to controls. Successful reversal learning is associated with intact striatal function. When dopamine was measured in striatal tissue from these animals, irradiated rats showed decreased oxotremorine-enhanced dopamine release, relative to nonirradiated rats; however, irradiated rats that consumed either the blueberry or strawberry diet showed no decrease in dopamine release, relative to nonirradiated controls. This effect was also observed when the test was conducted in the presence of H_2O_2 to simulate conditions of oxidative stress. Because striatal dopamine levels decline during aging, improvements in motor behavior, learning, and memory, observed following dietary supplementation with berries, may be attributable to protected dopamine regulation. Furthermore, the results of the behavioral testing indicate that blueberries and strawberries may exert their beneficial effects on cognition by acting in separate brain regions.

A further study by these authors (Rabin et al., 2009) examined the effects of berry supplementation on irradiation-induced deficits in object recognition. In this study, young Sprague-Dawley rats were irradiated with 0, 50, 80, 100, 150, or 200 cGy of 1 GeV/n high-energy ^{56}Fe particles and then allowed to recover for 1 week. Rats were then tested for novel object recognition as detailed previously (Goyarzu et al., 2004) except that rats experienced a 24 h delay between exposure to objects and testing with a novel object. Somewhat paradoxically, a U-shaped dose-response was observed with rats exposed to 50 cGy failing to show significant performance decrements, rats exposed to 80, 100, or 150 cGy showing significant impairments (chance performance), yet rats exposed to 200 cGy showing performance similar

to that of rats exposed to 0 or 50 cGy. The reason for this pattern of results is not clear. In a second experiment, rats were exposed to 150cGy of 1 GeV/n high-energy ^{56}Fe particles after consuming a modified NIH-31 diet or a diet containing 2% or 4% strawberry or blueberry for 2 weeks. Following irradiation, rats were tested for novel object recognition. Nonirradiated control rats spent more time exploring the novel object than the familiar one while irradiated rats consuming the control diet spent equivalent amounts of time exploring the familiar and novel object, indicating impaired object recognition. Rats consuming strawberry or blueberry at either the 2% or 4% diet showed no significant decrease in performance, relative to the nonirradiated controls. These studies show that dietary supplementation with berry fruit can protect against the deleterious effects of irradiation and prevent aging-like deficits in performance on tests of operant learning and both spatial and object memory.

Kainic acid, when administered intracranially, produces glutamate excitotoxicity, oxidative stress, and inflammation (Minami et al., 1991; Ueda et al., 2002) and provides another useful model for these age-related processes. In a recent study (Shukitt-Hale et al., 2008), young Fischer rats (4 months) were maintained on a diet containing either a control, 2% blueberry, or 0.015% piroxicam, a nonsteroidal anti-inflammatory drug, for 2 months. Kainic acid or vehicle was then microinjected into the hippocampus of the rats. After a 2 week recovery period, control rats injected with kainic acid took longer to locate a hidden platform in the MWM, relative to control rats injected with vehicle. Piroxicam and blueberry were able to counteract kainate-induced increases in latency to locate the hidden platform in the MWM with blueberry-fed rats showing somewhat better performance than those on piroxicam. Quantitative real-time polymerase chain reaction (qRT-PCR) revealed that supplementation with either piroxicam or blueberry counteracted kainate-induced elevations in the expression of IL-1β, TNF-α, and NF-κB in the hippocampus. Furthermore, while kainic acid increased insulin-like growth factor 1 (IGF-1), a growth factor involved in proliferation and cell survival, however, supplementation with blueberry but not peroxicam further increased levels of IGF-1.

In another study (Duffy et al., 2008), young Fischer 344 rats (4–5 months) were maintained on either a 2% blueberry or a modified NIH-31 control diet for 9–15 weeks prior to bilateral intra-hippocampal injection of either kainic acid or vehicle. Following a week-long recovery, rats received active avoidance training in which they learned to run down a narrow corridor to escape a mild scrambled foot shock (0.8 mA). Subsequently, rats were tested in a 14 unit T-maze in which rats learned to navigate through a series of maze sections to avoid a mild foot shock that initiated 10 s after entering each maze section. Rats that were injected with kainic acid made more maze errors and took longer to complete the maze than did rats injected with the vehicle only. Kainate-injected rats that had been maintained on a blueberry diet, however, showed only minimal increases in error rate or run time relative to vehicle-injected controls. Additionally, stereological analysis revealed that injection with kainic acid resulted in significant neuronal losses in the CA1 region of the hippocampus. Blueberry-fed, kainate-injected rats, however, showed no significant loss of neurons in CA1. Furthermore, FaO rat hematoma cells, pretreated in serum from blueberry-fed rats, also showed enhanced viability when challenged with H_2O_2, relative to cells pretreated with serum from controls.

Dietary blueberry's effects have also been investigated in mouse models of learning and memory. Barros et al. (2006) administered water or a blueberry extract to young Swiss mice (3 months). The blueberry extract contained either a low (0.6–1 mg/kg/day) or a high (2.6–3.2 mg/kg/day) concentration of anthocyanin, a polyphenol found in blueberry and other blue/purple fruits and vegetables. After 1 month on the diet, animals were tested on an inhibitory avoidance task in which mice are placed on a platform located amidst a metallic grid. Initially, when mice stepped down from the platform, they received a mild scrambled foot shock (0.4 mA). Mice that consumed blueberry extract waited on the platform without stepping down onto the metallic grid significantly longer than mice that consumed water when mice were subsequently tested 7 and 30 days after the initial training session, indicating enhanced long-term memory for the foot shock. Additionally, mice were tested in an elevated plus maze in which they are allowed to explore both open arms, where they are exposed, and arms that are walled in, where they are protected. Mice that consumed blueberry extract with a higher concentration of anthocyanin spent more time in the open arms of an elevated plus maze, relative to mice that consumed water, indicating reduced anxiety. This reduced anxiety may be protective as stress has been shown to exacerbate neurodegeneration in murine models of Alzheimer's disease (Green et al., 2006). Furthermore, when hippocampal and cortical sections were analyzed using an alkaline single cell electrophoresis (comet) assay, mice that consumed the blueberry extract with the higher concentration of anthocyanin also showed significantly less DNA damage following treatment with H_2O_2 than mice who consumed water.

Papandreou et al. (2009) administered vehicle or blueberry polyphenol extract at one of two doses (30 or 60 mg/kg; i.p.) to young Balb-c mice (3–4 months). On days 5 and 6 of administration, mice were tested on a step-through avoidance paradigm. During this test, animals are first (day 5) placed in a brightly lit (exposed) compartment with an aperture to an adjoining darkened (protected) compartment. When the mice crossed into the darkened compartment, a guillotine door was lowered behind them and they were administered a mild foot shock (0.3 mA). On the subsequent day (day 6), mice administered the higher dose of blueberry extract waited significantly longer before entering into the darkened compartment, indicating enhanced long-term memory for the foot shock. Additionally, brain homogenates from mice that were administered blueberry extract showed decreased levels of acetylcholinesterase in both salt- and detergent-soluble fractions, indicating that blueberry may be able to increase levels of acetylcholine, a neurotransmitter involved in learning and memory whose levels are compromised in Alzheimer's disease (Perry et al., 1978; Whitehouse et al., 1981).

In addition to neuroprotective effects and modulation of stress signaling, berry fruit also has more direct effects on the brain. Increases in neurogenesis have been observed among rats whose diet was supplemented with berry fruit. Casadesus et al. (2004b) maintained aged Fischer 344 rats (19 months) on a modified NIH-31 diet or the same diet containing 2% blueberry. After 2 months, rats were tested in the radial-arm water maze (RAWM) and blueberry-fed rats committed significantly fewer reference memory errors and showed a trend toward decreased working memory errors, relative to controls. Blueberry-fed rats also showed increased levels of IGF-1, IGF-1 receptors, and ERK 1/2, which were negatively associated with reference memory

errors in the RAWM. Additionally, bromodeoxyuridine (BrdU) staining revealed increased proliferation of precursor cells in the dentate gyrus of the blueberry-fed rats, relative to controls.

Similarly, Williams et al. (2008) maintained aged (18 months) Lister-hooded rats on either a modified NIH-31 diet containing 2% blueberry or the same diet containing vitamin C (2 mg/kg) to equate the antioxidant levels of the two diets. After 3 months, rats were tested in a plus maze in which they were first allowed entry into one arm of the maze to retrieve a food pellet and then, in a subsequent trial, rats were given the choice of two arms and were required to enter the opposite arm to receive a food reward. Aged blueberry-fed rats displayed fewer errors and shorter latencies than age-matched controls; this level of performance was similar to that of young rats (6 months). Anthocyanins and flavonols were detected in both the brains and plasma of blueberry-fed rats but not in rats consuming the control diet. Aged rats displayed decreased hippocampal levels of phosphorylated CREB and Akt, ERK 1/2, RSK2, activity-regulated cytoskeleton-associated protein, mammalian target of rapamycin (mTOR), brain-derived neurotrophic factor (BDNF), and proBDNF, relative to young controls; however, blueberry protected aged rats from these age-related declines in signaling.

Dietary berry fruit can alter neuronal morphology in aging rats. Normally, neurons are characterized by branching dendritic processes whose surface is covered with spines onto which other neurons' axons may synapse. During aging, these dendritic regions become less complex, displaying fewer branches and a lower density of spines. When Fischer 344 rats (19 month old) were maintained on either an NIH-31 control diet or a diet containing 2% blueberry for 2.5 months, Golgi staining revealed that blueberry-fed rats showed a significant 14% increase in basilar dendritic arborization among cortical neurons (layers II and III; Hwang et al., 2007), relative to age-matched controls. Furthermore, the density of dendritic spines located in the terminal tips was increased 11%, relative to age-matched controls (Mervis et al., 2007). These morphological alterations may increase the likelihood of synapse formation and potentially compensate for age-related synaptic losses. Within the striatum, however, a different pattern of morphological change was observed. When aged control-diet-fed rats were compared to a group of young rats (6 months) that were also fed the control diet, unlike in the cortex, a 30% increase in the complexity of dendritic arborization was observed among medium spiny neurons in the striatum (Kasimos et al., 2008). Paradoxically, rats that consumed the blueberry diet showed decreased dendritic arborization complexity in the striatum, relative to age-matched controls. Blueberry's ability to return rats to a morphology associated with younger rats may impact changes in motor ability and learning observed in aging. Dietary berry fruit may enhance neuronal structure and increase adult neurogenesis, thus allowing for cognition sparing adaptation in the face of age-relation neuronal atrophy.

CLINICAL TRIALS

While the accumulating animal evidence provides insight into the potential for neuroprotective effects of berry fruit, humans possess cognitive abilities and life spans that exceed those of rodent or even primate models. Clinical trials in humans are required to explore the effect of dietary interventions with berry fruit on human

cognition during aging. The beneficial effects of berry fruit on the brain have only recently been investigated in humans. While berries are known to affect serum antioxidant status (Kay and Holub, 2002; Mazza et al., 2002; Tulipani et al., 2009; Azzini et al., 2010), only two studies have investigated the effect of berry fruit on cognition. To explore the effect of grape juice on cognition, Krikorian et al. (2010a) recruited 12 nondemented older adults (8 men, 4 women; average age 78.2 years) with early memory decline. Participants then consumed either Concord grape juice (6–9 mL/kg; n = 5) or an isocaloric placebo (n = 7) in addition to their normal diet. Following 3 months of supplementation, participants that consumed the grape juice showed significantly improved acquisition of word lists on the California Verbal Learning Test (CVLT-II; Delis et al., 2000) as well as trends toward improvements in delayed verbal recall and spatial memory, as measured by the spatial paired-associate test (Krikorian, 1996), relative to participants that consumed the placebo beverage.

In a related study (Krikorian et al., 2010b), nine nondemented older adults (5 men, 4 women, average age 76.2 years) with mild cognitive impairment were recruited. Participants consumed either the placebo beverage used in the prior study or blueberry juice (6–9 mL/kg). Following 3 months, participants that consumed the blueberry juice showed significant improvement in word list recall on the CVLT-II and significant cumulative learning scores on the verbal paired-associate learning test (V-Pal), relative to baseline performance. Furthermore, V-PAL performance among participants that consumed the blueberry juice exceeded that of participants that consumed the placebo at study completion. While only preliminary, these studies show that moderate-duration dietary supplementation with berry fruit is capable of altering cognitive performance in humans, perhaps forestalling or reversing the effects of neurodegeneration in aging.

NUTS

A wide variety of nuts are consumed worldwide and between 11% and 33% of Americans consume nuts each day (King et al., 2007; Lin et al., 2001), frequently as a snack. As with berries, the common English usage of the term "nut" defies botanical classifications in that many hardened edible kernels are referred to as "nuts" (Black and Halmer, 2006). Tree nuts such as almonds (*Prunus dulcis*), cashews (*Anacardium occidentale*), macadamias (*Macadamia integrifolia* and *Macadamia tetraphylla*), pecans (*Carya illinoinensis*), pistachios (*Pistacia vera*), and walnuts (*Juglans regia*) are popular in the United States, as are peanuts (*Arachis hypogaea*), which, while actually legumes, are comparable in nutritional content to tree nuts. Nuts are generally good sources of fat, protein (Brufau et al., 2006), and phytosterols (Phillips et al., 2005); furthermore, many nuts have high phenolic content and thus considerable antioxidant capacity. For example, walnuts contain both ellagitannins and tocopherols (Anderson et al., 2001; Li et al., 2007) while pecans, almonds, and pistachios contain flavanoids. Pistachios and peanuts also contain the stilbene resveratrol (Tokusoglu et al., 2005). However, as much as half of the antioxidants are lost if nuts are consumed without the pellicle or "skin" (Blomhoff et al., 2006), and variations due to roasting, processing, and storage are to be expected. While the effect of nut consumption on cardiovascular disease risk has been studied extensively over the last 20 years

(Fraser et al., 1992; Albert et al., 2002; Sabaté and Wien, 2010), only recently have researchers begun to investigate the effect of nut consumption on the brain.

Only one study has examined the effect of nuts on neuronal health *in vitro*. Willis et al. (2010) exposed BV-2 murine microglial cells to LPS. Overnight incubation of cells with LPS more than doubled NO production and increased iNOS expression. However, cells pretreated overnight with a methanolic extract of English walnut (0.01–1.0 mg/mL) displayed significantly decreased NO production and normalized iNOS expression. Additionally, treatment with LPS significantly increased TNF-α release from the microglial cells, while cells pretreated with walnut released significantly lower levels of the cytokine in a dose-dependent manner. The ability of walnut extract to prevent microglial activation and mitigate expression of these inflammatory signaling compounds was further explored. Western blotting revealed that TLR4, receptors sensitive to both LPS and other pathogens, concentration was greater in cytoplasmic fractions than membrane fractions of walnut-treated cells, relative to untreated cells, indicating internalization of the receptor. Furthermore, treatment of microglial cells with n-butanol (0.3%) to inhibit phospholipase D2, an enzyme located in the cell membrane that modulates endocytosis and cytoskeletal reorganization (Colley et al., 1997), which is known to modulate TLR4 internalization (Lentschat et al., 2005), blocked the anti-inflammatory effects of walnut on nitrite production. Therefore, walnut's ability to dampen microglial activation and the subsequent expression of inflammatory intermediates may rely on phospholipase D2-mediated TLR4 internalization, thus preventing its activation.

This study was accompanied by an initial study of the effects of walnuts on cognitive behavior in aged rats (Willis et al., 2009). In that study, 19 month old Fischer 344 rats consumed diets containing 2%, 6%, or 9% ground English walnut (including skins) for 2 months. Animals consuming the 2% walnut diet showed slight improvement in balance while standing atop a narrow rod but failed to show improved behavior over age-matched controls on other tasks. Animals consuming the 6% walnut diet displayed improved balance on a narrow plank and located a hidden platform sooner in the working memory version of the MWM, relative to age-matched controls. However, animals consuming the 9% walnut diet showed impaired performance while balancing on a wide plank and took longer to locate a hidden platform in the MWM during the first of each pair of trials, relative to age-matched controls. While too much walnut seemed detrimental, the most beneficial dose is in line with the current FDA recommendations for nut consumption, 1 oz/day for humans. These studies suggest that walnuts, in addition to berries, may be neuroprotective against the effects of aging. While a number of studies have shown that consuming nuts can affect levels of antioxidants and inflammatory markers in serum (Reiter et al., 2005; Jiang et al., 2006; Sari et al., 2010; Hudthagosol et al., 2011), no studies have yet been conducted on the effects of other nut consumption on cognition.

CONCLUSIONS

In conclusion, berry fruits and nuts are nutrient dense and contain a variety of bioactive phytochemicals, specifically polyphenols. A growing body of literature describes preclinical research, using both *in vitro* and *in vivo* techniques, which show

beneficial effects of nut and berry consumption on the brain in the context of aging. These effects include reductions in oxidative stress and inflammatory mediators, reductions in cellular stress signaling, and improvements in behavioral measures of cognition and mobility in animal models. Furthermore, initial clinical studies demonstrate cognitive enhancement associated with as little as 3 months of regular berry consumption among older adults exhibiting age-related cognitive decline. While dementia is currently incurable, nutritional intervention, particularly during middle years, may be effective in forestalling the neurological changes associated with aging. Given the high individual and societal costs associated with dementia, berries and nuts are "easy pills to swallow."

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11 Brahmi
Traditional Botanical Medicine for Cognitive Decline

Carlo Calabrese and Amala Soumyanath

CONTENTS

INTRODUCTION

The use of complementary and alternative medicine (CAM) has been growing in Western countries. A 2007 survey by the National Center for Complementary and Alternative Medicine (Barnes et al., 2008) showed that nearly 40% of adults and 12% of children in the United States had used some form of CAM. This compared well with an earlier report of CAM use by 42% of the general population in the United States, 20% in the United Kingdom, and 65% in Germany (Ernst, 2000). In the United States, natural products, which largely consist of herbal or botanical products, accounted for about 18% of CAM therapies used (Barnes et al., 2008) and are sold as "dietary supplements." Total sales of dietary supplements in the United

States were estimated at U.S. $ 17 billion in 2002 (Burrill, 2002). Botanical dietary supplements are often used by customers for psychiatric or neurological conditions (Eisenberg et al., 1998; Druss and Rosenheck, 2000; Harnack et al., 2001; Sparber and Wootton, 2002). Ginkgo leaf (*Ginkgo biloba*), which is usually taken to improve cognitive functions, is among the top 10 natural products used in the United States (Barnes et al., 2008). Other popular products for neurological or psychiatric conditions are St John's wort herb (*Hypericum perforatum*) for the treatment of mild to moderate depression, valerian root (*Valeriana officinalis*) for insomnia, and kava root (*Piper methysticum*) to relieve anxiety. Numerous preclinical and human studies have been performed on these herbs, and critical reviews and meta-analyses of clinical studies on these botanicals are readily available in the literature.

Aging is frequently associated with cognitive decline even in the otherwise healthy. The increase in the aged demographic as the post-war "baby boomers" pass into their 60s represents a major emerging public health problem in terms of disability and economic impact. There is no current FDA-approved treatment for cognitive decline or any that significantly slow the progression of dementia. Safe agents that enhance and preserve cognitive function in the elderly will mitigate the social and personal losses associated with cognitive decline in aging.

Two botanical agents that show promise in improving cognitive function are *Bacopa monnieri* and *Centella asiatica*, hereinafter referred to as Bacopa and Centella. Both herbs originate from the ayurvedic system of medicine, where both have been referred to historically and colloquially by the Sanskrit name "Brahmi" (Kapoor, 1990). Authorities correctly distinguish them botanically and, ayurvedically, by specific function. Both Bacopa and Centella are regarded in ayurvedic medicine as "medhya rasayana" herbs, that is, they possess rejuvenating properties (rasayana) and are specific to brain (medhya) function (Singh et al., 2008). Brahmi has been loosely translated as giving knowledge of god or reality. Both Bacopa and Centella are readily available as dietary supplements in the United States. These two herbs have been less well studied clinically than those mentioned earlier in this chapter. Nevertheless, there is a growing body of evidence that supports their likely efficacy in improving cognitive function in humans, and specifically in cognitive decline in aging. The present state of knowledge on these two botanicals in relation to their neurological effects and potential active constituents is reviewed in this chapter.

Bacopa monnieri

The herb *Bacopa monnieri* (L.) Pennell (family Scrophulariaceae) has been used for millennia and is cited in ayurvedic medicine as a memory enhancer, sedative, and anti-epileptic, among other uses (Jain, 1994) and has retained the interest of modern phytomedicine researchers (Kidd, 1999; Russo and Borrelli, 2005). An Australian survey showed it to be one of the most popular supplements for memory among 60–64 year old consumers (Jorm et al., 2004). Folkloric use of Bacopa as a memory and cognitive aid in conjunction with good *in vitro*, animal and clinical trial evidence provide a reasonable possibility that there will be a beneficial effect in cognitive decline. The mechanisms suggest both improvement of present cognitive function and slowing of degeneration and progression to Alzheimer's disease (AD) and other forms of dementia.

Animal Studies

Animal studies have reported cognition-enhancing effects with various extracts of Bacopa showing improved motor learning (Prakash and Sirsi, 1962). A Bacopa alcohol extract improved the acquisition, consolidation, and retention of memory expressed in three acquired behavioral responses in albino rats: brightness discrimination, active conditioned avoidance, and Sidman continuous avoidance responses (Singh and Dhawan, 1997). The memory enhancing effects in animals have been attributed to saponins (bacosides, bacopasides, or bacopasaponins), which have been shown to exert facilitatory effects on mental retention in avoidance response in rats (Singh and Dhawan, 1997) and to reverse amnestic effects of neurotoxin, scopolamine, electroshock, and immobilization stress (Singh and Dhawan, 1997; Bhattacharya et al., 2000).

Double-Blind Randomized Controlled Trials

Nine double-blind randomized controlled trials (RCT) versus placebo in memory enhancement have been done with different but similarly standardized extracts of Bacopa in different populations. Five of these were done in the elderly, though only two in the cognitively impaired. The four in young and middle-aged adults include a double-blind placebo-controlled study in 46 healthy human volunteers 18–60 years old that evaluated performance on a battery of cognitive tests at baseline, after 5 and 12 weeks of 300 mg daily of Bacopa extract (Stough et al., 2001). Of 16 measures, there were statistically significant improvements in measures of the Rey Auditory Verbal Learning Test (AVLT), including learning rate, forgetting rate, and proactive interference as well as in inspection time (a measure of the speed of early information processing), and in state anxiety. A second more recent trial by the same investigators (Stough et al., 2008) in 107 subjects aged 18–60 confirmed these results. In another 3 month study in 76 adults 40–65 years old, results showed a significant effect of Bacopa on the retention of new information in delayed recall of word pairs (Roodenrys et al., 2002), appearing to investigators to be a reduction in information lost from memory. Two unpublished studies with an extract standardized to 50% bacosides (Bacognize®) also suggest benefit. In the first, 600 mg was given to 20 medical students aged 20–30 for 45 days in a cross-over study versus placebo. The extract significantly increased attention span and working memory during the forward and backward digit span tests and immediate recall of logical material in working memory tests.

Among the three trials in normal aged, we attempted to replicate Stough's results (Stough et al., 2001, 2008) in subjects over 65 years of age and reported elsewhere on its positive outcomes in cognition (Calabrese et al., 2008). The second unpublished Bacognize placebo-controlled study was done at the University of Montana in 2007 with 300 mg of the extract per day for 16 weeks in 20 older adults between the ages of 60 and 75. In this very small study, the Bacopa group showed trends of benefit in mood, attention, and working memory with no dropouts due to adverse events (AEs) (Geni Herbs, Noblesville, Indiana). A placebo-controlled trial by Morgan and Stevens (2010) in 103 normal subjects over the age of 55 confirmed the results by Calabrese et al. (2008) in older patients.

Raghav et al. (2006) performed an RCT evaluating age-associated memory impairment (AAMI) after 125 mg twice a day of an extract standardized to 55% bacosides in subjects 55–70 years old, recruited by advertisements. Subjects had complaints of memory loss in everyday activities fitting the category of AAMI. Forty subjects with Logical Memory II subtest scores <6 on the Wechsler Memory Scale were included; those scoring >24 on Mini-Mental State Exam (MMSE) were excluded; 35 subjects completed the study. Bacopa showed improvement from baseline on all eight of the subtests of the Wechsler Memory Scale and significantly so on four of the subtests, as well as on the total memory score at 16 weeks. Placebo showed improvement from baseline in three of the eight subtests, with one being significant.

Barbhaiya et al. (Barbhaiya et al., 2008) conducted a trial of 450 mg Bacopa (BacoMind®) provided daily to 65 subjects 60–75 years old with memory complaints for at least 1 year, but with an MMSE score of 24 or higher. Evaluation of these subjects by the AVLT and Wechsler showed significant interactions by group and time in several measures of delayed recall and attention.

Thus, nine well-designed, though small, studies (total n = 534) are in agreement on a cognitive benefit of Bacopa, and all with a primary measure in memory when the extract is given for 12–16 weeks. The most frequent finding is in measures of delayed recall, with memory enhancement appearing reliably, even in very small samples. All RCTs where Bacopa extract was given for more than 5 weeks showed some cognitive benefit.

In a single-dose study of Bacopa extract with neuropsychological testing performed 2 h after administration, no differences were found between groups (Nathan et al., 2001). A study by the same investigators with 4 weeks treatment by a combination of *Gingko biloba* 120 mg and Bacopa 300 mg in 85 healthy subjects also did not show cognitive enhancement (Nathan et al., 2004). Stough's study showed effects at 12 weeks but not at 5 weeks (Stough et al., 2001, 2008). Taken together, these studies suggest that cognitive effects with Bacopa may take months to appear.

There are several somewhat differently standardized commercial extracts of Bacopa including Bacopin® (Sabinsa); BacoMind, Baccdrix®, and Bacognize (Verdure, Geni Herbs); Bacopa55® (Laila Impex); and numerous purveyors of Bacopa. One manufactured combination (Mentat, Mind-Care or BR-16A) of 13 herbs of which Bacopa is the largest constituent has shown promise, but a combination of Bacopa with *Ginkgo biloba* (Membac, Memory Plus) has had mixed results (Nathan et al., 2004). Most extracts have manufacturer recommended doses of 300 mg/day while a few recommend up to 600 mg/day of concentrated extracts.

Bacopa: Multiple Mechanisms of Action

Several possible mechanisms may be operative in the time horizon of weeks to months. Proposed actions for Bacopa or its constituents have included cholinergic upregulation, modulation of gamma-aminobutyric acid (GABA), and possibly, protein synthesis in the hippocampus. Evidence shows that Bacopa affects the

cholinergic system including the modulation of (1) acetylcholine release (Agrawal, 1993; Bhattacharya et al., 2000), (2) inhibition of acetylcholinesterase (AChE), and (3) muscarinic cholinergic receptor binding (Bhattacharya et al., 2000). Stough showed a significant difference in inspection time after Bacopa treatment (Stough et al., 2001, 2008), a measure of early information processing mediated by the cholinergic system. In a 1966 experiment of Bacopa on selected cerebral neurotransmitter levels in mice (Dey and Datta, 1966), the extract profoundly increased the GABA level shortly after intraperitoneal administration. In this study, the GABA level gradually fell with a concomitant rise in glutamine level, and glutamine remained high over time. The authors concluded that Bacopa accelerates GABA synthesis from glutamate and/or inhibits GABA metabolism. Bacopa decreases NMDA R1 gene expression (Khan et al., 2008; Paulose et al., 2008), which may parallel the NMDA blocking mechanism of memantine, the only approved non-cholinergic AD drug. Studies that identified bacosides A and B as responsible for the facilitatory effect of Bacopa on learning also found them to be associated with more protein kinase activity and increased protein content in the hippocampus (Barbhaiya et al., 2008) suggesting increased metabolism.

In addition to the possibly shorter-acting mechanisms discussed earlier, longer-acting effects have been attributed to Bacopa, which may reduce progression of cognitive decline and conversion to AD. Oxidative damage plays an established role in AD (Beal 1995; Markesbery 1999; Christen 2000; Pratico et al., 2001). There is growing evidence that lipid oxidation precedes the appearance of amyloid plaques or neurofibrillary tangles and that oxidized lipid membranes can contribute to the formation of amyloid plaques (Cooper, 2003). Epidemiological studies on antioxidant intake yield mixed results but suggest an association between antioxidant intake and reduced risk of AD (Commenges et al., 2000; Engelhart et al., 2002; Morris et al., 2002). Treatment with antioxidants is an undemonstrated approach for slowing progression, however. One study reports that vitamin E supplementation in people with AD and moderately severe cognitive impairment delays the worsening of AD-related factors (Sano et al., 1997), but another showed no benefit for treatment in mild cognitive impairment (MCI) (Burrill, 2002). Bacopa contains the antioxidant flavonoids, luteolin and apigenin, and has shown potent antioxidant effects *in vitro* (Tripathi et al., 1996). Increases in the levels of the antioxidants superoxide dismutase, catalase, and glutathione peroxidase in the prefrontal cortex, striatum, and hippocampus followed chronic Bacopa administration in rats (Bhattacharya et al., 2000). The free radical scavenging capacity of Bacopa extract and its effect on DNA cleavage induced by hydrogen peroxide UV-photolysis showed a dose-dependent free radical scavenging and a protective effect on DNA cleavage in human fibroblasts (Russo et al., 2003). Bacopa has also demonstrated anticlastogenic activity in human lymphocytes, which was attributed to antioxidant properties (Deb et al., 2008). Because measures of lipid peroxidation have been correlated with measures of cognitive and functional impairment (Pratico et al., 2000), therapies that normalize lipid membrane composition and decrease oxidation could have the potential to either prevent or treat AD. A manifestation of oxidative stress on lipids is reflected in the increased concentrations of F2-isoprostanes in areas of the brain most affected by AD pathology

(Pratico et al., 1998). Concentration of F2-isoprostanes is an accepted approach to determining levels of oxidative damage *in vivo* (Morrow et al., 1992). The production of F2-isoprostanes is highly correlated with the antioxidant status of the body and responds to antioxidant approaches (Morrow et al., 1992; Richelle et al., 1999).

Inflammation has been implicated in the pathogenesis of AD and vascular dementia (Halliday et al., 2000). Oral doses (1–10 g/kg) of Bacopa suppressed experimentally induced inflammation in rodents comparably to indomethecin without gastric irritation (Jain et al., 1994). Bacopa extract selectively inhibited prostaglandin E(2)-induced inflammation in mice (Channa et al., 2006) and was shown to inhibit lipoxygenase and cyclooxygenase-2 enzymes *in vivo* and TNFα ex vivo in rats (Viji and Helen, 2008).

Bacopa shows increasing evidence of neuroprotection against a wide array of toxicities with a number of *in vivo* studies continuing to be published. It protected against phenytoin-induced cognitive deficit in epileptic patients (Vohora et al., 2000), nicotine-induced toxicity in mice (Vijayan and Helen, 2007), and aluminum salt neurotoxicity in rats (Jyoti et al., 2007). In mice, it exerted an antiamnesic effect in amnesia induced by diazepam (Prabhakar et al., 2008), scopolamine (Saraf et al., 2009; Zhou et al., 2009), and sodium nitrite (Kishore and Singh, 2005). Bacopa also ameliorated memory impairment after hypoxia (Hota et al., 2009) and protected against rotenone-induced oxidative stress and neurotoxicity(Hosamani and Muralidhara, 2009).

Amyloid-mediated neurotoxicity is an important mechanism in Alzheimer's pathogenesis. Bacopa shows a protective effect against beta-amyloid-induced cell death in primary cortical culture (Limpeanchob et al., 2008) and appears to affect the concentration of beta-amyloid in the brain. Beta-amyloid levels in the doubly transgenic presenillin amyloid precursor protein (PSAPP) AD mouse model were measured in the cortex and hippocampus by enzyme-linked immunosorbent assay (ELISA) and expressed as ng/mg of protein. Bacopa extract at 40 and 160 mg/kg dose-dependently decreased the levels of beta-amyloid 1–40 by 46% and 58% ($p \leq 0.001$) and beta-amyloid 1–42 by 48% and 62%, respectively ($p \leq 0.001$). In the cortex, Bacopa extract 40 and 160 mg/kg dose-dependently decreased the levels of beta-amyloid 1–40 by 35% and 44% ($p \leq 0.001$) and beta-amyloid 1–42 levels were reduced by 55% and 44% ($p \leq 0.001$), respectively (Dhanasekaran et al., 2004; Holcomb et al., 2006). The same team also showed that Bacopa contains polyphenols, which have been shown to disrupt amyloid aggregation (Dhanasekaran et al., 2007). A Thai group has shown Bacopa to provide protection from AF64A, a cholinergic neuron toxin, in rats (Uabundit et al., 2010).

Amyloid precursor and soluble amyloid beta proteins have been shown to localize to neuronal mitochondria, inducing free radical generation and beta-amyloid production and free radical cycling (Manczak et al., 2006; Reddy, 2006). This process leads to neuronal damage and degeneration. Rat brain mitochondria were protected from the effects of exposure to cigarette smoke (Anbarasi et al., 2005) and from nitric oxide–related toxicity (Russo et al., 2003) by Bacopa.

A central mechanism of Bacopa's effects remains unclear but the earlier discussed mechanisms may each independently, and in combination, contribute to the benefit seen after Bacopa administration. Where effects have been localized, they have been found in the prefrontal cortex, striatum, and the hippocampus.

Bacopa: Safety and Dosing

Data at present show Bacopa to be quite safe. A water extract of Bacopa given orally up to a dose of 5 g/kg did not show toxicity in rats and the LD_{50} of an oral alcoholic extract was 17 g/kg (Martis and Rao, 1992). A concentrated extract of Bacopa showed no evidence of oral toxicity in rats at up to 500 mg/kg for 90 days in clinical signs or on necropsy (Joshua Allan et al., 2007). Bacopa extract effect on plasmid DNA fragmentation tests *in vitro* showed no differences compared to vehicle (Dhanasekaran et al., 2007). Phase I clinical studies confirmed the safety of the pure bacosides in healthy male volunteers at single (20–300 mg) and multiple (100 and 200 mg) doses administered for 4 weeks (Singh et al., 1988; Asthana et al., 1996). Since the clinical extracts being considered here are standardized to about 55% bacosides, these would be the equivalents of twice the dose tested, that is, 40–600 mg of extract in a single dose and 200–400 mg for 4 weeks. A phase I study in 23 participants given 300 mg for 15 days and 450 mg for the next 15 days showed no untoward clinical, hematologic, biochemical, or electrocardiographic effects and any mild gastrointestinal effects subsided spontaneously (Pravina et al., 2007). Stough et al. found total AEs were similar in Bacopa and placebo groups although thirst, nausea, and muscle fatigue were more frequent with Bacopa at 12 weeks of a dose of 300 mg (Stough et al., 2001, 2008). Our studies, however, did not see an excess of these AE of the same length and dose in an older cohort, and we saw no attributable AE with a dose of 300 mg/day for 12 weeks (Calabrese et al., 2008). There were very few withdrawals due to AE in the other controlled trials reviewed earlier in this chapter; at 250–300 mg (six studies, n = 365) no dropouts, 450 mg (one study, n = 65) no AE reported, and at 600 mg (one study, n = 20) no dropouts. Nevertheless, Morgan (Morgan and Stevens, 2010) found excess mild gastrointestinal symptoms at 300 mg and, in a small unpublished study at 600 mg, we found gastrointestinal symptoms that were controllable simply by changing administration behaviors.

There are as yet no published pharmacokinetic (PK) data on Bacopa. The peak of the dose–absorption curve is unknown, although it appears that the onset of detectable activity is at more than 5 weeks with effects apparent at 12 weeks, as was noted in all the trials cited earlier.

Bacopa: Phytochemistry

Bacopa's many names, wide geographic distribution, and variety of possible extractions make phytochemical standardization essential to potential clinical application. Chemical characterization of Bacopa has been done by many researchers (Rastogi et al., 1994; Ahmed and Rahman, 2000; Mahato et al., 2000; Chakravarty et al., 2001, 2002; Hou et al., 2002; Deepak and Amit, 2004; Deepak et al., 2005; Sivaramakrishna et al., 2005; Bhandari et al., 2006, 2007; Pawar et al., 2007; Zhou et al., 2007). The majority of studies on *Bacopa monnieri* have been carried out on extracts made with methanol or different proportions of aqueous ethanol (Deepak et al., 2005). The major chemical constituents of such extracts of *Bacopa monnieri* are triterpenoid saponins based on dammarane aglycones. These saponins have been

variously named bacopasides, bacopasaponins, or bacosides, and differ in their aglycone, either jujubogenin or its stereoisomer pseudojujubogenin, and attached sugar chains (various combinations of glucose, sulfonyl-glucose, and arabinose in either pyranose or furanose form).

The various saponins isolated in pure form from *Bacopa monnieri* are listed in Table 11.1. Two different compounds have been assigned the name Bacopasaponin G by different researchers. Non-saponin glycosides found in alcohol extracts include monnierasides I to III (Chakravarty et al., 2002), plantainoside B (Chakravarty et al., 2002), and bacopasides A, B, and C (Hou et al., 2002), which are phenylethanoid glycosides based on hydroxybenzoic, caffeic, or ferulic acid. Glycosides of the flavones luteolin and apigenin are reported in Bacopa stems (Proliac et al., 1991) and whole plant (Deepak and

TABLE 11.1
Bacopa Saponins

Saponin	Aglycone	Reference
Bacopaside I	PS	Chakravarty et al. (2001)
Bacopaside II	PS	Chakravarty et al. (2001)
Bacopaside III	J	Chakravarty (2003)
Bacopaside IV	J	Chakravarty (2003)
Bacopaside V	PS	Chakravarty (2003)
Bacopaside VI	PS	Zhou (2006)
Bacopaside VII	J	Zhou (2006)
Bacopaside VIII	J	Zhou (2006)
Bacopaside N1	J	Sivaramakrishna et al. (2005)
Bacopaside N2	PS	
Bacopasaponin A	J	Garai (1996a)
Bacopasaponin B	PS	Garai (1996a)
Bacopasaponin C	PS	Garai (1996a)
Bacopasaponin D	PS	Garai (1996b)
Bacopasaponin E	J	Mahato et al. (2000)
Bacopasaponin F	J	Mahato et al. (2000)
Bacopasaponin G	J	Hou et al. (2002)
Bacopasaponin G	20-deoxy-PS	Pawar et al. (2007)
Bacoside A and B. A and B differ in sugar chains; basic building blocks (aglycones, sugars) are the same		Deepak and Amit (2004)
Bacoside A1	J	Jain et al. (1993)
Bacoside A2	PS	Rastogi and Kulshreshtha (1999)
Bacoside A3	J	Rastogi and Kulshreshtha (1994)
Bacoside A6	20-deoxy-J	Pawar et al. (2007)
Un-named	J	Sivaramakrishna et al. (2005)

J: jujubogenin, PS: pseudojujubogenin

Amit, 2004), while a sulfonyl glucoside of octanol has also been found (Proliac et al., 1991) in the roots. Other glycosidic compounds include bacosterol glycoside (Ahmed and Rahman, 2000) and four cucurbitacins (Chakravarty et al., 2001), which were reported in the aerial parts. Non-glycosidic constituents include the steroid bacosterol 1 and the lupane triterpenoid bacosine 2 (Ahmed and Rahman, 2000).

Bacopa products on the market and those used in clinical study reports vary widely. Many state standardization to a specific content of "Bacoside A" or "Bacoside A and B." However, as Deepak and Amit (Deepak et al., 2005) point out in their review, bacosides A and B are not single compounds. Ganzera et al. (2004) analyzed several samples of plant material, extracts, and commercial samples. All were found to have at least four of the seven reference saponins used, with bacoside A3 and bacopaside II as main components and lower levels of bacopasides IV and V. The total saponin content varied from 1.1% to 13%. These differences can arise due to different extraction methods and geographical sources of Bacopa. Deepak et al. (Deepak and Amit, 2004) extracted samples of plant material obtained from 14 different regions of India and found variations in total saponins (0.5%–1.8%) and in the relative proportions of the four bacoside A compounds they identified.

The foregoing demonstrates the vital importance of characterizing, quantifying, and documenting the constituents of products used in clinical studies. Total saponins can be quantified by acid hydrolysis to yield a breakdown product of the aglycones, known as ebelin lactone. Due to its conjugated double bond system, this can be measured spectrophotometrically at 278 nm (Pal and Sarin, 1992; *Indian Herbal Pharmacopoeia*, 1998). Individual saponins can be detected and quantified using high-performance liquid chromatography (HPLC). Suitable HPLC and detection methods have been described by Ganzera et al. (2004) and Deepak and Amit (2004). The saponins, which are poor chromophores, can be detected at a low wavelength of 205 nm (liquid chromatography–ultraviolet [LC-UV]). For higher sensitivity, mass spectral detection can be used (liquid chromatography–mass spectrography [LC-MS]). The advantage of the latter is that the mass spectrum of each peak can be obtained, and this will furnish information on the molecular weight of the substance if total fragmentation has not taken place. Masses of the fragments and of fragments of fragments (using LC-MS-MS) can give information on the sugar and aglycone moieties. Figure 11.1 illustrates the structure of Ebelin lactone, which is a resultant fragment after acid hydrolysis of bacoside A.

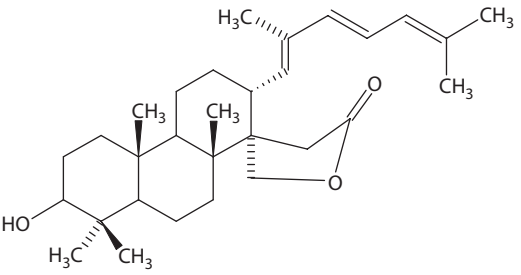


FIGURE 11.1 Ebelin lactone from Bacoside A after acid hydrolysis.

There are currently no published data on the bioavailability, pharmacokinetics, or metabolism of Bacopa's components.

Bacopa: Significance

Bacopa is a paradigmatic herbal medicine with a long history of documented safe use for the indication of cognitive decline. It appears to act via multiple constituents on multiple mechanisms, some perhaps improving cognitive function and some neuroprotective. If a sustainable Bacopa product is shown to have a magnitude of effect similar to current therapy in MCI as our data suggest, it will expand treatment options. If, in addition, it has both a better safety profile than current drugs and enhances cognition and slows decline, it could prove a rare advance. While the effect may not be large, Bacopa appears to produce reliable cognitive improvement, especially in memory, and to be safe at least in the short-term and with long-term use in India. It shows effects in heterogenous populations and it is promising as a safe and inexpensive treatment that can effectively and efficiently increase the area under the curve of long-term cognitive capacity. Other botanical agents may show a similar variety of potential mechanisms as well as traditional use, though often with less clinical evidence of effect. Thus, Bacopa is a low-hanging fruit among botanical candidates for the treatment of cognitive impairment.

Centella asiatica

Centella asiatica (L.) Urban, family Apiaceae, is highly regarded in ayurvedic medicine as a rasayana or rejuvenating herb (Kapoor, 1990). It is known in ayurveda as Mandukaparni, and sometimes as Brahmi, and is reputed to increase intelligence and memory (Kapoor, 1990). Traditionally, it is used to treat a variety of ailments including neurological conditions such as epilepsy and insanity (Kapoor, 1990). The herb has enjoyed increasing popularity in the United States and other Western countries, where the dried aerial parts are sold as the dietary supplement "gotu kola" (Newall et al., 1996). In the West, it is often used for venous insufficiency and wound healing (Brinkhaus et al., 1998). Centella is also promoted alone, or in combination with herbs such as *Ginkgo biloba*, as a general "nerve tonic" and memory booster. Centella has been extensively researched for many of the aforementioned conditions (Brinkhaus et al., 1998). This section will focus specifically on studies relating to its neurological effects.

Centella asiatica: Animal Studies

Animal studies have shown Centella to improve cognitive function in a number of rodent models. For example, aqueous, methanolic, and chloroform extracts of Centella were studied in rats at a dose of 200 mg/kg/day for 14 days (Veerendra Kumar and Gupta, 2003). Here, the aqueous extract improved learning and memory of rats in shuttle box, step-through paradigm, and elevated plus maze. These findings prompted the further study of Centella in a rat model of CNS

toxicity (intra-cerebroventricular streptozotocin). Aqueous extracts were dosed at 100–300 mg/kg/day for 21 days after the streptozotocin insult, and learning and memory were assessed with passive avoidance and elevated plus maze tests. The extracts showed a dose-dependent enhancement of performance in both paradigms (Veerendra Kumar and Gupta, 2002). Centella aqueous extract (100 and 300 mg/kg) also improved performance deficits in the passive avoidance test in pentylenetetrazole (PTZ) kindled rats (Gupta et al., 2003). Administering Centella aqueous extract to neonatal mice from day 15 to 30 postpartum (Rao et al., 2005) resulted in significant enhancement in learning efficiency (hole poking test) and spatial memory (radial arm test), with no differences seen from control in the open field test or dark/bright area test for locomotor function. Centella aqueous extract also improved learning and behavioral abnormalities in the Tg 2576 mouse model of AD (Soumyanath et al., 2012). Thus, studies in diverse *in vivo* settings have shown that Centella water extract has biological effects of relevance to memory and learning. A significant feature of these studies is that they all employed an oral route of administration, suggesting relevance to human consumption of Centella products as dietary supplements for the purpose of enhancing memory.

Apart from effects on cognitive function, Centella shows several other activities supportive of an effect on the central nervous system. An anti-depressant effect of a triterpene extract has been reported in mice (Chen et al., 2003) using the forced swimming test. Anxiolytic effects of Centella methanol and ethyl acetate extracts, and asiaticoside, were observed in rats using the elevated plus maze test paradigm (Wijeweera et al., 2006). Depression is a frequent concomitant and contributant to cognitive decline.

Centella asiatica: Human Studies of Cognition

Human studies of cognition after Centella administration have been few despite the wealth of animal and *in vitro* data. In one study, 30 mentally retarded children aged 7–18 years showed improvement in their general abilities after receiving 500 mg daily of dried herb for 3 months (Appa Rao et al., 1973). In a single group study, dried Centella leaves at 1000 mg/day for 6 months in 60 subjects aged 65 or older with MCI was associated with an improvement in the MMSE (Tiwari et al., 2008). A placebo-controlled study in 41 healthy men and women 35–50 years old showed enhancement of memory and other cognitive measures with 3 months of dried herb (50 mg/kg) (Dev et al., 2001). A randomized placebo-controlled trial by Wattanathorn et al. (2008) showed that an extract of Centella (250–750 mg daily for 2 months) improved cognitive performance and mood in healthy, elderly volunteers.

Centella asiatica: Multiple Mechanisms of Action

The effects of Centella extracts and their triterpene constituents have been studied in a number of *in vitro* models using primary or immortalized neuronal cells. Centella ethanol extract, but not water extract, increased neurite elongation in human SH-SY-5Y neuroblastoma cells *in vitro* (Soumyanath et al., 2005). Asiatic acid was found to contribute to some, but not all of the neurite elongatory activity of the extract.

Asiatic acid and/or asiaticoside displayed neuroprotective effects in cell culture models of glutamate-induced (Lee et al., 2000), and beta amyloid–induced (Mook-Jung et al., 1999; Jew et al., 2000), neurotoxicity. A Centella extract, as well as the triterpenes asiatic acid and madecassic acid were all found to enhance phosphorylation of cyclic AMP response element binding protein (CREB) in the mutant N2a neuroblastoma cell line expressing beta amyloid (1–42), as well as in rat embryonic primary cortical cell culture (Xu et al., 2008). The authors postulate that CREB phosphorylation may be central to the neurological activity of Centella, since p-CREB is reported to influence genes involved in memory formation, and also affect neural dendrite formation. Centella water extract also protected neuronal cell lines from toxicity due to exogenously added, or intracellularly generated, beta amyloid, possibly by inhibiting beta amyloid aggregation (Soumyanath et al., 2012).

In other *in vitro* tests, Centella extracts have shown anticholinesterase activity (Mukherjee et al., 2007), which would increase acetylcholine levels, and stimulatory effects on glutamic acid decarboxylase (Awad et al., 2007), an enzyme involved in the production of GABA. These activities suggest potential neurotransmission enhancing effects of Centella extracts, which may contribute to the improvements in memory observed *in vivo* in animals and humans.

Other studies on the possible mechanisms of Centella's effects include several demonstrations of anti-oxidant activity in the brain. Centella water extract (100–300 mg/kg/day for 2–3 weeks) reduced levels of malondialdehyde and increased levels of glutathione and catalase in rat brains (Veerendra Kumar and Gupta, 2002, 2003). Centella water extract (300 mg/kg/day for 60 days) was found to ameliorate age-related changes in lipid peroxidation, protein carbonyls, and anti-oxidant status in various rat brain regions (Subathra et al., 2005). Centella water extract (5 mg/kg/day for 10 days) protected various mouse brain mitochondria from oxidative damage induced by 3-nitropropionic acid (Shinomol and Muralidhara, 2008a,b). A chloroform:methanol extract caused improvements in antioxidant status (catalase, superoxide dismutase) and lipid peroxide levels in the hippocampal and striatal regions of rats treated with monosodium glutamate, a stimulant of neurodegeneration (Ramanathan et al., 2007).

In PSAPP mice expressing amyloid precursor protein and presenilin 1 mutations, long-term (8 months) administration of Centella not only displayed *in vitro* antioxidant effects but also reduced beta amyloid plaque burden *in vivo* (Dhanasekaran et al., 2009), suggesting additional effects on the amyloid cascade. These data are particularly relevant for their potential use in the treatment of Alzheimer's disease. Another reported effect of possible relevance to Centella's neurological effects is a reduction in cerebellar phospholipase activity in rats receiving Centella water extract containing asiaticoside (Barbosa et al., 2008).

Direct neurotropic effects of Centella extracts have also been reported. Oral administration of aqueous extract resulted in significant increases in dendritic arborization of apical and basal dendrites in the hippocampal neurons of neonatal mice (Rao et al., 2005) and both adult (Gadahad et al., 2008) and neonatal (Rao et al., 2006) rats. This may also help explain the effects of Centella on memory and learning.

As with Bacopa, Centella shows a number of effects *in vitro*, which may contribute to the overall effects seen *in vivo* and in humans. It is possible that the multiple effects are associated with different constituents of the herb, not all of which have been characterized.

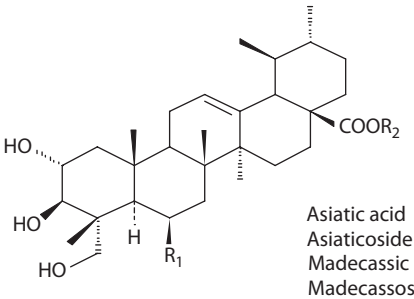
Centella asiatica: Safety and Dosing

Centella is an edible plant and the 1997 edition of *Botanical Safety Handbook* (McGuffin et al., 1997) classifies it as a Class 1 herb, that is, one that can safely be consumed when used appropriately. The recommended herbal dose of Centella is 0.5 and 1.5 g dried leaf daily (Kapoor 1990; Newall et al., 1996). No adverse effects were reported when a single 12 g dose of the dried herb was administered to healthy adults (Bradwejn et al., 2000), when 35 g leaves were taken daily for 4 days as part of a meal by adults (Liyanage et al., 1996), or in the four human studies on Centella reported earlier in this chapter. Despite widespread use of Centella, there is only one report of hepatotoxicity in three individuals taking botanical supplements containing Centella (Jorge and Jorge, 2005). Unfortunately, neither the original publication nor correspondence with the lead author shed any light on the nature of these extracts.

Centella asiatica: Phytochemistry

Centella contains a wide range of secondary metabolites (Newall et al., 1996; Brinkhaus et al., 1998). Of these, the compounds that have received most attention are triterpenoid saponins and their aglycones (Brinkhaus et al., 1998). The most important compounds in this group are madecassoside, asiaticoside, madecassic acid, and asiatic acid (Figure 11.2). Asiaticoside and asiatic acid, in particular, have been examined extensively for their bioactivity and bioavailability. Asiaticoside is metabolized to asiatic acid *in vivo* (*Botanical Safety Handbook*, 2006), and the bioavailability of asiatic acid has been demonstrated in human subjects administered either asiatic acid or asiaticoside orally (Grimaldi et al., 1990; Rush et al., 1993). Related compounds found in Centella include the madecassic acid isomer, terminolic acid, asiaticoside B, centellasaponins A, B, C, and D and the aglycone centellasapogenol A (Matsuda et al., 2001a,b), an unnamed novel triterpene, and a saponin based on ursolic acid (Yu et al., 2006, 2007).

Phenolic constituents in Centella include the flavonoids kaempferol and quercetin and their 3-O-glucosides (Satake et al., 2007). Two novel flavonoids, castilliferol 1 and castillicetin 2, which are esters of quercetin and kaempferol, have recently been isolated (Subban et al., 2008). Several compounds based on chlorogenic (Satake et al., 2007; Subban et al., 2008) or quinic acid have



	R_1	R_2
Asiatic acid	H	H
Asiaticoside	H	rhamnosyl-glucosyl-glucosyl-
Madecassic acid	OH	H
Madecassoside	OH	rhamnosyl-glucosyl-glucosyl-

FIGURE 11.2 Main triterpenoid compounds found in *Centella asiatica*.

been isolated from Centella (Satake et al., 2007) and are reported to have antithrombotic properties. A benzoic acid derivative, asiaticin, has also been found in Centella (Siddiqui et al., 2007). Long chain constituents reported in Centella include an octadecanyl hydroxypyrone (Srivastava and Shukla, 1997), the polyacetylene compound, cadiyenol (Govindan et al., 2007), and two novel acetylenic compounds, centellin and centellicin (Matsuda et al., 2001). The role of non-triterpene compounds in the biological activity of Centella has not been studied to any significant extent.

Centella asiatica: Significance

There is clear evidence from multiple models that Centella has a positive effect on learning and memory. Based on *in vitro* and *in vivo* studies, possible mechanisms by which it modulates these effects include neuroprotective effects, neurotropic effects mediated by CREB phosphorylation, antioxidant properties, and/or a reduction in brain beta amyloid levels or associated toxicity. It is also possible that it enhances the levels of the neurotransmitters acetylcholine and GABA. Such a wide range of biological activities is not unusual for botanical products. Botanicals typically contain a diverse range of chemical constituents that potentially treat a particular disease by influencing a range of mechanisms relevant to the condition. For example, while the triterpene components of Centella have been associated with neuroprotective and neurotropic effects, the phenolic constituents may contribute to its antioxidant properties. Surprisingly, there have been few human studies examining its cognitive effects in humans, but the studies that have been performed had positive outcomes. *Centella asiatica* is a botanical with significant potential in the treatment of cognitive impairment in humans, and future clinical studies in this area are clearly warranted.

Brahmi: CONCLUSIONS

Of ethnographic significance is that these two ayurvedic herbs, Bacopa and Centella with their overlapping ayurvedic indications in cognitive decline, were confused in nomenclature with each other in a name, Brahmi, which suggests clarity of mind. Current *in vitro*, animal and human studies are revealing that they have similar cognition-relevant mechanisms and similar cognitive outcomes. The parallels between these two plants in ancient use and in modern pharmacological assessment lend a little reassurance to the congruity of human and medical reality across the millennia.

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12 Bioavailability of Dietary Carotenoids in Humans

A Review of Results from Studies with Momordica cochinchinensis Spreng (Redmelon™) and of ^{14}C-Tracer Studies Using Accelerator Mass Spectrometry

Le Thuy Vuong

CONTENTS

INTRODUCTION

Carotenoids are ubiquitous in nature. There are more than 600 known carotenoids, and they are recognized by colors such as green in leaves and algae, orange in carrots, and red in hibiscus. Carotenoid pigments are also widespread among diverse animal species. Plants, and other organisms such as alga, bacteria, and fungus, can synthesize carotenoids while animals and humans obtain carotenoids from diets (DellaPenna and Pogson 2006). The key role of carotenoids in plants is in photosynthesis and in protecting the chlorophyll from photodamage (Von Lintig 2010).

There are two classes of carotenoids: oxygen-containing xanthophylls and carotenes containing only hydrocarbon. Carotenoids are lipophilic and destroyed if exposed to oxygen, light, and high heat, more so when taken from the original matrix. The roles of carotenoids in humans (and presumably animals) are well studied and documented (Cooper 2004). About 60 carotenoid pigments in nature have vitamin A activity, meaning they can be cleaved enzymatically in the body to yield vitamin A. Those carotenoids are named pro-vitamin A carotenoids. In theory, one molecule of β-carotene can be cleaved enzymatically to produce two molecules of retinol (Figure 12.1). However, the efficiency of the conversion depends on many factors, which will be discussed in the next section.

The most common carotenoids in diets are α-carotene, β-carotene, lutein, zeaxanthin, and lycopene, with the main sources being green leafy vegetables, carrots, mangos, yam, watermelons, tomatoes, and spinach. There are probably five or six pro-vitamin A carotenoids found in foods. They contribute to normal growth in children and to the prevention of night blindness and xerophthalmia, and are protective toward macula lutea degeneration (Olsen 1999). Several carotenoids show enhancement of the immune response; inhibition of mutagenesis; reduction of induced nuclear damage; and protection from various neoplastic events in cells, tissues, and whole animals (Olson 1999). Carotenoids also protect against photo-induced tissue damage (Offord et al. 2002; Stahl et al. 1989, 2006; Stahl and Sies 2012). They exhibit specific antioxidant activity and can influence signaling and gene expression at the cellular level (Sangeetha and Baskaran 2010; Rock et al. 1991, 1997). Some carotenoids,

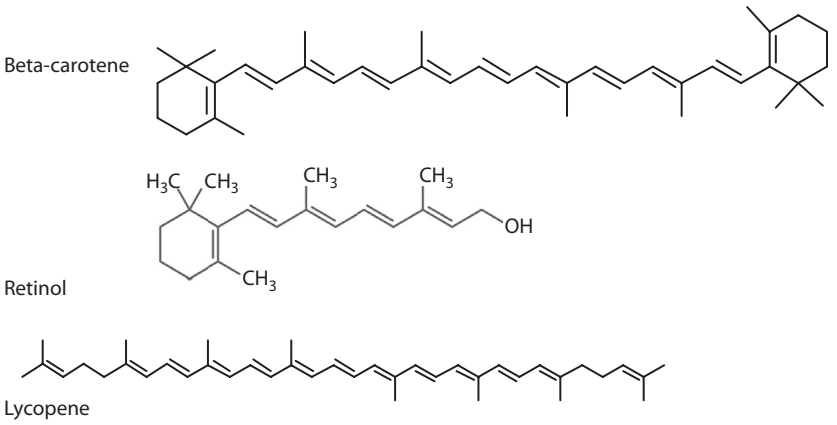

FIGURE 12.1 Chemical structures of β-carotene, retinol, and lycopene.

including β-carotene, quench highly reactive singlet oxygen under certain conditions and can block free radical-mediated reactions (Olson 1999). β-Carotene and lycopene (Figure 12.1) are among the most prominent members of this group of dietary antioxidants, perhaps due to their chemical structure and their abundance in nature and thus in the human diet. β-carotene and lycopene are also the dominating carotenoids in human blood and tissues. Beta-carotene has been found to comprise 15%–30% of total serum carotenoids. In epidemiological studies, the intake of carotenoid-rich fruits and vegetables has been correlated with protection from some forms of cancer, particularly lung cancer (Bendich and Olson 1989). Similarly lycopene has been found beneficial to the prevention of prostate cancer (Beilby et al. 2010; Llic et al. 2001). Due to their roles in human diet and health, β-carotene and lycopene are the carotenoids of interest, and selected results of studies will be discussed.

CAROTENOID BIOAVAILABILITY

Bioavailability of nutrient compounds is defined as the fraction of ingested amount that is available to the body. Bioavailability of dietary β-carotene is influenced by a multitude of factors:

- The species of carotenoids in the food impacts absorption. All *trans*-β-carotene is highly absorbed. However, food processing, especially heating, can increase the isomerization of β-carotene to the *cis*-isomer, which is less absorbable (Jensen et al. 1987).
- The composition of the food matrix affects the freeing of the β-carotene molecule, which makes it available for absorption. The simpler the matrix, the more effective the absorption (De Pee et al. 1995).
- β-carotene in fruits is located in the chromoplasts and is expected to be better absorbed than β-carotene located in the chloroplasts of green leafy vegetables (De Pee et al. 1998). It has been shown that β-carotene in carrots has low bioavailability (Bulux et al. 1994).
- The method of preparation is also important. β-carotene is destroyed by light and heat. Cooking on low heat and under cover was shown to minimize the loss of β-carotene (Rahman et al. 1990). Dincer and workers reported that β-carotene contents in baked and boiled sweet potatoes were lower than those in fresh potatoes, while antioxidant activity increased with treatments (Dincer et al. 2011).
- The particle size of foods is another factor, as homogenization of foods increases carotene absorption (Dimitrov et al. 1988; Bulux et al. 1994; Van Vliet 1996).
- Absorption modifiers such as fat or oil increase the bioavailability of β-carotene while other competitive carotenoids or fiber seems to have a negative effect on its bioavailability (Roels et al. 1958; Jialal et al. 1991; Rock and Swendseid 1991).
- The nutritional status of the host impacts bioavailability. Absorption of β-carotene is impaired in the conditions of fat malabsorption and protein-energy malnutrition (Combs 1992; Novotny et al. 1995; Slattery et al. 1995; Hebuterne et al. 1996; Castenmiller and West 1998; Wang et al. 1998).

- Bioconversion of β-carotene to retinal is dose dependent and ranges between 27% and 2% for a 6 and 126 mg dose, respectively (Schwedhelm et al. 2003).
- The uptake of lipophilic carotenoids into intestinal mucosal cells is aided by the formation of bile acid micelles. Because bile production is stimulated by dietary fat, genetic factors affecting fat absorption (such as metabolic disorders) can influence the bioavailability of carotenoids (McLaren and Zekian, 1971; Combs, 1992; Novotny et al. 1995; Slattery and Jacobs 1995; Hebuterne et al. 1996; Rock et al. 1997; Castenmiller and West 1998; Wang et al. 1998).
- Intakes of other nutrients, such as vitamin A, alpha-tocopherol (vitamin E), or ascorbic acid (vitamin C), also affect bioavailability (Zhang and Omaye 2000; Lemke et al. 2003; Yeum et al. 2000, 2009).
- The uptake of fat-soluble carotenoids is stimulated by dietary fat, as consuming carotenoids in a high-fat meal increases the efficiency of absorption (Stahl and Sies 1992).
- Intakes of certain fibers, fat substitutes, plant sterols, and cholesterol-lowering drugs have been shown to interfere with the incorporation of lycopene into micelles and can potentially decrease the efficiency by which this carotenoid is absorbed (Boileau et al. 2002).
- Bioavailability of lycopene is different than that of β-carotene. Lycopene is not metabolized to vitamin A, but oxidative metabolites of lycopene have been found in human serum. In human and animal tissues, lycopene exists mainly as *cis*-isomers; however, lycopene is found in most food sources primarily as the all-*trans*-isomer (Figure 12.1). The release of lycopene from the matrix is one important factor affecting lycopene bioavailability. It has been shown that heated and processed food products contain more bioavailable sources of lycopene than uncooked sources (Boileau et al. 2002) and that thermo-processing of food sources of lycopene result in a small increase in isomerization of all-*trans*-lycopene to *cis*-isomers (Schierle et al. 1997; Nguyen and Schwartz 1998).

There are two approaches of assessing bioavailability: (1) measure the portion of an ingested nutrient that crosses the intestinal barrier by sampling plasma (plasma response approach) or biological tissues and (2) measure the portion of an ingested nutrient that appears in the feces and then compare the mass of nutrient in feces to the total dose given (mass balance approach). Incorrect assessment arises in the plasma response approach when newly ingested nutrient mixes with endogenous circulating nutrient. In that case a label or tag is needed to identify the newly absorbed nutrient. In the mass balance approach, bioavailability might be overestimated if the nutrient is passed through the small intestine unabsorbed and becomes metabolized by the intestine flora. Bioavailability of some nutrients might be also underestimated if they are synthesized in the colon by intestinal bacteria. Radioisotope methodology solves some of the problem encountered by these other methods, as administering a labeled nutrient to an individual allows discernment of that nutrient from the endogenous nutrient in biological samples.

Bioavailability of Dietary β-Carotene and Lycopene: Studies with *Momordica cochichinensis* Spreng (Redmelon™)

Momordica cochinchinensis Spreng is the latin name of a fruit belonging to the Cucurbitaceae family, indigenous to Vietnam, China, Moluccas (Burma), Japan, India, Thailand, Laos, Cambodia, Philippines, Malaysia, Bangladesh, and Australia. Botanical and the use of the fruit are well published (Bailey 1937; Guichard and Bui 1941; Herklots 1972; Perry 1980; Vijay and Jalikop 1980; Shadeque and Baruah 1984; Nguyen 1998; Vu Dinh Trac 1986; Vo-Van-Chi 1997; Do 1991; World Health Organisation 1990). Fruits of *M. cochinchinensis* are large, densely aculeate, and green, turning to dark orange or red when ripe, resemblant to cantaloupes or honeydews. In Vietnam, the fruit is called gac. Some of the English names are Redmelon, spike gourd, sweet gourd, and spike fruited crow cucumber (Vuong 2000). For the remainder of this chapter, the name Redmelon will be used except in references to work in Vietnam or specific Vietnamese dishes without an equivalent English name. In such cases, the Vietnamese names will be mentioned.

In Vietnam, the seed pulp (aril) of Redmelon, the thick membrane covering a large seed, is used when the fruit is completely ripened. At this stage, the aril is bright red and used as rice colorant in a dish called "xoi gac." Seed pulp of the ripened fruit contains high concentrations of carotenoids, as high as 75,000 mg/100 g with β-carotene and lycopene as the dominant carotenoids. Concentrations of β-carotene in the aril are in the ranges of 18–35.5 mg/100 g, about four times higher than those in carrots. Lycopene contents of Redmelon aril can be as high as 340 mg/100 g, or seven times higher than those in tomatoes, which have been touted as the highest dietary source of lycopene (Figure 12.2). In addition, gac aril also contains a significant amount of oil (Vuong and King 2003; Ishida et al. 2004). Fatty acid analyses indicate that Redmelon aril contains 10,198 mg per 100 g of edible portion.

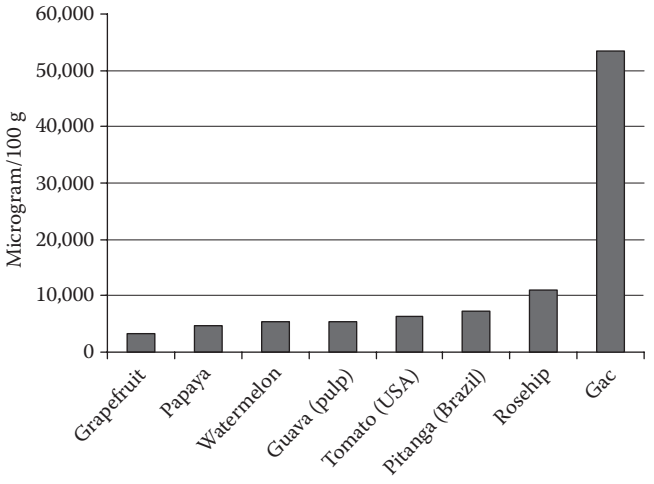

FIGURE 12.2 Lycopene concentrations in Redmelon™ (gac) in comparison with other fruits (Vuong, L.T., *Food Nutr. Bull*., 21, 173, 2000.).

TABLE 12.1
Antioxidant and Nutrient Profile of *M. cochinchinensis* (Redmelon™)

	mg/100 g Fresh Edible Aril	
α-carotene	3	
β-carotene	49	
Lycopene	341	
α-tocopherol	6.67	
Ascorbic acid	37.6	
Fatty acids	10,198	
	FA breakdown	%
	Omega-3	.2
	Omega-6	25.4
	Omega-9	46.2
	Trans-FA	0.1
	MUFA	47.4
	PUFA	25.6

Of the total fatty acids, 70% are unsaturated and 50% of these are polyunsaturated. The aril also contains quantifiable amounts of alpha-tocopherol and ascorbic acid. Concentrations of carotenoids and other nutrients of Redmelon aril are listed in Table 12.1. A 30 day feeding study of pre-schooled children of "xoi gac" showed significant increase in plasma retinol, in addition to lycopene and β-carotene (Vuong et al. 2002). Oil extract from the aril of Redmelon has been shown to contain a remarkable amount of lycopene and β-carotene, and the carotenoids are fairly stable in the oil matrix at room temperature and at low-oxygen, low-light conditions (Vuong and King 2003; Vuong et al. 2005).

Redmelon aril and oil extracted from the aril have an unequivocal nutrient profile: high concentrations of carotenoids, particularly β-carotene and lycopene, in combination with alpha-tocopherol, ascorbic acid, and a unique fatty acid profile containing 70% of essential fatty acids. The use of Redmelon in daily diet as an efficient source of antioxidants and pro-vitamin A is rational. In subsequent sections, data from studies of bioavailability and antioxidant capability of carotenoids in Redmelon aril will be presented.

In Vitro Digestion to Assess Bioavailability of Carotenoids from Redmelon™

A study assessing the bioavailability of carotenoids from Redmelon aril and oil using *in vitro* digestion was carried out. *In vitro* digestion is a method in which homogenized samples (meals) are subjected to simulated gastric and small intestinal digestion, as described by Garrett and Failla (Garrett et al. 1999, 2000). Aril and oil meals were prepared following published procedures. A flowchart

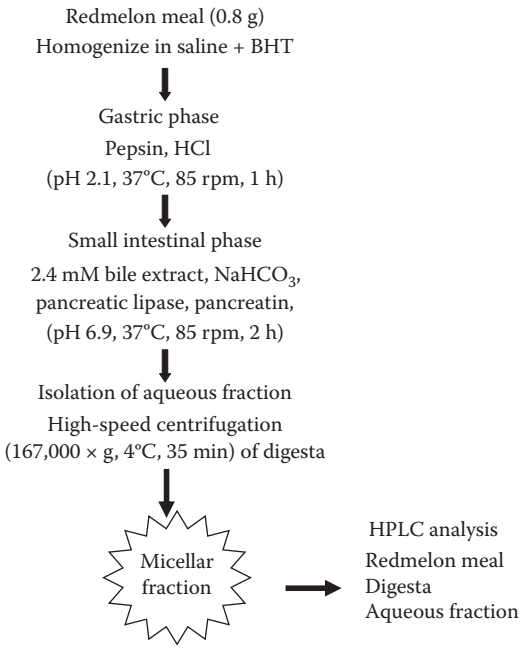

FIGURE 12.3 Flowchart of *in vitro* digestion procedure of Redmelon™ meal.

of the *in vitro* digestion procedure is provided in Figure 12.3. Results of the study are summarized as follows:

- Recovery of carotenoids from digested Redmelon meals was 65%–82%.
- Efficiency of micellarization from digested Redmelon meal was 22%–39% for all carotenoids except *trans*-lycopene, which was only 2%–10%.
- The isomeric profile of lycopene and β-carotene in micelles generated during simulated digestion of Redmelon aril remained constant during 4 h incubation in cell-free medium at 37°C and 5% CO_2.
- The transfer of micellarized *trans*-lycopene across the apical membrane of Caco-2 cells was about 50% that of *cis*-lycopene (Figure 12.4).
- Once accumulated, the intracellular profile of lycopene and β-carotene isomers remained stable during overnight incubation. This suggests that intracellular isomerization of lycopene and β-carotene isomers is quite limited.

The study concluded that β-carotene and lycopene from Redmelon meals are highly bioaccessible and that the more efficient micellarization and the greater apical uptake of micellar *cis*-lycopene compared to *trans*-lycopene contribute to the enrichment of *cis*- versus *trans*-isomers of lycopene in plasma and tissues compared to foods (Failla et al. 2008).

Antioxidant Activities of β-Carotene and Lycopene in Redmelon™

Antioxidant activities of dietary carotenoids have been well demonstrated with β-carotene and lycopene among the most prominent. Many radical oxidative species

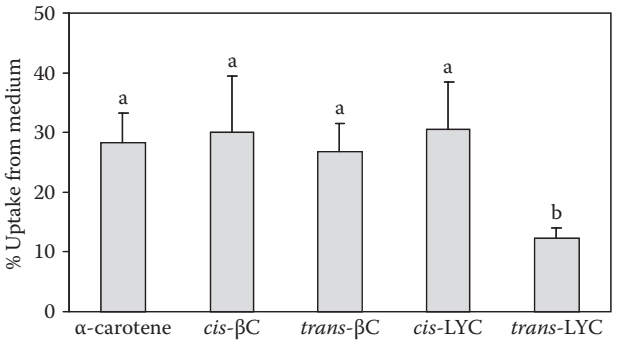

FIGURE 12.4 Apparent uptake of micellarized *trans*-lycopene is significantly less than that of other carotenoids from digested Redmelon™ aril. Data represent mean ± SD, n = 6. Means not sharing the same letters differ significantly by $p < 0.01$.

(ROS), including hydroxyl and peroxyl radicals, as well as superoxide anions, are known to cause damage to DNA, proteins, and other macromolecules. Damage to DNA that evades the DNA repair mechanisms of cells is thought to be an underlying cause of cancer and aging. Antioxidants aid cells in scavenging ROS and preventing intracellular damage. β-carotene, a potent ROS scavenger, has been shown to be effective against antioxidant imbalance of cystic fibrosis (Renner et al. 2001a, 2001b; Homnick et al. 1995; Rust et al. 1998, 2000). Lycopene is an efficient singlet oxygen quencher. Stahl and workers reported a significant decrease in the sensitivity of UV-induced erythema after ingestion of products rich in lycopene (Stahl et al. 2006). The aril of Redmelon contains high concentrations of both β-carotene and lycopene, and quantifiable amounts of alpha-tocopherol and ascorbic acid (Table 12.1). Synergistic effects of dietary antioxidants at physiological levels have been reported (Zhang and Omaye 2000; Yeum et al. 2009). A study was carried out to demonstrate the antioxidant capability of the major carotenoids β-carotene and lycopene and of the Redmelon aril using the total oxygen radical scavenging capacity (TOSC) and oxygen radical absorbance capacity assays. Antioxidant capacities were assessed by the overall abilities of the compounds to suppress peroxyl radicals generated from the thermal homolysis of 2,2´-azobis-amidinopropane. Stock solutions of all samples were diluted 1/100 and 1/1000. At a 1/100 dilution, lycopene exhibited the highest ability to scavenge peroxyl radicals, 2.4 and 1.4 times as much as β-carotene and the Redmelon aril, respectively (Figure 12.5a). However, at a lower concentration (dilution of 1/1000), the Redmelon aril provided the highest peroxyl radical scavenging capacity, 1.8 and 1.3 times as much as β-carotene and lycopene, respectively (Figure 12.5b).

Table 12.2 shows the values obtained from the TOSC analysis of Trolox (vitamin E analogue) and Redmelon aril (both samples were dissolved in DMSO). The comparative TOSC values (cTOSC) show that the Redmelon aril maintains a peroxyl radical scavenging capacity 5.4 times greater than Trolox. The experiments showed that the Redmelon aril, β-carotene, and lycopene all exhibit at least two times higher total antioxidant activity than Trolox, an analogue of Vitamin E. Lycopene was the most active, maintaining a peroxyl radical scavenging capacity 4.2 times greater than Trolox (Secrest et al. 2003).

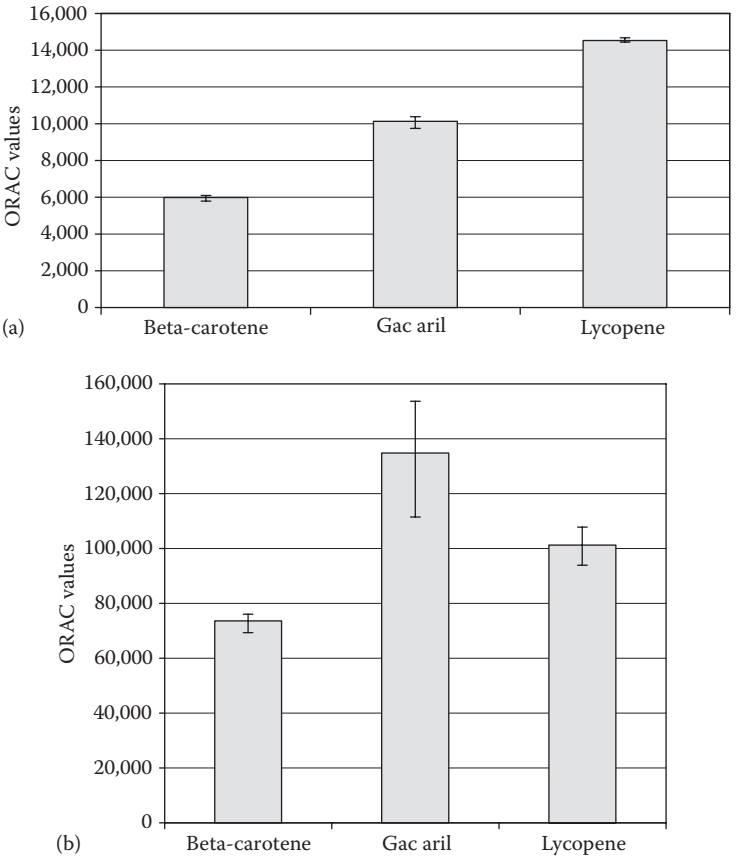

FIGURE 12.5 Graphical comparison of the values obtained using the ORAC assay with the Redmelon™ aril, β-carotene, and lycopene. (a) 1/100 dilution of the stock solutions of each antioxidant sample. (b) 1/1000 dilution of the stock solutions of each antioxidant sample. ORAC values were obtained using a Trolox standard linear regression line ($R^2 = 0.9885$, graph not shown).

TABLE 12.2
cTOSC Values (Both Samples Were Dissolved in DMSO)

Sample	cTOSC	rTOSC
Trolox	1.00	4.51
Redmelon aril	5.44	24.55

cTOSC showed that Redmelon™ aril maintains a peroxyl radical scavenging capacity 5.4 times greater than Trolox. Experiments showed that the Redmelon aril, β-carotene, and lycopene all exhibit at least two times higher total antioxidant activity than Trolox, an analogue of vitamin E.

Human Feeding Studies

Preliminary Study: 5 Day Feeding Trial

A study comparing bioavailability of β-carotene in "xoi gac" (rice colored by Redmelon aril) and β-carotene capsules was carried out. The study was a cross-over design with an 8 week washout period, and the subject was an adult female volunteer. Treatments were 15 mg/day of β-carotene in (1) "xoi gac" and (2) synthetic β-carotene capsules. All other intakes were kept constant, and the length of the treatment was 6 days. After the last dose, blood samples were collected at 0, 24, 48, 72, and 120 h and were analyzed for β-carotene and retinol concentrations. There were significant differences between the increases in plasma β-carotene from "xoi gac" and β-carotene capsules (Figure 12.6). There were also significant increases in plasma β-carotene after the 5 day feeding of Redmelon oil (unpublished data).

Multicenter, Randomized Controlled 30 Day Feeding Trial

A supplementation trial was conducted in northern Vietnam. The objective of the trial was to assess the efficacy of the traditional carotene-rich rice preparation known as xoi gac for improving vitamin A status of children in rural Vietnam. The length of the supplementation period was 30 days. The participants were 193 village children from 31 to 70 months of age. The children were selected from 711 village children in the aforementioned age groups. Selection criteria included a low hemoglobin concentration (100–120 g/L), which has been associated with vitamin A deficiency. The selected children were assigned to one of the three groups: (1) a fruit group that received rice cooked with Redmelon containing 3.5 mg carotene; (2) a powder group that received rice mixed with synthetic β-carotene powder containing 5 mg carotene; and (3) a control group that received rice without fortification. The usual vitamin A and carotenoid intakes were assessed by a food frequency questionnaire administered to the child's mother before and after the supplementation. Results of this study showed that the mean increases in plasma carotene concentrations among children in the fruit group were significantly higher than those of the control group. The increase in plasma lycopene concentration was significantly higher in the fruit group (940%) than in either the control group (99%) or the powder group (386%). Plasma retinol concentrations

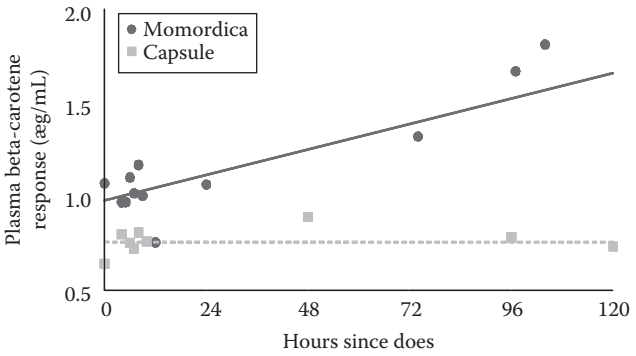

FIGURE 12.6 Plasma response of beta-carotene from Redmelon™ rice (momordica) and capsules in one adult volunteer.

TABLE 12.3
Significant Increase in Plasma Beta-Carotene and Lycopene in the Redmelon™ Group after 30 Day Supplementation

	ROH ± sd (µg/dL)	Beta-Car ± sd (µg/dL)	Lycopene ± sd (µg/dL)	Hb Conc. ± sd (g/dL)	Number of Children with ROH < 20 µg/dL
Before	25.70 ± 6	12.62 ± 11.85	4.6 ± 7.80	11 ± 4.5	10/55
After	29.79 ± 7.82	118.74 ± 50.90	47.85 ± 28.49	12 ± 8.3	3/55

increased significantly in all three groups compared to initial values (Table 12.3). The increase was significantly higher in the fruit group than in the other two groups. After supplementation, 52% of the children in the fruit group and 47% of those in the powder group reached an adequate hemoglobin concentration (120 g/L). The study concluded that β-carotene in Redmelon aril was highly bioavailable and that bioconversion of β-carotene in the aril into retinol was efficient due to oil content and alpha-tocopherol in the aril (Vuong et al. 2002).

BIOAVAILABILITY OF CAROTENOIDS IN HUMANS USING ^{14}C-LABELED TRACER AND ACCELERATOR MASS SPECTROMETRY

Accelerator mass spectrometry (AMS) was originally developed through the 1980s for high-sensitivity radiocarbon dating of organic residues from archaeology and earth sciences. In the 1990s, however, AMS emerged as a useful bio-analytical tool for the quantification of ^{14}C and (other long-lived isotopes) in biochemical labeling and tracing (Vogel et al. 1995). AMS uses a type of tandem isotope ratio mass spectrometer that measures the ratios of ^{14}C/C to parts per quadrillion or down to as few as 105 atoms of ^{14}C. In a typical AMS measurement, samples containing approximately 1 mg of carbon are combusted to CO_2, which is reduced to graphite off-line prior to analysis (Vogel 1992; Vogel and Love, 2005). AMS counts electrostatically accelerated nuclei in a simple particle detector. Molecular isobars are completely disassociated in the charge-changing process, and any atomic isobars are discriminated in the detector. The main difference between radioisotope quantitation by decay counting and by mass spectrometry is that, in decay counting, the efficiency of detecting (decay per time, e.g., dpm) is a function of the half-life of the isotope, while in mass spectrometry, this efficiency relates directly to the concentration of the isotope in the sample. The mean life (half-life/ln2) of ^{14}C is 8270 years, and the decay of 1% of ^{14}C in a sample requires 83 years. A sample decaying at 1 dpm (containing 0.45 picoCurie of ^{14}C) requires a week of counting (1% statistical precision), while a sample containing 7.2 fmol of ^{14}C in 1 mg of carbon would take 1 s to measure by AMS. In other words, the gain in sensitivity by AMS can be from 1000 to 1 billion for radioisotope quantitation. This gain can be used to reduce administered radioisotope tracers to below established safety levels of radiation exposures. This sensitivity also allows isolation of small and specific biochemical fractions for measurement, and tracing of compounds at physiological rather than pharmacological levels in all human populations. The sensitivity of AMS enables quantitative detection in samples that can be collected noninvasively, such

as saliva, expired air, mucosa membrane, hair, skin, urine, and feces. For blood samples, volumes as small as 10–15 μL or a few drops, which can be conveniently collected by finger prick or heel stick are sufficient for the detection of 14C. This allows easier participant recruiting and compliance. Due to the exquisite sensitivity and exceptional specificity, ^{14}C-AMS has been the method of choice for various groundbreaking nutritional studies (Vuong et al. 2004; Dueker et al. 2007).

Bioavailability of β-Carotene in Humans Using AMS

An advantage of radioisotopes is that whole-body measurements are possible to determine the retention of administered compounds, regardless of whether the compounds are metabolized or not. The route of labeled compounds through specific body tissues can be followed for as long as the tissues can be sampled. In single dose studies, the extent of isotopic enrichment of retinol and β-carotene in plasma is measured to enable the area under the curve of isotopic enrichment to be calculated (Dueker et al. 1994). Since the accuracy of the estimate of the bioavailability and bioefficiency depends on the frequency and timing of sampling of blood samples, a sufficient number of samples at representative time-points (during the absorption and during the elimination phase) must be collected and analyzed (van Lieshout et al. 2003). A study using β-[^{14}C]carotene coupled with AMS was carried out in one adult male volunteer. This was the first mass balance study applying AMS and ^{14}C-labeled tracer. Using this approach, the concentration-time course of a physiological (306 μg 200 nCi) oral dose of β-[^{14}C]carotene was determined for 209 days in plasma. Analytes included β-[^{14}C]carotene, [^{14}C]retinyl esters, [^{14}C]retinol, and several [^{14}C]retinoic acids. Cumulative urine and stool were collected for 17 and 10 days, respectively. Plasma kinetics of β-carotene in the subject were clearly described. There was a 5.5 h lag between dosing and the appearance of 14C in plasma. Labeled β-carotene and [^{14}C]retinyl esters rose and displayed several maxima with virtually identical kinetic profiles over the first 24 h period. Elevated [^{14}C]retinyl ester concentrations were sustained in the plasma compartment for >21 h postdosing. The appearance of [^{14}C]retinol in plasma was also delayed 5.5 h postdosing, and its concentration rose linearly for 28 h before declining. Analysis of stools reported that 57.4% of the dose was recovered within 48 h postdosing and that the stool was the major excretion route for the absorbed dose. The turnover times (1/k(el)) for β-carotene and retinol were 58 and 302 days, respectively. Area under the curve analysis of the plasma response curves suggested a molar vitamin A value of 0.53 for β-carotene, with a minimum of 62% of the absorbed β-carotene being cleaved to vitamin A (Dueker et al. 2000; Novotny et al. 1995, 1996). The study established AMS as a powerful tool for defining the *in vivo* metabolic behavior of β-carotene and related compounds at physiological concentrations.

Bioavailability of Lycopene in Skin Determined by AMS

The advantage of lycopene in protecting skin from photodamage has been proposed and investigated (Stahl et al. 1989; Stahl et al. 2006; Lopes and Reed 2009). It has been confirmed that the predominant long-chain carotenoids found in human skin are lycopene and β-carotene (Ermakov et al. 2004). In addition to the ethical, regulatory, and cost issues, the investigation of the benefits of dietary lycopene in humans

is complicated further since blood, plasma, and tissues of humans contain mainly the *cis*-isomers (5-*cis*, 9-*cis*, 13-*cis* and 15-*cis*; Dos Anios Ferreira et al. 2006). However, the major dietary source of lycopene for humans, tomatoes, contains mostly the *trans*-isomers, which are converted to the *cis*-isomers by cooking. ^{14}C-labeling coupled with AMS affords *in vivo* tracing of a single sub-toxic dose of [^{14}C]lycopene taken with a normal amount of food-source lycopene (tomato sauce) in humans. Exquisite sensitivity of AMS makes it also possible to detect lycopene in very small skin biopsies (4 mm). This approach was carried out in two adult male volunteers in the first clinical study with [^{14}C]lycopene described subsequently. The primary objective was to determine bioavailability of a single dose of [^{14}C]lycopene in human skin. Other objectives were to describe plasma kinetics of lycopene over a period of 6 weeks and to assess mass balance of the ^{14}C-labeled dose to achieve effective radioactive dose for future studies and gain insightful information on the metabolism of lycopene in humans. Each subject was given an oral dose of 10 mg of synthetic lycopene including ~6 μg of [6,6′,7,7′-^{14}C] lycopene (~30,000 Bq; 92% *trans*-lycopene). The appearance of ^{14}C in plasma, plasma triglyceride–rich fraction, urine, expired breath CO2, and skin biopsies was measured over 42 days. ^{14}C in lycopene isomer fractions from plasma and triacylglycerol-rich lipoprotein (TRL) fraction were measured to assess the isomerization of lycopene *in vivo*. Two main types of AMS instruments were used for ^{14}C measurements, graphite-fed AMS and gas-fed AMS. Separation and quantification of lycopene isomers were done by High Pressure Liquid Chromatography (HPLC). Identification of the isomers was determined by MS/MS analysis. Lycopene was extensively isomerized after dosing and rapidly metabolized into polar metabolites, which were excreted into urine. Metabolism of lycopene in humans involves β-oxidation, and its metabolites can be detected in skin for up to 42 days (Ross et al. 2011).

CONCLUSION

Bioavailability of dietary carotenoids is affected by many factors and can be enhanced in the presence of other antioxidants in the food. The fruit, *M. cochinchinensis* Spreng, or Redmelon, with high concentrations of β-carotene and lycopene, is a promising source of the highly bioavailable antioxidants called carotenoids, including pro-vitamin A carotenoids, and essential fatty acids. ^{14}C-labeled tracers coupled with AMS provide an unequivocal tool to study biokinetic and bioavailability of carotenoids in humans.

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