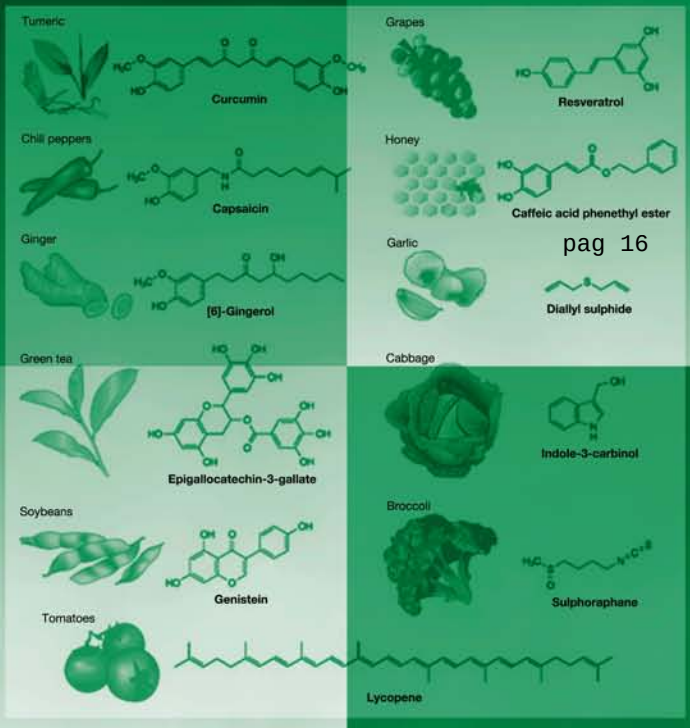
Dietary Modulation of Cell Signaling Pathways



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Dietary Modulation of Cell Signaling Pathways

OXIDATIVE STRESS AND DISEASE

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Series Preface

OXYGEN BIOLOGY AND MEDICINE

Through evolution, oxygen—itself a free radical—was chosen as the terminal electron acceptor for respiration. The two unpaired electrons of oxygen spin in the same direction; thus, oxygen is a biradical. Other oxygen-derived free radicals such as superoxide anion or hydroxyl radicals formed during metabolism or by ionizing radiation are stronger *oxidants,* that is, endowed with higher chemical reactivities. Oxygen-derived free radicals are generated during metabolism and energy production in the body and are involved in regulation of signal transduction and gene expression, activation of receptors and nuclear transcription factors, oxidative damage to cell components, antimicrobial and cytotoxic actions of immune system cells, as well as in aging and age-related degenerative diseases. Conversely, cells conserve antioxidant mechanisms to counteract the effects of oxidants; these *antioxidants* may remove oxidants either in a highly specific manner (e.g., by superoxide dismutases) or in a less specific manner (e.g., through small molecules such as vitamin E, vitamin C, and glutathione). *Oxidative stress* as classically defined is an *imbalance between oxidants and antioxidants*. Overwhelming evidence indicates that oxidative stress can lead to cell and tissue injury. However, the same free radicals that are generated during oxidative stress are produced during normal metabolism and, as a corollary, are involved in both human health and disease.

UNDERSTANDING OXIDATIVE STRESS

In recent years, the research disciplines interested in oxidative stress have grown and enormously increased our knowledge of the importance of the cell redox status and the recognition of oxidative stress as a process with implications for many pathophysiological states. From this multi- and inter-disciplinary interest in oxidative stress emerges a concept that attests to the vast consequences of the complex and dynamic interplay of oxidants and antioxidants in cellular and tissue settings. Consequently, our view of oxidative stress is growing in scope and new future directions. Likewise, the term *reactive oxygen species*—adopted at some stage in order to highlight nonradical oxidants such as H_2O_2 and O_2—now fails to reflect the rich variety of other reactive species in free radical biology and medicine encompassing nitrogen-, sulfur-, oxygen-, and carbon-centered radicals. With the discovery of nitric oxide, nitrogen-centered radicals gathered momentum and have matured into an area of enormous importance in biology and medicine. Nitric oxide or nitrogen monoxide (NO), a free radical generated in a variety of cell types by nitric oxide synthases (NOSs), is involved in a wide array of physiological and pathophysiological phenomena such as vasodilation, neuronal signaling, and

inflammation. Of great importance is the radical–radical reaction of nitric oxide with superoxide anion. This is among the most rapid nonenzymatic reactions in biology (well over the diffusion-controlled limits) and yields the potent nonradical oxidant, peroxynitrite. The involvement of this species in tissue injury through oxidation and nitration reactions is well documented.

Virtually all diseases thus far examined involve free radicals. In most cases, free radicals are secondary to the disease process, but in some instances causality is established by free radicals. Thus, there is a delicate balance between oxidants and antioxidants in health and disease. Their proper balance is essential for ensuring healthy aging.

Both reactive oxygen and nitrogen species are involved in the redox regulation of cell functions. Oxidative stress is increasingly viewed as a major upstream component in the signaling cascade involved in inflammatory responses, stimulation of cell adhesion molecules, and chemoattractant production and as an early component in age-related neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases, and amyotrophic lateral sclerosis. Hydrogen peroxide is probably the most important redox signaling molecule that, among others, can activate NFκB, Nrf2, and other universal transcription factors. Increasing steady-state levels of hydrogen peroxide have been linked to a cell's redox status with clear involvement in adaptation, proliferation, differentiation, apoptosis, and necrosis.

The identification of oxidants in regulation of redox cell signaling and gene expression was a significant breakthrough in the field of oxidative stress: the classical definition of oxidative stress as an *imbalance between the production of oxidants and the occurrence of cell antioxidant defenses* proposed by Sies in 1985 now seems to provide a limited concept of oxidative stress, but it emphasizes the significance of cell redox status. Because individual signaling and control events occur through discrete redox pathways rather than through global balances, a new definition of oxidative stress was advanced by Dean P. Jones (*Antioxidants & Redox Signaling* [2006]) as a disruption of redox signaling and control that recognizes the occurrence of compartmentalized cellular redox circuits. Recognition of discrete thiol redox circuits led Jones to provide this new definition of oxidative stress. Measurements of GSH/GSSG, cysteine/cystine, or $\text{thioredoxin}_{\text{reduced}}/\text{thioredoxin}_{\text{oxidized}}$ provide a quantitative definition of oxidative stress. Redox status is thus dependent on the degree to which tissue-specific cell components are in the oxidized state.

In general, the reducing environments inside cells help to prevent oxidative damage. In this reducing environment, disulfide bonds (S–S) do not spontaneously form because sulfhydryl groups are maintained in the reduced state (SH), thus preventing protein misfolding or aggregation. The reducing environment is maintained by metabolism and by the enzymes involved in maintenance of thiol/disulfide balance and substances such as glutathione, thioredoxin, vitamins E and C, and enzymes such as superoxide dismutases, catalase, and the seleniumdependent glutathione reductase and glutathione and thioredoxin-dependent hydroperoxidases (periredoxins) that serve to remove reactive oxygen species (hydroperoxides).

Also of importance is the existence of many tissue- and cell compartment-specific isoforms of antioxidant enzymes and proteins.

Compelling support for the involvement of free radicals in disease development originates from epidemiological studies showing that enhanced antioxidant status is associated with reduced risk of several diseases. Of great significance is the role that micronutrients play in modulation of redox cell signaling; this establishes a strong linking of diet and health and disease centered on the abilities of micronutrients to regulate redox cell signaling and modify gene expression.

These concepts are anticipated to serve as platforms for the development of tissue-specific therapeutics tailored to discrete, compartmentalized redox circuits. This, in essence, dictates principles of drug development-guided knowledge of mechanisms of oxidative stress. Hence, successful interventions will take advantage of new knowledge of compartmentalized redox control and free radical scavenging.

OXIDATIVE STRESS IN HEALTH AND DISEASE

Oxidative stress is an underlying factor in health and disease. In this series of books, the importance of oxidative stress and diseases associated with organ systems of the body is highlighted by exploring the scientific evidence and clinical applications of this knowledge. This series is intended for researchers in the basic biomedical sciences and clinicians. The potential of such knowledge for healthy aging and disease prevention warrants further knowledge about how oxidants and antioxidants modulate cell and tissue function.

Lester Packer
Enrique Cadenas

Preface

The fine-tuning of cellular signaling pathways is pivotal for maintaining homeostasis in the living organism. Therefore, disruption of any of the signal transduction pathways can lead to severe cellular dysfunction. Current research continues to increase its attention on the role of nutrition and diet in modifying oxidative and inflammatory damage in the progression of disease. *Dietary Modulation of Cell Signaling Pathways* reviews some of the latest research findings, focusing on nutrient–gene interactions with particular emphasis on the intracellular signaling network.

Dietary modulation of specific gene expression systems highlights underlying molecular and cellular mechanisms. Numerous reports have demonstrated the effects on health and disease of nutrition and specific dietary components, organic and mineral micronutrients, and other food-based and natural-source ingredients. Hence, dietary substances influence and modulate many vital aspects of cell regulation, such as the cell cycle, pathway of biosynthesis and degradation, metabolism, and apoptosis.

Much evidence shows that consumption of natural source substances confers chemopreventive and cytoprotectant activities. A now legendary report by Paul Talalay and colleagues (*Proceedings of the National Academy of Science USA*, vol. 77, pp. 5216–5220, 1980) demonstrated the induction of phase 2 enzymes like NAD(P)H:quinone reductase by dietary antioxidants.

Dietary ingredients that affect signaling are usually small molecules of plant origin with a broad range of molecular structures, including those having the properties of Michael acceptors (e.g., olefins or acetylenes conjugated to electron withdrawing groups). Some of these molecules like those with phenolic hydroxyl groups are also potent antioxidants, that is, are bifunctional cytoprotective substances. There is also direct evidence that sulfydryl groups of enzymes (e.g., phosphatases) and proteins (e.g., Keap 1) are critical targets modulated by oxidative and inflammatory conditions as well as dietary substances. Several chapters in this book describe nutrient actions on activation of antioxidant (Nrf2) and inflammatory (NF-κB) transcription factors, and induction of their target gene expression.

The coeditors gratefully acknowledge the initial stimulus for this book: a workshop on "Dietary Modulation of Cell Signaling Pathways" chaired by Young-Joon Surh during the twelfth annual meeting of the Oxygen Club of California (March 15–18, 2006, Santa Barbara) cosponsored by the Linus Pauling Institute. The editors thank the leading experts who have provided information

on state-of-the-art research of their own specialty. We hope this volume will contribute to the developments in this rapidly expanding and highly important area of biomedical research.

Young-Joon Surh
Zigang Dong
Enrique Cadenas
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Editors

Young-Joon Surh is a professor of biochemistry and molecular oncology at the College of Pharmacy, Seoul National University, and Head of the National Research Laboratory of Molecular Carcinogenesis and Chemoprevention supported by the Ministry of Science and Technology, South Korea. Surh earned his PhD at the McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, and completed his postdoctoral training at the Massachusetts Institute of Technology (MIT). In 1992, he was appointed as a tenure-track assistant professor at Yale University School of Medicine. Since relocating to Seoul National University in 1996, he has been leading the Chemoprevention Working Group, investigating the molecular mechanisms of cancer prevention with anti-inflammatory and antioxidative phytochemicals, with emphasis on intracellular signaling molecules as prime targets. He is currently a member of the editorial boards of more than 15 international journals, including *Carcinogenesis*, *Cancer Prevention Research, International Journal of Cancer, Molecular Carcinogenesis, Cancer Letters, Mutation Research, Food and Chemical Toxicology, Biofactors, Genes and Nutrition*, and *Molecular Nutrition and Food Research*. He is also coeditor of the books *Oxidative Stress, Inflammation and Health* (CRC Press), and *Molecular Targets and Therapeutic Use of Curcumin* (Springer-Verlag). Surh has published more than 200 papers in peer-reviewed international journals and more than 60 invited editorials, reviews, and book chapters. He published a seminal review article in *Nature Reviews Cancer* titled "Cancer Chemoprevention with Dietary Phytochemicals," which has been highly cited.

Zigang Dong received his medical training at Henan Medical University from 1978 to 1983. In the same medical school, he obtained his master's of science degree in 1986. In 1991, as a distinguished graduate, he obtained his doctor of public health degree from Mailman School of Public Health at Columbia University in New York. After postdoctoral training at the National Cancer Institute, he accepted a faculty appointment at the Hormel Institute, University of Minnesota in 1995. He was appointed as executive director and Hormel-Knowlton professor of the Hormel Institute in 1999. More recently, he received the University of Minnesota McKnight Presidential Professorship in Cancer Prevention, one of the highest honors bestowed by the university. He is one of very few professors holding two endowed professorships at the same time at the University of Minnesota. Dong has served as a member on many grant application review study sections with the National Institutes of Health (NIH). He is currently a member of the Cancer Etiology study section of NIH, a member of the expert panel of the National Nature Science Foundation of China, and a member of the selective

committee of the Chang-Jiang Scholar of the Minister of Education of China. Under his leadership, the funding from the government to the Hormel Institute has been tripled in the last few years.

Enrique Cadenas received his MD and PhD from the University of Buenos Aires. He served as a research fellow at the Universities of Dundee (Scotland), Pennsylvania (USA), and Dusseldorf (Germany). Prior to coming to the University of Southern California in 1989, Cadenas was an associate professor of pathology at the University of Linkoping (Sweden). Currently, he is a Charles Krown/Alumni Professor of Pharmaceutical Sciences and associate dean for Research Affairs at the University of Southern California School of Pharmacy and professor of biochemistry at the USC Keck School of Medicine.

Lester Packer received his PhD in microbiology and biochemistry from Yale University. For many years, he was professor and senior researcher at the University of California at Berkeley. Currently, he is an adjunct professor in the Department of Pharmacology and Pharmaceutical Sciences at the University of Southern California. Recently, he was appointed as distinguished professor at the Institute of Nutritional Sciences of the Chinese Academy of Sciences, Shanghai, China. His research interests are related to the molecular, cellular, and physiological role of oxidants, free radicals, antioxidants, and redox regulation in health and disease. Packer is the recipient of numerous scientific achievement awards, including three honorary doctoral degrees. He has served as president of the International Society of Free Radical Research (SRRRI), president of the Oxygen Club of California (OCC), and vice president of UNESCO—the United Nations Global Network on Molecular and Cell Biology (MCBN).

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1 Intracellular Signaling Molecules as Targets of Selected Dietary Chemopreventive Agents

*Joydeb Kumar Kundu, Hye-Kyung Na, and Young-Joon Surh**

CONTENTS

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1.1 CHEMOPREVENTION: A RATIONAL APPROACH TO FIGHT CANCER

Cancer is the end-stage manifestation of heterogeneous and chronic disease processes. Over the last several decades, a search for a cancer cure has largely been focused on developing chemotherapeutic agents, radiotherapy, and surgical intervention. Nonetheless, the incidence and mortality of cancer, in general, are still increasing. The number of cancer-related deaths is expected to double in the next 50 years despite current advances in cancer prevention and treatment [1]. In fact, the aforementioned conventional strategies, largely implemented after the diagnosis of cancer at the advanced stage, have been proven unsatisfactory to cure cancer. This is because the malignant lesions exhibit heterogeneity in terms of genotypic and phenotypic characteristics, which makes it impractical to find a specific molecular target for the defined cure for cancer. Moreover, many of the clinically approved cytotoxic drugs exert deleterious effects on the normal tissues, thereby devastating the quality of life [2]. In this context, chemoprevention has recently attracted much attention as an alternative strategy for the management of cancer. *Chemoprevention*, the term coined by Michael B. Sporn, refers to the use of nontoxic chemical substances of natural or synthetic origin to inhibit, retard, or even reverse the specific stage of carcinogenesis [3]. A wide spectrum of preclinical and clinical studies put a strong scientific basis on the success of the chemoprevention strategy in reducing the global burden of cancer. Based on the advances in chemoprevention research in the past three decades, there is now a paradigm shift from cancer treatment toward cancer chemoprevention [2,4,5].

The success of chemoprevention lies in the understanding of the molecular basis of carcinogenesis. The transformation of a population of normal cells into malignant cancer apparently involves three distinct phases: initiation, promotion, and progression. Tumor initiation, a rapid and irreversible process, begins with the genotoxic damage of cellular DNA upon exposure to endogenous or exogenous carcinogens. The initiation stage of tumorigenesis involves the metabolic activation of carcinogens and subsequent covalent modification of genomic DNA, leading to activation of oncogenes and inactivation of tumor suppressor genes. Tumor promotion is recognized as a reversible process undergoing clonal expansion of initiated cells to form a solid mass of proliferating preneoplastic cells. Progression, the final stage of neoplastic transformation, involves the growth of a tumor with invasive and metastatic potential [5,6]. Currently, chemoprevention strategies have been grouped into three categories: primary, secondary, and tertiary prevention. The primary chemoprevention strategy aims to prevent carcinogenesis in healthy individuals, who are

usually referred to as a low-risk group. Secondary chemoprevention refers to the blockade of the progression of premalignant lesions to complete neoplasia. Tertiary prevention of cancer means the avoidance of recurrent primary tumors to develop in patients already cured for their premalignant lesions [1,7,8].

According to the somatic mutation theory, cancer is considered to be a genetic disorder involving the acquisition of multiple mutations in key genes that govern cellular proliferation, programmed cell death, and genetic stability [9]. Genetic mutation leads to conversion of cellular protooncogenes to oncogenes, usually referred to as the *gain-of-function* event and/or functional inactivation of tumor suppressor genes, resulting in *loss-of-function* of these genes. In the postgenomic era, the epigenetic basis of carcinogenesis has evolved. The term *epigenetics* refers to the changes in gene expression that do not result directly from mutational changes in the DNA sequences. Epigenetic events, including alterations of cellular signal transduction pathways, have been observed in various cancers from early initiation to subsequent promotion and progression stages. Such altered biochemical events disrupt the control of cellular protein repertoire at either the transcriptional or the translational level, thereby perturbing cellular homeostasis. Since epigenetic changes are reversible, restoration of the altered cellular signal transduction pathways by targeting component signaling molecules is considered as a rational strategy to achieve molecular target-based chemoprevention [10].

1.2 MECHANISTIC ASPECT OF CHEMOPREVENTION

The recent progress in molecular biology of cancer has identified key components of the intracellular signaling network, especially protein kinases and transcription factors, that function abnormally during the course of cellular transformation and malignancy. In response to carcinogenic stimuli, the intracellular signaling network becomes disrupted, thereby favoring premalignant and malignant transformation of cells. Therefore, the modulation of inappropriate cell signaling cascades might be a realistic approach in achieving chemoprevention. Mechanistically, the process of carcinogenesis can be prevented in both the initiation and promotion stages by targeting signal transduction pathways involved in carcinogen detoxification, cellular proliferation, inflammation, apoptosis, and angiogenesis (Figure 1.1) [5,8].

1.2.1 Enhancement of Cellular Antioxidant/Detoxification Capacity

Oxidative stress and the inflammatory microenvironment of the tissue act as predisposing factors to multistage carcinogenesis [1,11,12]. Reactive oxygen species (ROS), such as superoxide anion, hydroperoxyl radical, hydrogen peroxide, and hydroxyl radical, are constantly generated in cells as unwanted by-products of aerobic metabolism. Although a low physiologic level of ROS is scavenged efficiently by the cellular antioxidant defense system, an imbalance between the generation of ROS and cellular antioxidant capacity turns into a state of "oxidative stress" that contributes to carcinogenesis [13–15]. Oxidative stress contributes to

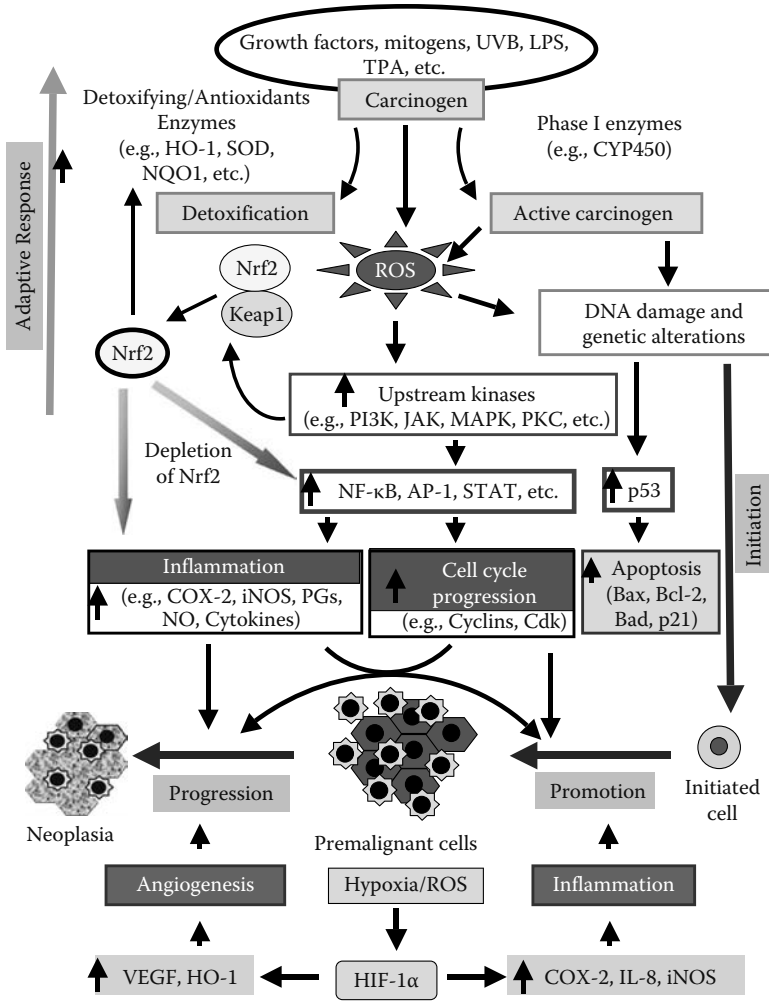

FIGURE 1.1 (See color insert following page 74.) Major molecular events in multistage carcinogenesis.

tumorigenesis by multiple mechanisms involving damage to critical biomolecules—such as DNA, RNA, proteins, and membrane lipids—and modulation of cellular signal transduction pathways [13,16,17]. Certain environmental insults (e.g., microbial infection and ultraviolet radiation) and chemical carcinogens (e.g., polycylic aromatic hydrocarbon and phorbol ester) act as tumor initiators and/or promoters by inducing steady-state increases in the generation of ROS, which contributes to carcinogenesis by modulating cellular signaling pathways [11].

In response to oxidative stimuli or electrophilic carcinogens, cells turn on the antioxidant/detoxification arsenal as the first line of defense. The phase 2 detoxifying/antioxidant enzymes are involved in deactivation or elimination of

electrophilic carcinogens, thereby protecting cellular macromolecules from oncogenic insults [18,19]. The redox-sensitive transcription factor nuclear factor-erythroid-2-related factor-2 (Nrf2) interacts with the antioxidant/electrophile response element (ARE or EpRE), located on the promoter region of genes encoding antioxidant or detoxifying enzymes [12]. Major Nrf2/ARE-regulated gene products are NADP(H):quinine oxidoreductase-1 (NQO1), superoxide dismutase (SOD), glutathione *S*-transferase (GST), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL), and catalase (CAT): Several studies have demonstrated the role of Nrf2/ARE-regulated genes in carcinogenesis [20-25]. For example, mouse epidermal JB6 C41 cells transfected with manganese superoxide dismutase (MnSOD) exhibited a slower growth rate and a reduced rate of colony formation in soft agar upon exposure to a prototype tumor promoter 12-*O*-tetradecanoylphorbol-13 acetate (TPA) [23]. The overexpression of MnSOD was shown to suppress mouse skin papilloma formation [22]. Moreover, mice lacking copper–zinc superoxide dismutase (CuZnSOD) experienced higher numbers of liver nodules, either as hyperplasia or hepatocellular carcinoma, than their wild-type counterparts [20]. In addition, the incidence and the multiplicity of chemically induced mouse skin papillomas were higher in GST-π-null [21] and NQO1-null [24,25] mice in comparison to their wild-type littermates.

In resting cells, Nrf2 resides in the cytoplasm by forming an inactive complex with the repressor Kelch-like ECH-associated protein 1 (Keap1). Dissociation of Nrf2 from the inhibitory protein Keap1 is a prerequisite for nuclear translocation and subsequent DNA binding of Nrf2 [12,19]. After forming a heterodimer with small Maf protein inside the nucleus, the active Nrf2 binds to *cis*-acting ARE or EpRE (alternatively known as Maf recognition element, MARE) located in the promoter region of genes encoding antioxidant/detoxifying enzymes [19,26]. Multiple mechanisms of Nrf2 activation by signals mediated via one or more of the upstream kinases, such as mitogen-activated protein (MAP) kinases, phosphatidylionositol-3-kinase (PI3K)/Akt, and protein kinase C (PKC), have recently been reviewed [18,19,27]. Besides the dissociation of Nrf2-Keap1 complex by upstream kinase-mediated signals, covalent modification of multiple cysteine residues on Keap1 by electrophiles or inducers of detoxifying enzymes may release Nrf2 from the Keap1 repression [28].

The *Nrf2*-null mice failed to induce genes responsible for carcinogen detoxification and protection against oxidative stress [19,26,27,29,30]. The significance of Nrf2 activation as a chemoprevention strategy was evident from the higher burden of benzo[*a*]pyrene-induced gastric neoplasia in *Nrf2*-deficient mice, which was less responsive to the chemopreventive agent oltipraz [31]. Besides its role in regulating carcinogen detoxification and cellular antioxidative defense, the down-regulation of Nrf2 signaling triggered inflammatory responses [32–34]. In a recent study, Khor et al. [35] reported that the increased severity of dextran sulfate sodium-induced colitis in *Nrf2*$^{-/-}$ mice was associated with decreased expression of HO-1, NQO-1, UGT1A1, and GSTμ-1 [35]. In addition, levels of proinflammatory mediators such as COX-2, iNOS, interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α), were significantly increased in the colonic tissues of

Nrf2$^{-/-}$ mice as compared to their wild-type counterparts [35]. Therefore, targeted activation of Nrf2 and Nrf2-regulated gene products is considered as a timely approach for chemoprevention with dietary phytochemicals (Table 1.1).

TABLE 1.1
Activation of Nrf2 Signaling and Modulation of Detoxifying/Antioxidant Enzymes by Dietary Chemopreventive Phytochemicals

Compound	Molecular Targets	Cells/Tissues and Stimuli	References
Resveratrol			
	↑ NQO1 activity	Hepa1c1c7cells	[98]
	↑ Expression and activity of HO-1 via activation of Nrf2	PC12 cells	[100]
Curcumin			
	↑ p38 MAP kinase, ↑ dissociation of Nrf2-Keap1, ↑ Nrf2 binding to *ho-1*-ARE, and ↑ expression and activity of HO-1	NRK-52E cells and LLC-PK$_i$ cells	[147]
	↑ Nuclear translocation and DNA binding of Nrf2-ARE	HBE1 cells	[148]
	↑ GCL mRNA and protein, ↑ GSTP1 mRNA, ↑ Nrf2-ARE-regulated GSTP1 promoter activity	HepG2 cells	[149]
	↑ PKC and p38 MAP kinase, ↑ Nrf2 activation, and ↑ expression of HO-1	HepG2 cells	[150]
	↑ Nrf2-ARE-mediated induction of HO-1	Rat vascular smooth muscle cells	[151]
EGCG			
	↑ MAP kinases, ↑ ARE luciferase activity	HepG2 cells	[179]
	↑ GST, GPx, SOD, and CAT	DMBA-treated mouse skin	[178]
Sulforaphane			
	↓ p38 MAP kinase, ↑ Nrf2-ARE activity, ↑ HO-1 expression	HepG2 cells	[221]
	↑ NQO1, ↑GCL, ↑GST	Nrf2 wild-type mice	[222]
	↑ mRNA expression of NQO1 and UGT1A1	Caco2 cells	[223]
Capsaicin			
	↑ ROS, PI3K/Akt expression, ↓ NQO1 expression and activity, ↑ Activation of Nrf2	HepG2 cells	[247]

1.2.2 Suppression of Abnormally Activated Proinflammatory Signaling Pathways

The generation of ROS, tissue injury, or infection can create a state of inflammation, which is causally linked to tumorigenesis. Accumulating evidence suggests that chronic inflammation acts as a predispoding factor for cancers of different organs and tissues including stomach, colon, breast, skin, prostate, and pancreas [36–39]. Examples of inflammation-associated malignancies are the development of carcinomas of stomach, liver, gallbladder, prostate, and pancreas from *Helicobacter pylori*-induced gastric inflammation, chronic hepatitis, cholecystitis, inflammatory atrophy of the prostate, and chronic pancreatitis, respectively [40,41]. Proinflammatory mediators, such as cytokines, chemokines, prostaglandins (PGs), nitric oxide (NO), and leukotrienes, promote neoplastic transformation of cells by altering normal cellular signaling cascades [42]. For example, IL-6 and TNF-α, two proinflammatory cytokines, have been implicated in tumor promotion [43]. Moreover, the incidence and the multiplicity of mouse skin papillomas have been shown to be significantly reduced in TNF-$\alpha^{-/-}$ animals as compared to TNF-$\alpha^{+/+}$ mice [44].

One of the molecular links between inflammation and cancer is cyclooxygenase-2 (COX-2), which is aberrantly upregulated in premalignant and malignant tissues [45–49]. The role of inappropriately elevated COX-2 in experimental tumorigenesis has been widely investigated. For example, transgenic mice overexpressing *cox-2* in mammary glands, skin, or stomach develop malignancies of these organs [50–52], whereas genetic ablation of *cox-2* suppresses the development of intestinal tumors [53] or skin papillomas [54]. Overexpression of COX-2 has also been associated with an elevated expression of antiapoptotic Bcl-2, thereby providing a survival advantage to transformed cells [55]. In response to inflammatory stimuli, COX-2 is transiently induced and catalyzes the biosynthesis of PGs. Recent studies suggest that overproduction of specific PGs is functionally related to tumor promotion [56–59]. The levels of COX-2 protein and PGs are elevated in certain tissues in response to various external stimuli such as proinflammatory cytokines, bacterial lipopolysaccharide (LPS), ultraviolet (UV) radiation, ROS, and phorbol ester [60,61]. Possible mechanisms by which COX-2 and PGs contribute to carcinogenesis include promotion of cellular proliferation, suppression of apoptosis, and enhancement of angiogenesis and invasiveness [62]. Another proinflammatory mediator, NO, produced by inducible nitric oxide synthase (iNOS), has also been implicated in mouse skin tumorigenesis [63].

Although the precise molecular mechanism underlying the expression of COX-2, iNOS, and various proinflammatory cytokines has not been fully elucidated, distinct roles of aberrant activation of cellular signaling mediated via a panel of upstream kinases and transcription factors in the induction of various pro inflammatory genes have been well documented [60,61,64,65]. Ras, Raf, MAP kinases, PKC, Janus-activated kinase (JAK), PI3K, Akt/protein kinase B (PKB), glycogen synthase kinase (GSK), and the downstream transcription factors, such as nuclear factor-kappaB (NF-κB), activator protein

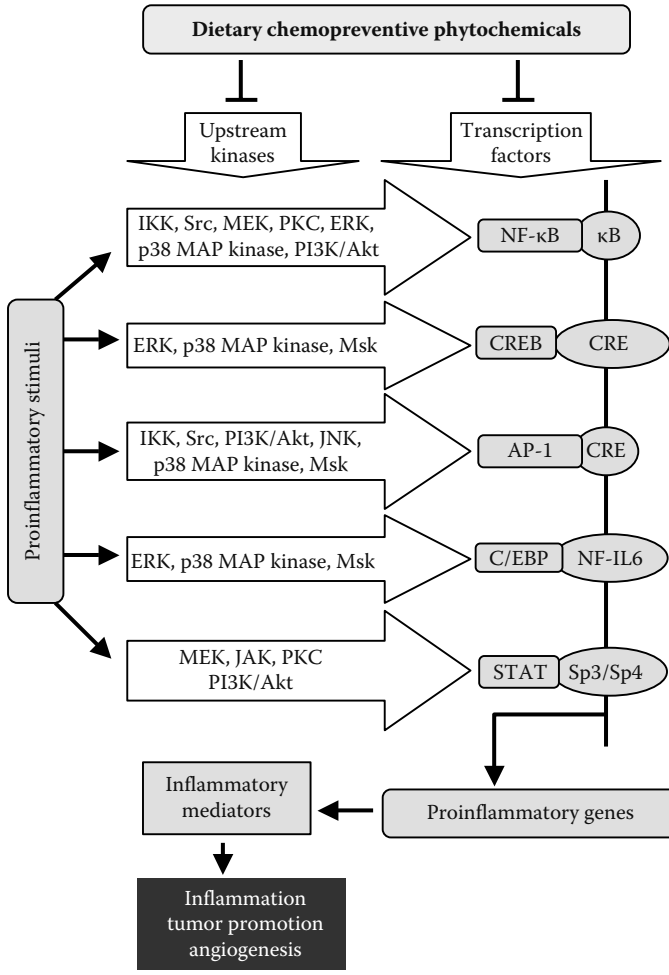

FIGURE 1.2 (See color insert following page 74.) Some representative upstream kinases and transcription factors as potential targets of dietary chemopreventive phytochemicals.

(AP-1), cyclic adenosine monophosphate-response element binding protein (CREB), signal transducer of activated transcription (STAT), and CCAAT/enhancer binding protein (C/EBP) are major components of proinflammatory signaling pathways. Targeting components of proinflammatory signaling pathway represents a practical dietary chemoprevention strategy (Figure 1.2).

1.2.3 Targeting Cell Cycle Regulatory Proteins

The growth of eukaryotic organisms depends on cell division, which is controlled by intricate and evolutionarily conserved cell cycle machinery. Thus, growth arrest of

abnormally proliferating cells that comprise premalignant or malignant tumors by the modulation of cell cycle progression is another important strategy for chemoprevention as well as therapy [4,66]. A cell cycle consists of four different phases: gap I (G1), synthesis (S), gap 2 (G2), and mitosis (M). During the G1 phase, cells grow in size and get prepared for DNA replication. The S phase involves the replication of chromosomes, and the G2 phase checks the completion of DNA replication and prepares cells to undergo mitosis. In the M phase, segregation of chromosome and cytokinesis results in mitosis to produce daughter cells, which further enter into the G1 phase of a new cell cycle or exit from the cell cycle to remain in a resting phase (G0) [66,67]. Cells might also escape from entering a new cell cycle in cases of programmed cell death (apoptosis) and differentiation. Intracellular signaling pathways comprising various cyclins, cyclin-dependent kinases (Cdk), Cdk inhibitors, and check point kinases (Chk 1 and Chk 2) in association with tumor suppressor gene products, such as retinoblastoma (Rb) and p53, regulate cell cycle and, hence, maintain a homeostatic balance between cell growth, apoptosis, and differentiation [66,67].

Members of the Cdk family of serine/threonine kinases regulate transition of dividing cells through different phases by forming active complexes with various cyclins in a stage-specific manner. In response to growth stimulatory signals, the entry of cells into the G1 phase is favored by a complex of cyclin D (D1, D2, D3) proteins with Cdk4 and Cdk6 in a tissue-specific manner. At the end of the G1 phase, a complex of cyclin E-Cdk2 initiates DNA replication and centrosome duplication. The cyclin E-Cdk2 complex causes hyperphosphorylation of Rb, thereby releasing E2F transcription factor. The Rb, in its unphosphorylated state, represses the E2F-mediated transcription of genes required for entry into the S phase and DNA replication. Once cells enter the S phase of the cell cycle, a complex of cyclin A-Cdk2 inactivates Rb, thus ensuring a unidirectional passage of cells toward the G2 and subsequent M phase, which is regulated by a complex of cyclin B-Cdk1. The cyclin-Cdk complex-mediated cell cycle progression is positively regulated by Chk 1 and Chk2. Chks indirectly activate cyclin-Cdk complexes by dephosphorylation of CDC25 phosphatase, which inactivates Cdks. Cellular components that negatively regulate cyclin-Cdk complex include inhibitor of Cdk4 (INK4) family members (e.g., $p16^{Ink4a}$, $p18^{Ink4c}$, $p19^{Ink4d}$) and Cdk interacting protein/kinase inhibiting proteins (CIP1/KIP1) including $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$. Among the CIP1/KIP1 family proteins, $p21^{WAF1/CIP1}$, an extensively investigated p53-regulated protein, inhibits phosphorylation of Rb by suppressing cyclin-Cdk complexes [66–68]. Multiple intracellular signaling pathways initiated by growth factors, mitogens, and UV destabilize cell cycle regulatory proteins, thereby promoting cellular proliferation. Components of such signaling pathways include enzymes (e.g., PI3K/Akt and GSK-3) and transcription factors (e.g., AP-1, NF-κB, and β-catenin) [69–72]. The suppression of abnormal proliferation by downregulating cyclin-Cdks and Chks along with triggering Cdk inhibitors via modulation of upstream kinases and transcription factors may provide an ample scope to intervene in the multistage carcinogenesis by dietary phytochemicals (Table 1.2).

TABLE 1.2
Components of Cell Cycle Machinery as Targets of Dietary Chemopreventive Agents

Compound	Molecular Targets	Cells/Tissues and Stimuli	References
Resveratrol			
	↓ Cyclin D1, D2, D3, ↓ Cdk-2,-4,-6 expression and activity, ↑ p21	A431 cells	[103]
	↓ E2F, ↓ phosphorylation of Rb	A431 cells	[104]
	↓ Cyclin D1 and D2, ↓ Cdk-2,-4,-6 expression and activity, ↑ p21, ↑ p53	UVB-irradiated mouse skin	[105]
	↓ Cyclin D1 and Cdk4, ↑ p21, ↑ p53, ↓ Cyclin E-Cdk2 expression and activity	DU-145 cells	[106]
	↓ Cyclin D1 and Cdk4, ↑ p21, ↑ p53	MCF-7 cells	[107]
	↑ p53, ↑ p21, ↓ phosphorylation of Rb	A549 cells	[108]
	↓ Cyclin A, B1 and D1	SW480 cells	[109]
	↓ D1 and Cdk4 expression and complex formation, ↑ cyclin E and A	Caco2 and HCT-116 cells	[110]
Curcumin			
	↓ Cyclin B1, ↓ Cdc2, ↑ p21 and ↑ p27	ECV034 cells	[153]
	↓ NF-κB, ↓ iNOS, ↑ p53, ↑ p21, p27, and Chk2	Human melanoma cells	[154]
	↓ Cyclin A, ↑ p27	T24 cells	[155]
EGCG			
	↓ Rb, ↓ pRb, ↓ E2F, ↓ DP1, ↓ DP2	A431 cells	[183]
	↓ HER-2 phosphorylation, ↓ STAT-3 activation, ↓ c-cyclin D1 ↓ Bcl-xl	Human head and neck, breast	[184]
Sulforaphane			
	↓ Cyclin D1, ↓ Cdk4, ↓ pRb, ↑ p21	DU-145 cells	[226]
	↓ Cyclin D1, ↓ cyclin A, ↓ c-Myc, ↑ p21	HT-29 cells	[227]
[6]-Gingerol			
	↓ Cyclin A, ↓ Cdk, ↓ pRb, ↓ c-Myc, ↑ p21	BxPC-3 and HPAC cells	[244]
Capsaicin			
	↓ Cdk2 and cyclin E complex	HL-60 cells	[257]

1.2.4 Induction of Apoptosis in Precancerous or Malignant Cells

Apoptosis, an important protective mechanism against neoplastic transformation, involves elimination of a damaged cell or suppression of the outgrowth of transformed cells. Characteristic features of apoptosis, in general, are chromatin

condensation, nuclear fragmentation and cell shrinkage, plasma membrane blebbing, and formation of membrane-bound cellular fragments known as "apoptotic bodies" [73]. The induction of apoptosis in precancerous or malignant cells is considered as one of the promising strategies that can be applied for practice of chemoprevention. Apoptosis occurs primarily through two well-characterized signaling pathways: intrinsic (mitochondria-dependent) and extrinsic (death receptor-mediated) signaling [74]. In its simplest form, the intrinsic pathway of apoptosis involves the localization of proapoptotic Bax in the mitochondrial membrane, depolarization of the mitochondrial membrane resulting in a decrease in the mitochondrial transmembrane potential, release of cytochrome *c*, alterations in the ratio of antiapoptotic Bcl-2 to Bax, activation of caspases, and the cleavage of poly(ADP)ribosyl polymerase (PARP). In the extrinsic pathway, activation of death receptors, such as tumor necrosis factor receptor-1 (TNFR-1), Fas (alternatively known as CD95), and TNF-related apoptosis inducing ligand (TRAIL) receptors by TNF, FAS-ligand (or CD95-L), and TRAIL, respectively, causes activation of procaspase 8 and 10, which then execute cell death by triggering activity of effector caspase molecules (caspase 3, 6, 7). Cells are also enriched with a set of antiapoptotic proteins such as Bcl-2, Bcl-xl, Mcl, cMyc, IAP, XIAP, and FLIP. Recent advances in dissecting apoptotic signaling also revealed a set of new apoptosis-inducing proteins such as Bak and Bim. Expression of these proteins in cells is under the transcriptional control by a panel of transcription factors, such as NF-κB, AP-1 and p53, and their upstream kinases, including MAP kinases and PI3K/Akt. Tumor suppressor genes involved in the induction of apoptosis include *p53*, *p19ARF*, *Rb*, *PTEN*, *TRAIL*, and *CD95/Fas*. On the contrary, genes inhibiting inherent controls of apoptosis include *Bcl-2*, *MDM2*, *IAPs*, *NF-κB*, *Akt*, *PI3K*, *Ras*, *Myc*, and *FLIP*. A wide variety of dietary phytochemicals have been shown to trigger the apoptotic signaling pathways in transformed cells, while downregulating components of survival signaling pathways [4,74,75]. Table 1.3 summarizes the key molecules of the apoptotic signaling pathway that are targeted by selective dietary chemopreventive phytochemicals.

1.2.5 Inhibition of Angiogenesis

Angiogenesis is a physiological process of forming new blood vessels. The induction of angiogenesis is essential for the growth and survival of solid tumors, and their progression to invasive phenotypes. The concept of angiogenesis as a mechanism of growth and survival of tumor cells was first introduced by Judah Folkman, who proposed that tumor cells could sense their distance from the normal vasculature and release angiogenic signals [76]. Later, it has been shown that the growth of tumor implants is dramatically impaired if the nearby capillaries are physically blocked to reach the implant [77]. Moreover, the blocking of angiogenesis can lead to the induction of apoptosis in tumor cells [78]. One of the critical factors responsible for angiogenesis is the presence of hypoxia within the solid tumors. Due to increased metabolic activities and oxygen consumption by rapidly proliferating cells, hypoxia occurs in most solid tumors [79,80].

TABLE 1.3
Major Molecular Targets of Selected Dietary Chemopreventive Phytochemicals to Induce Apoptosis

Compound	Molecular Targets	Cells/Tissues	References
Resveratrol			
	↑ Activation of ERK, p38 and JNK, ↑ p53 phosphorylation	JB6 cells	[117,118]
	↑ Expression of p53 responsive genes: *p53*, *p21*, *p300/CBP*, and *Apaf-1*	LNCaP cells	[115]
	↑ Intranucleosomal DNA fragmentation, ↓ Bcl-2 expression	HL-60 cells	[262]
	↑ MAP kinase, ↑ phosphorylation of p53, ↑ p53 DNA binding	DU-145 cells	[263]
	↓ Bcl-2 expression, ↑ Bax expression colocalization of Bax with mitochondria	Esophageal cancer cells EC-9706	[111]
	↓ Mitochondrial membrane potential, activation of caspase 3 and 9	HCT-116 cells	[120]
	↑ Expression of pERK, p53, p21, c-Fos, c-Jun; ↑ *c-fos* and *c-jun*	Human papillary thyroid carcinoma cells	[264]
	↓ NF-κB activity; ↑ cytochrome *c* release, activation of caspase-3	Human pancreatic cancer cells	[265]
Curcumin			
	↑ Bax, p53, Bak, PUMA, NOXA, and Bim, ↓ Bcl-2 and Bcl-xl; ↑ translocation of Bax and p53 to mitochondria, ↑ Phosphorylation and acetylation of p53; ↑ ROS, ↓ mitochondrial membrane potential, ↑ cytochrome *c* release, ↑ activation of caspase 3	LNCaP cells	[156]
	↑ Bax and p53, Bak, ↓ Bcl-2, ↑ ROS, ↓ Mitochondrial membrane potential, ↑ p21, ↑ cytochrome *c* release, ↑ Activation of caspase 3	Colo205 cells and HL-60 cells	[157,158]
	↑ Nuclear translocation and DNA binding of p53, ↑p21 and Gadd45	Human basal cell carcinoma cells	[159]
EGCG			
	↑ p53 phosphorylation and stabilization, ↑ MDM2, ↓ NF-κB, ↓ Bcl-2, ↑ Bax, ↑ p21, ↑ Bax/Bcl-2, ↑ caspase activation, ↑ PARP cleavage	LNCaP cells	[185]
	↑ Caspase-9, ↓ survivin, ↓ Akt	MCF-7 cells	[186]
	↓ Phosphorylation of EGFR and HER-2^{neu}, ↓ pERK, ↓ pAkt, ↓ Promoter activity of NF-κB, AP-1, and cyclin D1	HT-29 cells	[187]

TABLE 1.3 (continued)
Major Molecular Targets of Selected Dietary Chemopreventive Phytochemicals to Induce Apoptosis

Compound	Molecular Targets	Cells/Tissues	References
Sulforaphane			
	↓ Bcl-2, ↑ caspase activation	DU 145 cells	[226]
	↑ Bax, ↑ cytochrome *c* release, ↑ Caspase activation	HT-29 cells	[228]
	↑ Fas-FasL signaling, ↑ caspase 8 and 3, ↑ PARP cleavage	MDA-MB-231 cells	[229]
	↓ Bcl-2, ↑ cytochrome *c* release, ↑ Caspase 9 and 3	MCF-7 cells, T47D cells	[229]
	↑ Bax and Bak, ↓ NF-κB activity, ↓ IAPs, ↑ Apaf-1	Prostate cancer cells	[230,231]
	↑ ERK and JNK, ↑ AP-1	PC-3 cells	[232]
[6]-Gingerol			
	↓ NF-κB activity, ↓ cIAP1, ↑ caspase 7 and 3	Gastric cancer cells	[241]
	↑ ROS, ↓ Bcl-2	HL-60 cells	[243]
Capsaicin			
	↑ ROS, ↓ mitochondrial membrane potential, ↑ cytochrome *c* release, ↑ caspase 3	HL-60 cells	[257]
	↑ ROS, ↑ ERK, ↑ JNK	PC-3 cells	[256]
	↑ Cytochrome *c* release, ↑ caspase 3, ↑ PARP cleavage, ↓ Bcl-2	B16-F10 melanoma cells	[255]
	↑ ROS, ↑ caspase 3	Esophagus epidermoid carcinoma cells	[258]

Tumor cells adapt to the hypoxic microenvironment by inducing hypoxia-responsive genes encoding proteins involved in cell proliferation and apoptosis, glucose metabolism, pH regulation, iron metabolism, extracellular matrix metabolism, erythropoesis, inflammation, and angiogenesis [81,82].

One of the key transcription factors that regulate expression of hypoxia-responsive genes is the transcription factor hypoxia inducible factor (HIF), which acts as a master regulator of cellular oxygen homeostasis [83]. The HIF is induced at early stages of carcinogenesis and often correlated with increased angiogenesis in progressing tumors [80]. The HIF family of transcription factors includes four members: HIF-1α, HIF-1β, HIF-2α, and HIF-3α. While HIF-1α and HIF-2α are overexpressed in many human cancers, a splice variant of HIF-3α acts as an inhibitor of HIF-1α [79,81]. An increase in HIF-1α protein has been recorded in cancers of breast, prostate, lungs, and pancreas [81]. The induction of HIF-1α has also been observed in spontaneously generated epidermal squamous cell

carcinomas in human papilloma virus-16 transgenic mice [84]. Several studies have reported that HIF-2α is upregulated in tumor parenchymal cells and in tumor-associated macrophages (TAM), which are recruited to the hypoxic, avascular regions of tumors by growth factors and chemokines resulting in tumor progression [81,85,86]. A number of HIF-regulated angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), VEGF receptor (VEGFR), IL-8, iNOS, and angiopoietins, are released by macrophages [81,87]. Many of these factors further accelerate inflammatory angiogenic process, thereby triggering tumor growth [88].

Mechanisms underlying the elevated expression of HIF and HIF-regulated inflammatory and angiogenic gene products in tumors includes control of mRNA expression, protein stability, and activity of HIF [79]. Under hypoxic conditions, HIF-1α protein escapes prolylhydroxylase/von Hippel Lindau (VHL)-dependent proteasomal degradation, thus, forming a heterodimer with HIF-1β and subsequent binding to the hypoxia response elements (HRE) located in the promoter of target genes [79]. Under normoxic conditions, the transcriptional activity of HIF-1α is regulated by factor inhibiting HIF-1α (FIH), which prevents the binding of HIF-1α with transcriptional coactivator p300/CBP by hydroxylation of an asparagene residue located in the C-terminal domain of HIF-1α [89,90]. The activation of extracellular signal-regulated protein kinase (ERK) enhances transcriptional activity of HIF-1α by promoting phosphorylation and subsequent nuclear localization of HIF-1α [91,92]. Moreover, loss-of-function of tumor suppressor genes, such as VHL, p53, and PTEN, results in increased HIF-1α and HIF-2α activity [79]. On the other hand, gain-of-function of oncogene products, such as Ras, vSrc, epidermal growth factor receptor (EGFR), and HER-2^{neu}, and subsequent signaling through PI3K/Akt and MAP kinase pathways lead to HIF-1α accumulation, facilitating angiogenesis [79,81]. As presented in Table 1.4, dietary chemoprevention can be achieved by targeting proangiogenic factors, especially HIF-1α and VEGF.

TABLE 1.4
Selected Dietary Chemopreventive Phytochemicals Targeting Proangiogenic Molecular Switches

Compound	Molecular Targets	Cells/Tissues and Stimuli	References
Resveratrol			
	↓ VEGF binding to HUVEC	HUVEC	[266]
	↓ Expression of HIF-1α and VEGF	OVCAR-3 cells	[139]
	↑ Proteasomal degradation of HIF-1α, ↓ Akt, ↓ MAP kinase, ↓ RSK, ↓ expression of HIF-1α and VEGF	Hypoxia-stimulated SCC-9 cells, HepG2 cells	[140]

TABLE 1.4 (continued)
Selected Dietary Chemopreventive Phytochemicals Targeting Proangiogenic Molecular Switches

Compound	Molecular Targets	Cells/Tissues and Stimuli	References
	↑ Proteasomal degradation of HIF-1α, ↓ Akt, ↓ ERK, ↓ Secretion of VEGF in culture media	MDA-MB-231 cells	[141]
Curcumin			
	↓ Expression and activity of HIF-1α,	Hep3B hepatoma xenografted tumor in mice	[173]
	↓ Expression of erythropoetin and VEGF, ↓ expression and activity of HIF-1α, ↓ Expression of VEGF	Hypoxia-stimulated vascular endothelial cells and HepG2 cells	[174]
EGCG			
	↓ Expression of HIF-1α and VEGF, ↑ Proteasomal degradation of HIF-1α, ↓ PI3K/Akt, ↓ ERK1/2	Hypoxia- and serum-stimulated HeLa cells and HepG2 cells	[213]
	↓ VEGF, ↑ AMPK	HT-29 cells	[193]
Sulforaphane			
	↓ Angiogenesis and microtubule polymerization	VEGF-impregnated matrigel plug bearing Balb/c mice	[236]
	↓ mRNA levels of HIF-1α, VEGF and c-Myc	Hypoxia-stimulated human microvascular endothelial cells	[237]
[6]-Gingerol			
	↓ VEGF-induced capillary-like tube formation	HUVEC	[245]
	↓ Sprouting of endothelial cells	Rat aorta	
Capsaicin			
	↓ VEGF expression	Human multiple myeloma (U266) cells	[261]

1.3 CELL SIGNALING MOLECULES AS TARGETS OF SELECTED DIETARY CHEMOPREVENTIVE PHYTOCHEMICALS

Accumulating evidence from population-based and laboratory studies indicates an inverse relationship between regular consumption of fruits and vegetables

and the risk of cancer [5]. The National Cancer Institute (NCI) of the United States has identified about 40 plant-based foods that possess chemopreventive properties. Attention has recently been focused on a vast reservoir of nonnutritive phytochemicals present in fruits, vegetables, spices, and beverages as potential chemopreventive agents. It is now estimated that more than 1000 different phytochemicals possess chemopreventive activities [5]. Examples of chemopreventive dietary phytochemicals are epigallocatechin gallate (EGCG) from green tea, curcumin from turmeric, genistein from soybeans, sulforaphane from broccoli, proanthocyanidins from grape seeds, indole-3-carbinol from cabbage, resveratrol from grapes, lycopene from tomatoes, organosulfur compounds from garlic, gingerol from ginger, and caffeic acid phenethyl ester (CAPE) from honey bee propolis [5]. The following section will focus on an overview of the modulation of cell signaling molecules by selected dietary chemopreventive phytochemicals.

1.3.1 Resveratrol

Resveratrol (*trans*-3,5,4′-trihydroxystilbene), an extensively investigated dietary chemopreventive phytochemical present in grapes and other plant species, exerts antioxidant, anti-inflammatory, and chemopreventive activities by modulating various components of cellular signaling pathways [93]. John M. Pezzuto and colleagues first reported the ability of resveratrol to interfere with initiation, promotion, and progression stages of carcinogenesis [94]. Subsequent studies demonstrated that resveratrol prevented chemically induced tumorigenesis in different animal models [95–97]. Resveratrol exerts chemopreventive effects primarily by enhancing cellular antioxidant/detoxifying enzymes [98]. The compound induced NQO1 activity in Hepa1c1c7 cells [98]. Recent studies have demonstrated that the compound can induce HO-1 expression and activity in human aortic smooth muscle [99] and rat pheochoromocytoma (PC12) cells [100] via activation of NF-κB and Nrf2, respectively.

Resveratrol exhibited antiproliferative effects by interfering with DNA synthesis [101] and various stages of cell cycle progression [102]. Resveratrol arrested the growth of human epidermoid carcinoma (A431) cells via upregulation of $p21^{WAF1/CIP1}$, downregulation of the expression of various cyclins (D1, D2, and E), and inhibition of the protein expression and catalytic activities of Cdk (2, 4 and 6), thereby imposing artificial checkpoints at the G1/S transition of the cell cycle [103]. In addition, the antiproliferative effect of resveratrol in A431 cells was associated with a decrease in the hyperphosphorylated form of Rb protein and subsequence inhibition of the expression of E2F transcription factor [104]. Similarly, resveratrol suppressed the expression of Cdks (2, 4, 6) and cyclins (D1 and D2), and upregulated the expression of $p21^{WAF1/CIP1}$ and p53 in SKH-1 hairless mouse skin stimulated with UV radiation [105]. Likewise, resveratrol elicited an antiproliferative effect by targeting cyclin D1 and Cdk4 in human prostate cancer (DU-145) [106] and human breast cancer (MCF-7) [107]

cells, which was associated with the induction of p53 and $p21^{WAF1/CIP1}$. Moreover, resveratrol blocked the formation of cyclin E-Cdk2 complex in DU-145 cells without changing the protein levels [106]. Treatment of human lung carcinoma (A549) cells with resveratrol resulted in the S-phase arrest, which was associated with the inhibition of Rb phosphorylation and induction of $p21^{WAF1/CIP1}$ and p53 [108]. The proteins of the cell cycle regulatory process were differentially targeted by resveratrol in a cell-specific manner. While resveratrol inhibited the expression of cyclin B1 in a variety of human cancer cells (MCF-7, SW480, HCE-7, Seg-1, Bic-1, and HL-60), it downregulated the expression of cyclin A, cyclin B1, and cyclin D1 in human colon cancer (SW480) cells [109]. Although resveratrol attenuated the expression of cyclin D1 in SW480 cells, it failed to inhibit the cyclin D1 promoter activity [109]. Resveratrol inhibited the expression as well as complex formation of cyclin D1 and Cdk4, but increased the expression of cyclin E and cyclin A in human colon cancer (Caco2 and HCT-116) cells [110].

One of the underlying molecular mechanisms of chemoprevention with resveratrol is the induction of apoptosis in precancerous or malignant cells. Resveratrol has been shown to induce apoptosis in various transformed or malignant cell types by activating p53 and specific caspases, stimulating cytochrome *c* release, upregulating proapoptotic Bax, downregulating antiapoptotic Bcl-2, and inducing DNA fragmentation [102,107,111]. The induction of apoptosis in human promyelocytic leukemia (HL-60) and breast cancer (T47D) cells by resveratrol was mediated via activation of the CD95-CD95L signaling pathway [112]. In contrast, resveratrol-induced apoptosis in SW480 cells was not mediated through modulation of Fas/FasL interaction, but was attributable to caspase activation and increased localization of Bax and Bak to mitochondria [113]. Huang et al. [114] demonstrated that resveratrol induced apoptosis in human embryonic fibroblast cells that express wild-type p53, but not in p53-deficient cells, suggesting a p53-dependent induction of cell death by this phytochemical. The upregulation of p53-responsive genes such as $p21^{WAF1/CIP1}$, *p300/CBP*, and *Apaf1* by resveratrol led human prostate cancer (LNCaP) cells to undergo apoptosis [115]. Similarly, resveratrol induced apoptosis in human hepatocellular carcinoma (HepG2) cells by a p53-dependent increase in Bax and p21 [116]. Resveratrol increased the MAP kinases-mediated phosphorylation of p53 at serine 15 residue, thereby inducing apoptosis in mouse epidermal JB-6 Cl 41 cells [117,118]. Alternatively, a p53-independent mechanism for resveratrol-induced apoptosis of human colon carcinoma (HCT-116) cells was reported [119,120]. Therefore, resveratrol induced apoptosis in various cancer cells in a cell type-specific manner, being p53-dependent in certain cells, while p53-independent in others.

Molecular mechanisms underlying anti-inflammatory and antitumor-promoting activities of resveratrol include the inhibition of cytokine release and proinflammatory gene expression, downregulation of intracellular signal transduction molecules and transcription factors that regulate expression of proinflammatory genes [93,121]. The induction of proinflammatory gene products such as COX-2 and iNOS by diverse stimuli, including LPS, TPA, and

interferon-γ, was attenuated by resveratrol [93,122]. Resveratrol significantly inhibited expression of COX-2 in LPS-, TPA- or H_2O_2-stimulated mouse peritoneal macrophages [123], LPS plus interferon-γ (IFN-γ)-treated RAW 264.7 macrophages [122], and TPA-stimulated mouse skin [124]. Besides the protein expression, resveratrol also downregulated the expression of *cox-2* mRNA transcripts in *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors in F344 rats [97] and the production of PGE_2 in peripheral blood leukocytes stimulated with LPS plus IFN-γ [125]. The expression and activity of another proinflammatory enzyme iNOS were also diminished by resveratrol [122,126]. The compound inhibited the expression of iNOS protein and mRNA, and reduced NO generation in LPS-activated RAW 264.7 cells by blocking IκBα phosphorylation and NF-κB activation [126].

Resveratrol suppressed activation of NF-κB in Jurkat-T and human cervical carcinoma (HeLa) cells exposed to various proinflammatory stimuli including TPA, LPS, H_2O_2, okadaic acid, and ceramide [127]. In response to TNF-α, LPS, TPA, or UVB, resveratrol modulated the intracellular signaling through the IKK-IκBα-NF-κB pathway [124,127–130]. It has been recently reported that topical application of resveratrol attenuated TPA-induced NF-κB activation in mouse skin by blocking the activation of IKK, phosphorylation of IκBα and p65, nuclear translocation of p65, and the interaction of p65 with a transcriptional coactivator cyclic AMP-response element binding protein–binding protein (CBP) [124]. The AP-1 is another molecular target of resveratrol in suppressing inflammation, cell proliferation, and tumor promotion [131]. Resveratrol decreased TPA-induced transcriptional activity of AP-1 in human mammary epithelial cells [132], but failed to suppress AP-1-driven transcriptional activity in LPS-stimulated THP-1 cells [130]. However, subsequent studies revealed that resveratrol negated TPA-induced DNA binding of AP-1 in U937 cells [133] and mouse skin [131].

The activation of a panel of upstream kinases is also modulated by resveratrol. The compound suppressed TPA-induced activation of MAP kinases in HeLa cells [134] and mouse skin *in vivo* [124,135]. Besides MAP kinases, the activation of other upstream signaling kinases such as protein tyrosine kinase and PKC was also inhibited by resveratrol [132,136]. Resveratrol inhibited H_2O_2-induced NF-κB activation in HeLa cells partly by blocking activation of PKCμ, alternatively known as protein kinase D [137]. The phosphorylation of another upstream kinase Akt in MCF-7 cells was abrogated by resveratrol [138].

Resveratrol inhibited the expression of HIF-1α and VEGF in human ovarian cancer (OVCAR-3) cells through multiple mechanisms involving inhibition of Akt and MAP kinases, inhibition of protein translational regulators (e.g., ribosomal protein S6 kinase (RSK)-1, eukaryotic initiation factor 4E-binding protein-1, and eukaryotic initiation factor 4E), and enhancement of proteasomal degradation of HIF-1α protein [139]. Moreover, resveratrol significantly reduced hypoxia-induced HIF-1α protein accumulation and VEGF expression in human tongue squamous cell carcinomas (SCC-9) and HepG2 cells, without affecting HIF-1α mRNA levels, partly by inhibiting activation of ERK and Akt and

promoting proteasomal degradation of HIF-1α [140]. According to a recent study, resveratrol reduced tumor growth and angiogenesis in ERα/ERβ(+) human breast tumors (MDA-MB-231) xenografts in nude mice and reduced extracellular levels of VEGF *in vitro* [141].

1.3.2 Curcumin

Curcumin, a polyphenol isolated from the rhizomes of turmeric (*Curcuma longa* Linn, family-Zingiberaceae), has been shown to inhibit chemically induced carcinogenesis in various animal models *in vivo* [142–145] and transformation of cultured cells [146]. Curcumin targets multiple intracellular signaling molecules involved in the initiation and promotion stages of carcinogenesis. Curcumin activates the Nrf2-ARE signaling pathway to induce phase 2 detoxifying enzymes. Balogun et al. [147] reported that curcumin disrupted the Nrf2–Keap1 complex, leading to increased Nrf2 binding to ARE and subsequent increases in the expression and activity of HO-1 in porcine renal epithelial proximal tubule (LLC-PK_1) cells and rat kidney epithelial (NRK-52E) cells via activation of p38 MAP kinase. Curcumin also enhanced the expression of GCL at mRNA and protein levels in immortalized human bronchial epithelial (HBE1) cells by promoting nuclear translocation and DNA binding of Nrf2-ARE [148]. The treatment of HepG2 cells with curcumin resulted in the elevation of GSTP-1 mRNA and Nrf2-ARE-regulated GSTP1 reporter gene activity [149]. At a nontoxic concentration, curcumin (10 μM) induced HO-1 expression by activating Nrf2 via ROS generation, activation of PKC and p38 MAP kinases, and the inhibition of protein phosphatase activity in human hepatoma cells [150]. In a recent study, Pae et al. [151] reported that curcumin inhibited serum- and TNFα-induced growth of rat vascular smooth muscle cells (rVSMC) and human aortic smooth muscle cells, respectively by activating Nrf2-ARE-mediated induction of HO-1 and increasing the expression of p21, while its hydrogenated analogue tetrahydrocurcumin failed to induce such effects. Moreover, cotreatment of rVSMC cells with curcumin and tin protoporphyrin, a HO-1 inhibitor, partially abolished curcumin-induced $p21^{WAF1/CIP1}$ expression and growth inhibition, suggesting that the antiproliferative effect of curcumin is mediated via upregulation of HO-1. Structurally, curcumin contains an α, β unsaturated carbonyl moiety and can hence act as a Michael reaction acceptor, which may cause thiol modification of Kaep1, thereby facilitating Nrf2 nuclear translocation.

The antiproliferative effects of curcumin are reflected in its ability to induce growth arrest and apoptosis in various premalignant and malignant cells. Curcumin diminished cyclin D1 protein and cyclin D1-dependent promoter gene expression, at both transcriptional and posttranscriptional levels, in a variety of human cancer cells [152]. Curcumin induced G0/G1 and/or G2/M phase cell cycle arrest by upregulating $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and p53, and downregulating cyclin B1 and Cdc2 in immortalized human umbilical vein endothelial cells [153]. Similarly, curcumin inhibited proliferation of melanoma cells by arresting cell growth at the G2/M phase of the cell cycle in association with upregulation

of p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and Chk2 [154]. This study also demonstrated that curcumin inhibited the activation of NF-κB and the expression of iNOS [154]. The G2/M phase arrest in human bladder carcinoma (T24) cells by curcumin treatment was associated with downregulation of cyclin A and upregulation of $p21^{WAF1/CIP1}$ [155].

The suppression of growth and induction of apoptosis in LNCaP cells by curcumin were associated with the inhibition of the PI3K/Akt-mediated cell survival pathway resulting in downregulation of Bcl-2 and Bcl-xl, and upregulation of p53, Bax, Bak, PUMA, Noxa, and Bim [156]. According to this study, treatment of LNCaP cells with curcumin resulted in translocation of Bax and p53 to mitochondria, phosphorylation and acetylation of p53, production of ROS, drop in the mitochondrial membrane potential, release of cytochrome *c*, and activation of caspase-3 [156]. Similarly, induction of apoptosis in colon cancer (Colo 205) and HL-60 cells with curcumin was dependent on the generation of ROS, induction of Bax and p53, reduced mitochondrial membrane potential, release of cytochrome *c*, upregulation of $p21^{WAF1/CIP1}$, downregulation of Bcl-2, and finally the activation of caspase-3 [157,158]. Curcumin also induced apoptosis in human basal cell carcinoma cells by increasing nuclear translocation and DNA binding of p53, inducing p53-regulated gene products such as $p21^{WAF1/CIP1}$ and Gadd45, but did not interfere with the cell cycle regulatory process as well as the expression of Bax and Bcl-2 proteins [159]. The compound decreased the basal levels of Ets-1 and Bcl-2 in human endometrial adenocarcinoma (HEC-1-A) cells and induced an apoptosis-like morphological changes [160].

The antitumor-promoting effects of curcumin have largely been attributed to its ability to modulate proinflammatory signal transduction pathways. Curcumin inhibited mediators of inflammation such as NF-κB, AP-1, COX-2, and iNOS by blocking the activation of upstream signal transduction molecules including IKK, MAP kinases, and PI3K. Curcumin inhibited expression of COX-2 and generation of PGE_2 in TPA-stimulated mouse skin [161] and human pancreatic cancer cells [162]. The compound inhibited activation of AP-1 and NF-κB in TPA-stimulated mouse skin *in vivo* as well as in cultured HL-60 cells [161,163]. The nuclear translocation of p65 was suppressed by curcumin via blockade of phosphorylation-dependent degradation of IκBα and phosphorylation of ERK [161,164]. The inhibition of IκBα degradation via downregulation of NF-κB-inducing kinase (NIK) and IKK appeared as the mechanism of curcumin inhibition of TNF-α-induced *cox-2* transcription and NF-κB activation in human colonic epithelial cells [165]. Moreover, curcumin blocked the activation of IKK in *Helicobacter pylori*-treated gastric epithelial (AGS) cells [166], multiple myeloma cells [167], and pancreatic cancer cells [162]. According to a recent study, curcumin reduced trinitrobenzene sulfonic acid-induced rat colon inflammation by suppressing the expression of COX-2 and iNOS via blockade of p38 MAP kinase [168]. Curcumin inhibited the expression of c-Jun and c-Fos proteins and their mRNA transcripts as well as AP-1 DNA binding in NIH3T3 cells [169] and diminished the expression of c-Jun and c-Fos proteins in CD-1 mouse skin after treatment with TPA [170]. Treatment of multiple myeloma cells with curcumin resulted in the

inhibition of constitutive as well as IL-6-induced STAT-3 phosphorylation [171]. Curcumin diminished TNF-α-induced phosphorylation of Akt, activation of NF-κB, and expression of NF-κB-regulated gene products such as COX-2, iNOS, Bcl-2, Bcl-xl, IAP, and cyclin D1, which was reversed by glutathione (GSH), suggesting that curcumin exerted anti-inflammatory and apoptosis-inducing effects by modulating cellular redox status [172].

Curcumin downregulated HIF-1α activity and the expression of HIF-1α-regulated angiogenic factors, such as erythropoetin and VEGF, in Hep3B hepatoma-xenografted tumors in mice [173]. Under hypoxic conditions, curcumin inhibited the expression and activity of HIF-1α and decreased the expression of VEGF in HUVEC and HepG2 cells [174].

1.3.3 EGCG

One of the most extensively investigated dietary sources of chemopreventive agents is green tea. EGCG, the major active component of green tea, has been known to possess antioxidant, anti-inflammatory, and chemopreventive properties [175,176]. Topical application of EGCG protected human skin from UV radiation-induced oxidative damage by restoring depleted GSH level and GPx activity [177]. EGCG also attenuated lipid peroxidation and restored the reduced levels of detoxification enzymes, namely GST, GPx, SOD, and CAT in mouse skin treated with 7,12-dimethylbenz[a]anthracene (DMBA) [178]. At a low concentration, EGCG increased ARE-luciferase activity and expression of ARE-regulated genes in HepG2 cells by activating MAP kinases [179]. A recent study from the authors' laboratory revealed that EGCG enhanced the expression of GCL and HO-1, nuclear translocation and ARE binding of Nrf2 in human mammary epithelial (MCF-10A) cells (Na and Surh, unpublished observations).

General mechanisms by which EGCG modulates deregulated cell cycle progression include reduced expression of cell cycle regulatory proteins (e.g., cyclin D1, cyclin E, Cdk2, Cdk4, and Cdk6), inhibition of cyclin-Cdk complex formation, induction of Cdk inhibitors (e.g., $p21^{WAF1/CIP1}$, $p27^{KIP1}$, p16, and p18), and suppression of phosphorylation of Rb [175,180,181]. EGCG inhibited growth of ovarian cancer cells (SKOV3, OVCAR and PA1) through induction of apoptosis and cell cycle arrest by modulating cell cycle regulatory proteins [182]. While EGCG arrested the cell cycle at the G1 phase in SKOV-3 (p53 negative cells) and OVCAR-3 (p53 mutant) cells, it arrested the cell cycle at the G1/S phase in PA-1 (expressing wild-type p53) cells [182]. The compound caused G1/S phase arrest in LnCaP and DU-145 cells irrespective of p53 status [180]. EGCG induced G0/G1 arrest and apoptosis in A431 cells by decreasing the total level of Rb, phosphorylation of Rb at serine 780 residue, downregulating expression of other Rb family proteins, namely p130 and p107, and suppressing the expression of E2F (1 ~ 5) family of transcription factors and their heterodimeric partners DP1 and DP2 [183]. The growth of human head and neck squamous carcinoma and human breast carcinoma cells was suppressed by EGCG through

inhibition of the phosphorylation of HER-2^{neu}, blockade of the activation of STAT-3, inhibition of promoter activity of *c-fos* and *cyclin D1*, and a decrease in the cellular levels of the cyclin D1 and Bcl-xl proteins [184].

Hastak et al. [185] demonstrated that EGCG induced apoptosis in LNCaP cells by stabilizing p53 via phosphorylation of critical serine residues, blocking p14/ARF-mediated inhibition of murine double minute-2 (MDM2) protein, and negatively regulating the NF-κB activity, thereby decreasing the expression of the NF-κB-regulated antiapoptotic protein Bcl-2. EGCG-induced stabilization of p53 protein resulted in the upregulation of p21$^{WAF1/CIP1}$ and Bax, thereby positively changing the ratio of Bax/Bcl-2, activating initiator and effector caspases and PARP cleavage, leading to the induction of apoptosis [185]. The inhibition of growth and induction of apoptosis in MCF-7 cells by EGCG were mediated via suppression of signaling through survivin and Akt, and activation of caspase 9 [186]. The G1 arrest and apoptosis of colon cancer (HT-29) cells resulted from EGCG inhibition of EGFR and HER2^{neu} protein phosphorylation and subsequent decrease in the phosphorylated forms of the ERK and Akt proteins [187]. The study also revealed that EGCG inhibited the transcriptional activity of AP-1, NF-κB, and cyclin D1 promoters [187]. Moreover, EGCG inhibited cell proliferation and blocked cell cycle transition at the G1 phase by decreasing cyclin D1 expression in Ha-*Ras*-transfected intestinal epithelial (RIE-1) cells [188].

EGCG blocks tumor promotion by targeting components of the proinflammatory signaling pathway. Major targets of EGCG in this pathway are COX-2, iNOS, proinflammatory cytokines and their upstream regulators such as MAP kinases and PI3K/Akt, and various transcriptional regulators. EGCG suppressed COX-2 expression in cells or tissues exposed to diverse stimuli including TPA [189], IL-1β [190], and NMBA [191], as well as in various malignant cells [192–194]. EGCG also decreased the production of PGE_2 [190,191]. The expression of TNF- α mRNA in macrophage 264.7 cells [195], keratinocytes [196], and BALB/3T3 cells [197] stimulated with LPS, UVB, and okadaic acid, respectively, was blunted by EGCG. The compound also attenuated the gene expression and release of IL-8 in normal human keratinocytes [198] and human airway epithelial (A549) cells [199] stimulated with TNF-α and IL-1β, respectively. In addition, EGCG inhibited the expression of iNOS and production of NO in human osteoarthritic chondrocytes [190,200] and murine peritoneal macrophages [201] stimulated with IL-1β and LPS, respectively.

NF-κB, AP-1, and CREB are major molecular targets of EGCG to block proinflammatory signaling. EGCG inhibited the activation of AP-1 in mouse epidermal JB6 cells stimulated with TPA [202] and UVB [203] or transformed with H-*ras* [204] and in the epidermis of transgenic mice bearing an AP-1-driven luciferase reporter gene [205]. The compound diminished TPA-induced activation of CREB in mouse skin by blocking p38 MAP kinase [206]. EGCG also diminished the activation of NF-κB in TPA-stimulated JB6 cells [207] and mouse skin *in vivo* [206] by blocking phosphorylation-dependent degradation of IκBα. The inactivation of NF-κB by EGCG was associated with inhibition of IKK

activity [199,208,209], enhancement of phosphorylation-dependent degradation of IκBα and subsequent increases in nuclear translocation of p65 protein [199,208]. Besides interference with the IKK-IκB signaling, EGCG showed inhibitory effects on the activation of MAP kinases [189,210]. A hydrophilic cream containing EGCG prevented UVB-induced phosphorylation of ERK, p38 MAP kinase, and c-Jun N-terminal kinase (JNK) in SKH-1 hairless mouse skin [211]. Moreover, EGCG inhibition of NF-κB signaling was linked with suppression of Akt phosphorylation [194,208,212].

EGCG significantly inhibited hypoxia- and serum-induced HIF-1α protein expression in HeLa and HepG2 cells by blocking PI3K/Akt and ERK1/2 signaling pathways, and enhancing the proteasomal degradation of HIF-1α [213]. The suppression of HIF-1α by EGCG resulted in a dramatic decrease in VEGF mRNA and protein expression [213]. The compound also suppressed VEGF expression by activating AMP-activated protein kinase (AMPK) in HT-29 cells [193]. Treatment with EGCG suppressed VEGF-induced mitogenesis in human umbilical arterial endothelial cells (HUAEC) by blocking DNA synthesis, cell proliferation, autophosphorylation of VEGFR, phosphorylation of ERK1/2, and mRNA expression of the early growth response factor-1 [214]. Thus, the inhibition of signaling mediated via HIF-1α and VEGF appears as the mechanism of antiangiogenic effects of EGCG.

1.3.4 Sulforaphane

Sulforaphane [1-isothiocyanato-(4*R,S*)-(methylsulfinyl)butane] and its analogues, commonly known as isothiocyanates, are promising chemopreventive entities present in broccoli sprouts and mature broccoli [215]. Sulforaphane suppressed chemically induced carcinogenesis in experimental animals [216,217]. It has been reported that sulforaphane induces phase 2 enzymes by activating certain MAP kinases and Nrf2 [73,218–220]. The p38 MAP kinase negatively regulates Nrf2 activation by promoting phosphorylation of Nrf2 and its interaction with inhibitory protein Keap1 [221]. Sulforaphane induced HO-1 expression in HepG2 cells by downregulating p38 MAP kinase, thereby activating Nrf2-ARE signaling [221]. The compound upregulated the expression of detoxifying enzymes including NQO1, GST, and GCL in the small intestine of *Nrf2* wild-type mice, while the *Nrf2*-null mice displayed lower levels of these enzymes [222]. Sulforaphane elevated the mRNA expression of NQO-1 and UGT1A1 in human colon adenocarcinoma (Caco-2) cells by activating PI3K/Akt- and MEK/ERK-mediated signaling [223]. Besides the modulation of upstream kinases, the mechanism of Nrf2 activation by sulforaphane involves a direct covalent binding of sulforaphane with cysteine residue on Keap1 leading to dissociation of Nrf2 from Keap1 [224].

The antiproliferative and apoptotic activity of sulforaphane have been studied extensively [73]. Sulforaphane caused G1 cell cycle arrest in LNCaP cells by inhibiting the expression of cyclin D1 and DNA synthesis, and induced apoptosis

through caspase activation [225]. When androgen-independent DU-145 cells were treated with sulforaphane, there was a decrease in the expression of cyclin D1 and Cdk4, reduced phosphorylation of Rb proteins and induction of Cdk inhibitor $p21^{WAF\text{-}1/CIP\text{-}1}$ resulting in growth inhibition [226]. In addition, sulforaphane induced apoptosis in DU-145 cells by downregulating Bcl-2 and activating caspases [226]. Sulforaphane induced G1 cell cycle arrest in HT-29 cells by downregulating cyclin D1, cyclin A, and c-Myc at both mRNA and protein levels and increasing the expression of p21 in a ROS- and MAP kinase-dependent, but p53-independent manner [227].

Similarly, sulforaphane induced apoptosis in HT-29 cells by upregulating Bax, mitochondrial release of cytochrome *c*, and PARP cleavage without affecting p53 expression [228]. Sulforaphane induced G2/M phase arrest in several human breast cancer cells (MDA-MB-231, MDA-MB-468, MCF-7, and T47D) and induced apoptosis following both mitochondria-dependent and -independent mechanisms [229]. While apoptosis induced by sulforaphane in MDA-MB-231 cells was initiated through induction of Fas-FasL resulting in the activation of caspase-8, caspase-3, and PARP cleavage, the apoptosis in the other breast cancer cells was associated with a decrease in Bcl-2 expression, release of cytochrome *c*, and activation of caspase-9 and caspase-3 [229]. Sulforaphane was found to cause apoptosis in prostate cancer cells by mechanisms involving the activation of proapoptotic proteins Bax and Bak, inhibition of the NF-κB activity, downregulation of IAP family proteins, and induction of Apaf-1 [230,231]. The compound also induced apoptosis in human prostate cancer (PC-3) cells by activating ERK, JNK, and AP-1 [232].

Besides modulation of biotransformation enzymes and cell cycle regulatory proteins, sulforaphane targets molecules involved in proinflammatory signaling pathways. Sulforaphane inhibited LPS-induced expression of COX-2 and iNOS and the secretion of PGE_2 and TNFα in Raw 264.7 murine macrophages by blocking the activation of NF-κB via cysteine thiol modification [233]. Khor et al. [35] demonstrated that sulforaphane upregulated a set of proapoptotic genes, while downregulated the expression of prosurvival gene cyclin-D2 and COX-2 in $Apc^{Min/+}$ mice. Sulforaphane and structurally related phenylethylisothiocyanates significantly inhibited the NF-κB transcriptional activity and expression of NF-κB-regulated genes such as *VEGF*, *cylcin D1*, and *Bcl-xl* by blocking phosphorylation of IKK and degradation IκBα, and nuclear translocation of p65 in PC-3 cells [234]. The UVB-induced AP-1 transcriptional activity in HaCaT cells stably transfected with AP-1-luciferase reporter plasmid was diminished by sulforaphane [235].

Sulforaphane targets various proangiogenic factors including VEGF. Administration of sulforaphane to female Balb/c mice bearing VEGF-impregnated Matrigel plugs revealed that the compound suppressed angiogenesis and disrupted endothelial mitotic progression and microtubule polymerization [236]. Moreover, sulforaphane exhibited a time- and concentration-dependent inhibitory effect on hypoxia-induced expression of HIF-1α, VEGF, and c-Myc mRNA in human microvascular endothelial cells [237].

1.3.5 [6]-Gingerol

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) is a widely used condiment and contains a variety of anti-inflammatory phytochemicals. [6]-Gingerol, one of the major pungent principles of ginger, inhibited chemically induced tumorigenesis, TPA-induced ornithine decarboxylase activity, and TNF-α production in mouse skin [238]. The induction of AP-1 and neoplastic transformation of mouse epidermal JB6 cells stimulated with epidermal growth factor were attenuated by [6]-gingerol [239]. Topical application of [6]-gingerol inhibited TPA-induced COX-2 expression in mouse skin by suppressing the degradation of IκBα and nuclear translocation of p65 via blockade of phosphorylation of p38 MAP kinase [240]. [6]-Gingerol also diminished NF-κB transcriptional activity in TPA-treated mouse skin by blocking the interaction of phosphorylated p65 with the CBP/p300 [240].

The apoptosis-inducing effects of [6]-gingerol in tumor cells have been reported [241–243]. [6]-Gingerol facilitated TRAIL-induced apoptosis in gastric cancer cells by suppressing the activation of NF-κB and expression of cIAP1, thereby activating caspase-3 and -7 [241]. The compound also induced ROS-dependent apoptosis in HL-60 cells by inhibiting the constitutive expression of Bcl-2 [243]. Treatment of human pancreatic cancer cells (wild-type p53-expressing HPAC and mutant p53-expressing BxPC-3 cells) with [6]-gingerol inhibited cell growth through G1 phase arrest by inducing $p21^{WAF1/CIP1}$, down-regulating the expression of cyclin A and Cdk, and subsequently suppressing the phosphorylation of Rb protein [244]. Moreover, the induction of apoptosis by [6]-gingerol was observed in BxPC-3 cells, but early signs of apoptosis in HPAC cells were absent, suggesting that [6]-gingerol exerts a cytotoxic effect in mutant p53-expressing cells and a cytostatic effect in wild-type p53-expresing cells [244]. [6]-Gingerol inhibited both the VEGF- and bFGF-induced proliferation of human endothelial cells and caused cell cycle arrest in the G1 phase [245]. It also blocked VEGF-induced capillary-like tube formation by endothelial cells and sprouting of endothelial cells in the rat aorta, and formation of new blood vessels in the mouse cornea [245].

1.3.6 Capsaicin

The chemopreventive potential of capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), a pungent principle of hot chili pepper (*Capsicum annuum* L., Solanaceae), has been reviewed earlier [246]. Capsaicin induced expression of HO-1 in HepG2 cells by activating PI3K/Akt-mediated Nrf2 signaling in a ROS-dependent manner [247]. This study hypothesizes that a quinone metabolite or other reactive forms of capsaicin might covalently modify NQO-1, thereby suppressing the expression and activity of NQO-1, which leads to production of ROS and subsequent PI3K/Akt-mediated activation of Nrf2 [247].

As underlying molecular mechanisms of its antitumor-promoting activity, capsaicin was found to inhibit the activation of NF-κB by blocking IκBα degradation

and p65 nuclear translocation in mouse skin [248]. Duvoix et al. [249] demonstrated that treatment of K562 and U937 leukemia cells with capsaicin significantly inhibited TNF-α- and TPA-induced DNA binding of AP-1 and NF-κB. Moreover, capsaicin inhibited LPS- and TPA-induced COX-2 expression and PGE_2 production as well as LPS-induced NF-κB and AP-1 activation in Raw 264.7 cells [250]. Similarly, capsaicin blocked COX-2 enzyme activity, production of PGE_2, and expression of iNOS by inhibiting IκBα degradation and NF-κB activation in LPS-stimulated peritoneal macrophages [251]. Capsaicin also inhibited LPS-induced TNF-α production in Raw 264.7 cells [252].

A decrease in the NF-κB activation by capsaicin was accompanied by the growth arrest of human T-cell leukemia virus type 1 (HTLV-1)-associated adult T-cell leukemia (ATL) cells [253]. Moreover, capsaicin inhibited constitutive activation of NF-κB in malignant melanoma cells, leading to the induction of apoptosis [254]. Capsaicin induced G0/G1 phase arrest in HL-60 cells by blocking Cdk2 and the cyclin E complex. The induction of apoptosis in various transformed cells by capsaicin was mediated via generation of intracellular ROS and Ca^{2+} [255–258]. Capsaicin induced apoptosis in HL-60 cells, human esophagus epidermoid carcinoma cells, and B16-F10 melanoma cells, which was accompanied by the decrease in levels of mitochondrial membrane potential, enhanced cytochrome *c* release, and increased activation of caspase-3 [255,257,258]. Treatment of HT-29 cells with capsaicin caused apoptosis via upregulation of AMPK [259].

Capsaicin caused G1 arrest in endothelial cells that was correlated with the downregulation of the expression of cyclin D1-Cdk-4 and reduced phosphorylation of Rb protein. Moreover, capsaicin inhibited VEGF-induced expression of p38 MAP kinase and Akt in endothelial cells [260]. Capsaicin also inhibited the constitutive or IL-6-induced expression of STAT-3-regulated gene products, such as cyclin D1, Bcl-2, Bcl-xl, survivin, and VEGF in human multiple myeloma (U266) cells, by downregulating JAK-STAT-3-mediated signaling [261].

1.4 CONCLUSION

Over the past several decades, advances in biotechnology and multidisciplinary research led us to have better insight into the cellular and molecular events associated with the pathophysiology of cancer. In the early stage of tumor initiation, the disruption of cellular defense mechanisms comprising a battery of detoxifying/antioxidant enzymes would make cells/tissues more vulnerable to DNA damage by environmental carcinogens. Beyond the demarcation of stage-specific multistep carcinogenesis, biochemical processes such as abnormal cell proliferation, resistance to programmed cell death and angiogenesis, and associated improper intracellular signaling pathways play a central role in the journey to cancer. The reversal or blockade of inappropriate signaling pathways and the restoration of the normal cellular signaling orchestra may provide the basis of developing cancer chemopreventive agents.

Considering the burden of health care costs as a major global concern, the dietary chemoprevention strategy provides an inexpensive, readily applicable, and easily accessible approach to cancer control and management. Since the inception

of chemoprevention research, numerous dietary phytochemicals have been shown to be effective in preventing malignant transformation of cells *in vitro* and tumorigenesis *in vivo*. As discussed in previous sections, chemoprevention with a particular dietary phytochemical results from a sum of multiple mechanisms. The extreme complexity of the intracellular signaling network, especially some still unidentified cross talk between signaling molecules, appears to be a major barrier to simplifying the mechanistic basis of chemoprevention. However, the inhibition of well-defined prosurvival pathways and induction of apoptotic signaling cascades appear as common mechanisms of chemoprevention with dietary phytochemicals. Among the cell signaling molecules, Nrf2 and HIF-1α appear as critical targets for chemoprevention. Although a direct link between Nrf2 and HIF-1α is yet to be established, existing literature suggests that targeted induction of Nrf2 and inhibition of HIF-1α by edible phytochemicals may provide molecular basis of dietary chemoprevention. Besides the modulation of cell signaling molecules, the current advances in assessing the single nucleotide polymorphism have made it possible to identify the specific genes that contribute to individual differences in cancer susceptibility. Thus, it would not be an overenthusiastic thought to provide the high-risk group of people carrying altered cancer-related genes with a cocktail of phytochemicals as a "personalized chemopreventive regimen" in the near future.

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2 Modulation of Cell Signal Transduction by Tea and Ginger

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CONTENTS

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2.1 INTRODUCTION

Research findings clearly indicate that the multifaceted, intricate process of carcinogenesis involves changes in a plethora of genes and gene products that are critical in the regulation of a copious number of cellular functions. Elucidating the molecular and cellular mechanisms involved in cancer development is crucial in the development of effective, nontoxic agents to prevent this deadly disease. Many natural or dietary phytochemicals are believed to have potent anticancer activity with very few adverse side effects and thus have received intensive research attention. Accumulating research evidence suggests that many of these phytochemicals may be used alone or in combination with traditional chemotherapeutic agents to prevent or treat cancer. Therefore, identifying the specific signal transduction pathways, protein and gene targets, and mechanisms explaining the purported anticancer activity of various phytochemicals may provide effective alternatives or additions to traditional methods of cancer prevention (i.e., chemoprevention) or cancer treatment (i.e., chemotherapy).

Signal transduction is the process by which information from a stimulus outside the cell is transmitted through the cell membrane (e.g., through its receptor) into the cell and along an intracellular chain of signaling proteins to stimulate a response. Cancer is believed to be a multistage process and includes an initiation stage that can be relatively short and irreversible. The promotion stage is a long-term process that requires chronic exposure to a tumor promoter and may therefore be considered as rate limiting to the overall process of cancer development, which makes promotion an ideal target for intervention. Thus, understanding the molecular mechanisms of promotion is crucial for development of effective anticancer agents. Our laboratory has demonstrated strength in examining the signal transduction pathways associated with tumor promotion and in identifying novel mechanisms explaining the antitumor promotion activities of numerous phytochemicals, including the various components of green and black tea and [6]-gingerol. Phytochemicals and dietary factors have attracted a great deal of interest because of their perceived ability to act as highly effective chemopreventive agents. In addition to being professed as generally safe, they appear to have efficacy as anticancer agents by preventing or reversing premalignant lesions and/or reducing second primary tumor incidence [1]. One of the most vital outcomes of our research investigations has been the clarification of signal transduction pathways induced by tumor promoters in cancer development. The widespread opinion today is that cancer may be prevented or treated by targeting specific cancer genes, signaling proteins, and transcription factors. Significantly, the molecular mechanisms explaining how normal cells undergo transformation to cancer cells induced by tumor promoters are rapidly being refined. In particular, the mitogen-activated protein (MAP) kinase signaling pathways are activated differentially by various tumor promoters (reviewed in Refs. [2–6]). MAP kinases are activated by translocation to the nucleus, where they phosphorylate a variety of target transcription factors that are important in tumor development. A transcription factor is comprised of one or more proteins that bind to a specific DNA sequence in a gene and

act to initiate transcription of that target gene resulting in production of the protein gene product. Transcription factors with a proven role in carcinogenesis include activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB) [7–10], which in turn may activate the transcription of a variety of cancer-related genes such as *cyclooxygenase-2* (*COX-2*). AP-1 is a well-characterized transcription factor composed of homodimers and/or heterodimers of the Jun, Fos, ATF (activating transcription factor), and MAF (musculoaponeurotic fibrosarcoma) protein families [11,12]. AP-1 plays a major role in cell transformation and is crucial in tumor promotion, progression, and metastasis [13–15]. Notably, neoplastic transformation and TPA-induced cancer progression are blocked by inhibiting tumor promoter-induced AP-1 activity [14,16–19].

In many cell lines, tumor promoters also induce activation of the transcription factor NF-κB. NF-κB is a rapidly induced stress-responsive transcription factor that activates the transcription of a variety of genes including cytokines, growth factors, and acute response proteins [20]. NF-κB activation is also linked to MAP kinase signaling pathways [21]. NF-κB is found in the cytosol in its inactive form bound to a protein known as inhibitory kappa B (IκB). When stimulated, IκB is phosphorylated by an IκB kinase (IKK) resulting in its dissociation from NF-κB and subsequent degradation. Following separation from IκB, NF-κB translocates into the nucleus where it activates gene transcription by binding to its target DNA sequence found in specific genes. Importantly, NF-κB activation is associated with initiation or acceleration of tumorigenesis [22], and in JB6 cells, inhibition of NF-κB also blocked tumor promoter-induced cell transformation [23]. Therefore, similar to AP-1, NF-κB appears to be another potential target for chemopreventive agents.

The MAP kinases include the extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38 kinases. ERKs generally transmit signals initiated by tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) [24]. The JNKs/SAPKs and p38 kinases are strongly stimulated by stresses such as ultraviolet (UV) irradiation [9] and arsenic [25]. The activation of these signaling cascades can result in a multitude of cellular responses including apoptosis, proliferation, inflammation, differentiation, and development. The purpose of this review is to examine the scientific evidence supporting the effectiveness of tea components and [6]-gingerol as chemopreventive or chemotherapeutic agents in modulating signal transduction pathways associated with cancer development.

2.2 MODULATION OF SIGNAL TRANSDUCTION BY COMPONENTS OF TEA

2.2.1 Signal Transduction and Tea Components

Signal transduction molecules induced by tumor promoters appear to be prime targets for nutritional or dietary factors, and especially for the components of tea

including the catechins, theaflavins, and caffeine. Several recent reviews have provided excellent overviews of the biological activities of tea [26–31].

Green, oolong, and black teas are all derived from the *Camellia sinensi* plant and are distinguished according to their level of oxidation [32]. Green tea is processed immediately from fresh leaves and is protected from oxidation, oolong tea has been partially oxidized, and black tea has been fully oxidized. Green and black teas contain several active polyphenols collectively known as catechins and theaflavins, respectively. The catechins include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC) [33,34]. Theaflavins give black tea its characteristic color and taste and include theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate [34]. Tea also contains caffeine, another important bioactive compound. More and more evidence suggests that tea may possess powerful anticancer activity [34–37]. Topical application or oral consumption of green tea, black tea, and tea polyphenol preparations has been reported to inhibit skin, lung, esophagus, stomach, liver, duodenum and small intestine, pancreas, and colorectal cancers in rodent models [34–38] and to reduce pancreatic, breast, and stomach cancer risk in humans [39–41]. Results from a very recent study suggested that pancreatic cancer (PANC-1) cells treated with EGCG can limit PANC-1 cell proliferation [42], breast cancer cell proliferation and invasiveness [43], and growth of androgen-dependent LNCaP human prostate cancer cells [44]. Moreover, green tea polyphenols, black tea water extract, EGCG, and theaflavins all reduced growth of implanted androgen-sensitive human CaP CWR22Rnu1 cells in athymic nude mice and also caused a substantial regression of established tumors [45]. Unfortunately, reports of anticancer activity of tea polyphenols in humans are less dramatic. Phase I and II clinical trials have been performed to test the anticancer effects of oral administration of green tea but results were inconclusive [46,47]. Thus more comprehensive research data are needed that provide possible mechanisms explaining the chemopreventive effects of tea in cell and animal models for translation to humans. One important issue to be considered in translating results from cell and animal models to humans may be related to the bioavailability of EGCG or other tea components. Notably, the achievable tissue concentrations of EGCG appear to be in the low micromolar range and therefore results observed with much higher concentrations *in vitro* may not be relevant to the anticarcinogenic process [35,48].

2.2.2 Green Tea Catechins

EGCG is the major active polyphenol in green tea and may account for 50%–80% of the total catechins found in tea [4,34,49,50]. A great deal of data suggest that this green tea catechin appears to exert many of its anticancer effects by suppressing the activation of AP-1 or NF-κB transcription factors with consequences on subsequent target gene expression. In addition, EGCG has been reported to induce or suppress apoptosis and has been shown to interact directly with numerous proteins directly involved in the carcinogenic process.

2.2.2.1 AP-1 Transcriptional Activation Inhibited by EGCG

EGCG has been reported to inhibit tumor promoter-induced MAP kinase and AP-1 activation and cell transformation of JB6 [51,52], A172, and NIH 3T3 cells [53]. UVB exposure of mouse skin epidermis or cultured skin cells has been shown to cause a marked increase in AP-1 activation and the activation is substantially suppressed by treatment with EGCG or theaflavins [54,55]. Suppression of UVB-induced AP-1 activation may very likely be related to EGCG's reported inhibition of UVB-induced transcriptional activation of the *c-fos* gene and accumulation of the c-Fos protein [56], an important component of the AP-1 protein complex.

The Ras pathway is critical in the activation of AP-1 and mutations of the *Ras* gene occur frequently in many cancers and are associated with uncontrolled growth. Chung et al. [57] found that the H-*Ras*-activated AP-1 pathway was a major growth stimulant in transformed mutant *H-Ras* JB6 cells. Treatment of cells with green or black tea polyphenols strongly inhibited cell growth and phosphorylation of ERKs, c-Jun, Fra-1, and AP-1 activity [57]. Recent work indicated that in intestinal epithelial cells (RIE-1), EGCG inhibited *Ras*-induced cell proliferation apparently by suppressing cyclin D1 expression, which resulted in G1 arrest [58]. Notably, the inhibitory effects of EGCG were more pronounced in transformed cells than in nontransformed cells [58]. Similarly, EGCG has been shown to have no effect on normal human fetal colon cells compared to its potent inhibition of the growth of a variety of colon cancer cell lines, including Caco2, HCT116, HT29, SW480, and SW837 [59]. One marked difference between normal colon cells and the colon cancer cell lines used in these studies was the overexpression and constitutive activation of the EGF receptor (EGFR) and HER2 proteins in the cancer cell lines [59]. The inhibition by EGCG was accompanied by a decreased phosphorylation of EGFR, HER2, ERKs, and Akt proteins and an induction of G1 arrest and apoptosis. The effects appeared to be mediated through a suppression of AP-1, c-fos, NF-κB, and cyclin D1 activities [59]. Phorbol 12-myristate 13-acetate (PMA) has been revealed to induce cell invasiveness, matrix metalloproteinase (MMP)-9 expression, and transcriptional activity in human gastric cancer AGS cells [60]. EGCG was reported to suppress these effects along with PMA's activation of ERKS, JNKs, and AP-1 [60]. In support of these results, EGCG was again later shown to downregulate the expression of MMP-9 and decrease tumor invasion in 95-D cells and in this case, the decreased expression of MMP-9 corresponded with a diminished nuclear translocation of NF-κB [38]. Others have very recently demonstrated similar results where EGCG pretreatment resulted in a suppression of cigarette smoke condensate (CSC)-induced phosphorylation of IκBα and activation and nuclear translocation of NF-κB/p65 in normal human bronchial epithelial cells [61]. These effects corresponded with a downregulation of several NF-κB-regulated proteins, including cyclin D1, MMP-9, IL-8, and iNOS; a decreased phosphorylation of ERKs, JNKs, and p38; and reduced expression of PI-3/Akt and mTOR signaling molecules [61]. All of these results seem to suggest that EGCG may prevent malignant transformation by suppressing MAP

kinase activation of AP-1 and/or NF-κB transactivation. However, in marked contrast to these results, EGCG has also been shown to activate ERKS, JNKs, and p38 and induce phosphorylation of c-Jun (Ser63/73) with subsequent activation of AP-1 in HT-29 human colorectal cancer and Caco-2 adenocarcinoma cells [62]. These effects were suggested to be associated with an EGCG-induced production of superoxide and other reactive oxygen species [62], but the possible implication for cancer development is not clear.

2.2.2.2 NF-κB, PI-3 Kinase/Akt, and COX-2 Inhibited by EGCG

As suggested earlier, NF-κB activation has been reported to be associated with initiation or acceleration of tumorigenesis [22]. EGCG and theaflavins appear to suppress the NF-κB pathway mainly by preventing the release and degradation of IκBα, the primary negative regulator of NF-κB activation. Suppression of IκBα phosphorylation results in an accumulation of IκBα and subsequent inactivation of NF-κB. For example, EGCG treatment was shown to inhibit UVB-induced phosphorylation and degradation of IκBα in normal human keratinocytes [63] and suppressed both UVB- and TPA-induced IκBα phosphorylation (Ser32) in JB6 cells [64]. In Jurkat T cells, EGCG inhibited activity of the 20S proteasome, which normally targets IκBα for degradation, and the inhibition resulted in cell growth arrest and an accumulation of IκBα [65]. Furthermore, EGCG was shown to inhibit IκBα degradation in cancer cells at much lower doses than were required for inhibition in normal cells [66], which again suggests a differential response of normal cells and tumor cells to treatment with EGCG. This differential targeting of cancer cells enhances the potential for EGCG use in chemopreventive or chemotherapeutic protocols.

EGCG treatment was reported to dramatically inhibit NF-κB nuclear translocation and IL-6 secretion induced by UVB (20, 30 mJ/cm^2) or UVA (10, 20 J/cm^2) [67]. Topical application of EGCG (1 mg/cm^2 skin) has also been shown to prevent UV-induced carcinogenesis in wild-type (C3H/HeN) mice but not in IL-12 knockout (KO) mice [68]. EGCG also had a protective effect on resolving UVB-induced DNA damage in control mice but not in IL-12 KO mice, suggesting that EGCG prevented UV-induced skin cancer through an EGCG-induced IL-12-dependent DNA repair mechanism [68]. EGCG has also been shown to possess antigenotoxic potential in human peripheral leucocytes [69].

2.2.2.2.1 PI-3 Kinase/Akt

Phosphatidylinositol-3 (PI-3) kinase is activated by numerous oncogenes and this pathway, which includes the PI-3 kinase downstream target, Akt/PKB (protein kinase B), is deregulated in many human cancers [70]. A chronic 4-week exposure of V79 Chinese hamster lung fibroblasts to H_2O_2 (30 μM) caused a constitutive phosphorylation of Akt (Ser473, Thr308) and phosphorylation of IKK and transcriptional activation of NF-κB and these effects were mostly prevented by concurrent exposure to 4.5 μM EGCG [71]. EGCG and theaflavins have been

shown to decrease UVB-induced PI-3 kinase activity, resulting in decreased phosphorylation of Akt (Thr308, Ser473) and another downstream target, p70S6K (Thr389, Thr421/Ser424) [72]. Inhibition of PI-3 kinase/Akt signaling to NF-κB and subsequent suppression of basal Her-2/neu receptor tyrosine phosphorylation by EGCG was reported to result in decreased viability of N639 breast tumor cells [73]. Pretreatment of the immortalized, nontumorigenic breast cell line, MCF10A, and the invasive breast carcinoma cell line, MDA-MB-231, with 0.3 μM EGCG inhibited heptocyte growth factor (HGF)-induced activation of Akt and ERKs [74].

EGCG has also been reported to suppress vascular endothelial growth factor (VEGF)-induced Akt activation, and decrease PI-3 kinase-dependent activation and DNA-binding ability of NF-κB and proangiogenic cytokine interleukin IL-8 production [75]. EGCG has also been shown to inhibit hypoxia-inducible factor-1-alpha (HIF-1alpha) and its downstream target, vascular endothelial growth factor (VEGF) protein expression in human cervical carcinoma (HeLa), and hepatoma (HepG2) cells [76]. These effects were related to an EGCG-mediated suppression of PI-3-kinase/Akt/mammalian target of rapamycin (mTOR) and ERK1/2 signaling pathways [76]. EGCG was shown to inhibit EGF-dependent activation of EGFR and EGFR-dependent activation of ERK1/2 and Akt. Notably, the inhibition of ERK1/2 and Akt was associated with increased levels of p53, $p21^{Waf-1}$, and $p27^{Kip-1}$, and decreased levels of cyclin E and Cdk2 kinase activity resulting in G1 arrest and eventual apoptosis [77].

In somewhat of a contrast, EGCG was shown to increase ERK1/2 levels but decrease PI-3 kinase and Akt protein levels in DU145 (androgen-unresponsive prostate carcinoma cells) and LNCaP (androgen-responsive prostate carcinoma cells) [78]. Moreover, heme oxygenase-1 (HO-1) is a cytoprotective enzyme activated by various phytochemicals and EGCG has been shown to induce HO-1 expression that was associated with activation of Akt and ERK1/2 [79]. Some cancer cells have been reported to develop resistance to EGCG with chronic exposure and activation of the MAP kinase pathways has been suggested to mediate this resistance in, for example, breast cancer cells that overexpress Her-2/neu [80].

2.2.2.2.2 COX-2

COX has two well-known isoforms, denoted COX-1 and COX-2. COX-1 is constitutively expressed in almost every cell type, whereas COX-2 is induced by stresses, including inflammation. Overexpression of COX-2 has been implicated in cancer development [81–83], including cancers of the colon [84–86], breast [87,88], lung [89,90], stomach [91] and gastric system [92], head and neck [93–95], pancreas [96], uterine [97], cervix [98], urinary bladder [99], gall bladder [100], and skin [101–105]. Studies utilizing mice deficient in COX-1 or COX-2 develop fewer tumors when subjected to the DMBA/TPA mouse skin tumorigenesis protocol [106], but overexpression of COX-2 in mouse skin also led to fewer tumors in a similar protocol [107]. Research findings generally suggest

that development of compounds, which can block COX-2 expression preferably without affecting COX-1, is highly interesting to cancer researchers.

NFκB activation has been linked to increased COX-2 expression and generally, EGCG is believed to target COX-2 and not COX-1. For example, EGCG was shown to inhibit COX-2 without affecting COX-1 expression at both the mRNA and protein levels in androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate carcinoma cells [108]. Research studies have shown that EGCG significantly inhibited COX-2 activity in lipopolysaccharide (LPS)-activated Raw 264.7 macrophage cells [109], whereas others have shown that in Raw 264.7, COX-2 expression and activity and prostaglandin production were increased by EGCG treatment and were associated with the activation of both the ERKs and protein-tyrosine phosphatase signaling pathways [110].

Topical pretreatment with green tea extract has been shown to block the acute COX-2 response to UVB in mice or humans [103]. In one study, a single topical application of EGCG to Skh-1 hairless mice inhibited lipid peroxidation, UVB-induced COX, and ornithine decarboxylase activities [63]. Application of EGCG has also been reported to substantially inhibit TPA-induced COX-2 expression in mouse skin and also in TPA-induced human mammary epithelial cells [111]. This inhibition corresponded with a suppression of TPA-stimulated ERKs and p38 activities, but did not affect TPA-induced AP-1 DNA binding [111]. In other animal models, decreased expression of COX-2 induced by EGCG corresponded with significantly less N-nitrosomethylbenzylamine (NMBA)-induced rat esophageal tumor development and tumor incidence [112].

A number of studies have shown that EGCG has an anti-colon cancer effect that appeared to be related to a suppression of COX-2. EGCG was recently reported to inhibit COX-2 expression and prostaglandin E2 secretion in HT-29 colon cancer cells [113] and HT-29, and HCA-7 colon cancer cells treated with EGCG exhibited significant suppression of constitutive COX-2 mRNA and protein overexpression [114]. The effects of EGCG appeared to occur through a down-regulation of ERKs and Akt and a decreased COX-2 promoter activity through the inhibition of NF-κB activation [114]. Similar results were reported in SW837 human colon cancer cell lines that express not only high levels of COX-2 but also high and constitutive activation of the receptor tyrosine kinase HER3 [115]. In this case, treatment of cells with EGCG (20 μg/ml) caused a decrease in the phosphorylated forms of the EGFR, HER2, HER3, ERKs, and Akt in the cellular levels of both COX-2 protein and mRNA, and eventually induced apoptosis [115].

2.2.2.3 Modulation of Apoptosis by EGCG

Literally hundreds of research studies have investigated the effect of EGCG on cell death and EGCG has been reported to either induce or suppress apoptosis in a variety of cancer cell lines. Importantly, EGCG's proapoptotic effects appear to target only cancer cells with little effect on normal cells suggesting that EGCG can be used in combination with traditional chemotherapeutic agents to enhance cancer cell death without harming normal cells.

EGCG treatment has been shown to induce apoptosis in human colorectal carcinoma HT-29 cells [116], cervical adenocarcinoma cells (OMC-4, TMCC-1) [117], human prostate carcinoma LNCaP cells [118], liver cancer cells (HepG2) [119], human osteogenic sarcoma cells [120], various melanomas [121], breast cancer cell lines T-47D [122], MDA-MB-231 [123], MDA-MB-468, HEY and OVCA 433 ovarian cancer cells [124], and MIA PaCa-2 pancreatic carcinoma cells [125], just to name a few. Many of the associated effects included stabilization/phosphorylation of p53, downregulation of the antiapoptotic Bcl-2 protein, upregulation of proapoptotic Bax, activation of caspases, and cell cycle arrest at G1 [118–120,122,123]. In most cases, the effects of EGCG seem confined to cancer cells with little effect on normal cells. For example, apoptotic and cell cycle effects induced by EGCG have been observed in A-375 amelanotic malignant melanoma and Hs-294T metastatic melanoma cell lines but not in normal human epidermal melanocytes [121]. Others [66] have also found that treatment with EGCG (10–100 μM, 24 h) caused growth inhibition, G1-phase arrest, and apoptosis in human epidermoid carcinoma cells (A431) but not in the normal cells.

The effects of EGCG have not only been observed in cell culture models but also in animal models. Most recently, treatment of nude mice inoculated with human breast cancer MDA-MB-231 cells delayed tumor incidence as well as reduced tumor burden compared to untreated control mice [123]. Growth of HEY ovarian carcinoma xenografts was significantly inhibited by oral administration of green tea [126]. EGCG treatment of athymic nude mice implanted with androgen-sensitive human CaP CWR22Rnu1 cells resulted in decreased growth that was associated with a reduction in serum PSA levels [45].

Combining EGCG with some anticancer agents also seems to be beneficial in enhancing cancer cell death. For example, treatment of PC-9, A549, or ChaGo K-1 lung cancer cells with EGCG plus celecoxib, a COX-2 inhibitor, synergistically induced apoptosis [127]. Neither EGCG nor celecoxib was effective alone or with other anticancer agents such as N-(4-hydroxyphenyl)retinamide or aspirin [127]. EGCG was also shown to increase the toxicity of the cheomotherapeutic drug, cisplatin, by three- to sixfold in SKOV3, CAOV3, and C200 ovarian cancer cells and showed IC_{50} values for EGCG in the μM range even for C200, which is known to be resistant to cisplatin [128]. Furthermore, the combination of EGCG with radiotherapy was recently suggested to improve the efficacy of ionizing radiation in treating glioblastoma cells [129].

EGCG (1–20 μM) or theaflavins (1–20 μM) has also been shown to inhibit apoptosis under certain conditions, such as exposure to arsenic, hypoxia, or UV. Arsenic's toxicity may be due to its ability to induce abnormal apoptosis and green tea has been used in Chinese medicine for detoxification of arsenite-associated toxicity. EGCG or theaflavins have been shown to block arsenite-induced apoptosis of JB6 cells [51]. In normal human keratinocytes, EGCG also inhibited UV-induced apoptosis by two mechanisms involving phosphorylation of the Bad protein through the ERKs and Akt pathways and by changing the Bcl-2/Bax ratio [130]. The molar ratio of Bcl-2 to Bax has been shown to establish whether

apoptosis is induced or inhibited in many tissues [131]. Another study indicated that EGCG inhibited UVB-induced apoptosis, which was characterized by a recovery of UV-induced loss of an antiapoptotic component, Bcl-2, and inhibition of the UV-induced apoptotic component, Fas ligand, expression [67]. Finally, EGCG has been reported to prevent apoptosis induced by hypoxia that was associated with increased levels of Bax and caspase-3 activity in HepG2 cells [132].

2.2.2.4 Specific Molecular Targets of EGCG

Identifying the EGCG "receptor" or high affinity proteins that bind to EGCG is the first step in understanding the molecular and biochemical mechanism of this polyphenol's anticancer effects. A few proteins that have been reported to directly bind with EGCG include several plasma proteins [133], insulin growth factor-1 receptor (IGF-1R) [134,135], fatty acid synthase [136], laminin [137] and the 67-kDa laminin receptor [138,139], and more recently vimentin [140] and the glucose-regulated protein 78 (GRP78) chaperone protein [141]. Identification of new EGCG-binding proteins should facilitate the design of new strategies to prevent cancer and hopefully help translate the effectiveness of EGCG observed in cell and animal models to humans.

The IGF/IGF-1R system includes the IGF, IGF-1R, and IGF-binding proteins (IGFBPs) and plays an important role in the development and growth of numerous types of cancer. This conclusion is based on the results of numerous studies [142] showing that impairment of IGF-1R signal results in inhibition of cell transformation in soft agar and tumor formation in athymic nude mice. Many colon cancer cell lines, including Caco2, HT29, SW837, and SW480, have high or constitutive expression of IGF-1R, and EGCG (20 μg/ml) has been reported to decrease the phosphorylated or activated form of IGF-1R [134]. EGCG was recently demonstrated to be a small molecule inhibitor of IGF-1R activity and kinetic studies showed that EGCG inhibited IGF-1R kinase activity by an ATP-competitive mechanism [135]. EGCG also abrogated anchorage-independent growth induced by IGF-1R overexpression and prevented human breast and cervical cancer cell phenotype expression through inhibition of IGF-1R downstream signaling [135].

EGCG (IC_{50} 52 μM) and epicatechin gallate (ECG; IC_{50} 42 μM) have been shown to inhibit animal fatty acid synthase, which has been reported as a potential therapeutic target for obesity and cancer [143]. Both compounds appear to interact with the beta-ketoacyl reductase (KR) domain of fatty acid synthase. Catechin gallate (CG) was also reported to have an inhibitory effect but does not interact with the KR domain [143]. Unfortunately, the high IC_{50} values suggest that targeting fatty acid synthase *in vivo* may not be physiologically achievable based on what is known regarding EGCG's bioavailability [34,144,145].

On the other hand, the binding interaction of EGCG with the metastasis-associated 67-kDa laminin receptor (67LR) was reported to occur at physiologically relevant concentrations [138]. Recently, the intermediate filament protein, vimentin, which has an important functional involvement in cell division

and proliferation [146], was identified as a novel EGCG-binding protein [140]. Vimentin displayed a high affinity (K_i – 3.3 nM) for binding with [^{3}H]EGCG. EGCG specifically interfered with the Cdc2 kinase-mediated phosphorylation of vimentin (IC_{50} 17 μM) serines 50 and 55 [140]. EGCG has been reported to inhibit cell proliferation of a variety of cell lines [73,145,147–150], and when vimentin expression was suppressed with siRNA techniques, cell growth was inhibited suggesting that EGCG's association with vimentin can have a regulatory role in controlling cell proliferation.

EGCG has very recently been shown to directly interact with the glucose-regulated protein 78 (GRP78), which is associated with the multidrug resistance phenotype of many types of cancer cells [141]. EGCG directly interacted with GRP78 at the protein's ATP-binding site and regulated its function by competing with ATP binding resulting in the inhibition of ATPase activity. EGCG binding caused the conversion of GRP78 from its active monomer to the inactive dimer and oligomer forms [141]. Further findings indicated that EGCG interfered with the formation of the antiapoptotic GRP78-caspase-7 complex, which resulted in an increased etoposide-induced apoptosis in cancer cells and suppression of the transformed phenotype of breast cancer cells treated with etoposide [141]. Overall, these results strongly suggested that EGCG could prevent the antiapoptotic effect of GRP78, which usually suppresses the caspase-mediated cell death pathways in drug-treated cancer cells, contributing to the development of drug resistance [141].

Besides interacting with proteins, EGCG was recently shown to bind with both DNA and RNA molecules, including single-stranded 18mers of DNA and RNA and double-stranded (AG-CT) oligomers of various nucleotide lengths [151]. These data suggested that multiple binding sites of EGCG are present in DNA and RNA oligomers [151], however, the full physiologic relevance of this binding is still to be determined.

2.2.3 Theaflavins

Theaflavins give black tea its characteristic color and taste, and are differentiated by the gallate group. They include theaflavin (TF), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-digallate (TF-3) [144]. Research data suggest that theaflavins may have even more potent anticancer activity than EGCG and may act by different mechanisms.

EGCG and TF-3 were compared for their effects on the MAP kinase signaling pathways and both inhibited phosphorylation of c-Jun and ERKs, but only TF-3 inhibited p38 kinase [57]. Further studies [152] confirmed that either EGCG or TF-3 decreased phosphorylation of ERKs and MEKs, but TF-3 acted at 15 min compared to 60 min for EGCG. In addition, TF-3 decreased Raf-1 protein levels and EGCG decreased the association of Raf-1 with MEK1 [152]. In a comparison of the effects of EGCG and theaflavins on UVB-induced AP-1 activation, the theaflavins were stronger inhibitors of UVB-induced AP-1 activation [55]. Others have reported that theaflavins, and especially TF-3, also inhibited IκB kinase

activity, which prevented the phosphorylation and degradation of IκB and subsequent activation of NFκB [153]. Theaflavins at very low concentrations (0.5 μM) were shown to inhibit UVB-induced phosphorylation of JNKs [154], and very recently, theaflavins (20 μM), and especially TF-3, were reported to induce EGFR downregulation in JB6 Cl41 mouse epidermal and A431 human EGFR-overexpressing epidermoid carcinoma cells [28]. Further, TF-3 inhibited EGFR-induced phosphorylation and downstream signaling to ERKs and AP-1 resulting in an inhibition of EGF-induced cell transformation [28].

In vivo, theaflavins induced a substantial reduction in the incidences of aberrant crypt foci, the preneoplastic lesion formed during colon carcinogenesis, which corresponded with a reduced expression of COX-2 and iNOS [155]. Theaflavins have also been associated with a lowered incidence and delayed onset of preinvasive lung lesions in mice [156].

2.2.4 Caffeine

Another component of tea and coffee that may be important in cancer prevention is caffeine, and the activities of this compound have been recently and extensively reviewed [157]. Caffeine may very well be the most frequently ingested neuroactive drug in the world [158]. Caffeine has been reported to affect cell cycle function, induce programmed cell death or apoptosis, and perturb key cell cycle regulatory proteins, including the tumor suppressor protein p53 [159,160]. This compound has been used experimentally in numerous cell types under a variety of conditions at concentrations ranging from micromolar to high mM. Physiologically, achieving a 2 mM blood level of caffeine would require the simultaneous consumption of over 100 cups of coffee [161]. Therefore, the relevance of experimental data obtained by using greater than 1 mM caffeine is not clear and may account for some of the discrepancies found in the literature. At concentrations of less than 1 mM, caffeine has been reported to induce p53 phosphorylation and p53-dependent apoptosis associated with increased expression of proapoptotic Bax and caspase-3 [160]. On the other hand, low concentrations have also been reported to have aberrant effects on nervous system development [162]. At concentrations of 1–2 mM, caffeine may induce G1 arrest [163–165], whereas concentrations of 2–4 mM appear to block G1 arrest [166,167] and induce apoptosis [168]. Furthermore, depending on concentration and status of p53 expression, caffeine has been reported to induce [159,160,163,169–172] apoptosis, not induce [173] or even protect against apoptosis [174–178].

When DNA damage occurs, p53 is phosphorylated (Ser15) by the ataxia telangiectasia mutated (ATM) protein and the AT-related homolog, ATR [179], which results in p53 stabilization and accumulation [166]. ATM and ATR also prevent p53 degradation through their phosphorylation of mouse double minute 2 (MDM2) [180], which disrupts MDM2's association with p53 thereby preventing p53 ubiquitination and degradation. These two proteins, ATM/ATR, have been widely accepted as the primary protein targets of caffeine [181], but this idea has been disputed [182,183].

Although exceptions have been noted [184], most concentrations of caffeine almost always reverse the DNA-damage induced G2/M block [170,172,178,181, 182,185,186]. However, higher concentrations of caffeine can directly induce G2 arrest [159]. Usually, but not always [169], the effect of caffeine is most potent in p53 deficient cells [174,178,187–189] making it an attractive agent for treating p53 deficient cancers.

Caffeine at various concentrations generally seems to enhance the toxicity of ionizing radiation and DNA-damaging chemical agents, but exceptions have been noted [190–192]. Unfortunately, the high levels of caffeine required may be too toxic for humans. This suggests that the development of caffeine analogs with biologic activity similar to the parent compound, but with less toxicity, could be very useful in combination with DNA-damaging agents for treating cancers.

2.3 MODULATION OF SIGNAL TRANSDUCTION BY COMPONENTS OF GINGER

2.3.1 Background

One of the most popular and highly consumed dietary substances in the world is derived from the plants of the ginger (*Zingiber officinale* Roscoe, Zingiberaceae) family [193]. The oleoresin or oil from ginger root contains [6]-gingerol [1-(4′-hydroxy-3′-methoxyphenyl)-5-hydroxy-3-decanone], which is the major pharmacologically active component, and a variety of other gingerols, gingerdiols, paradols, and zingerones. Based on results of both experimental and clinical data, ginger has been suggested to be safe for therapeutic use [194]. However, some clinicians and researchers advise caution because of the lack of a complete understanding of its mechanisms of action [195–197]. Ginger has been reported to have a variety of powerful therapeutic effects and has been used for thousands of years for treatment of numerous ailments, including colds, nausea, arthritis, migraines, and hypertension. The medicinal, chemical, and pharmacological properties of ginger have been extensively reviewed [198–200]. Numerous research studies have reported that components of ginger suppress cancer cell growth [201–204]. Reports suggest a variety of indirect mechanisms by which ginger compounds exert their antitumorigenic effects and several aspects of the chemopreventive effects of ginger have been recently reviewed [205,206]. Ginger appears to exert chemopreventive activity through a combination of antioxidant and proapoptotic activities. Although COX-2 and NF-κB are clearly affected by various components of ginger, specific interacting protein targets for binding with ginger have not been clearly identified as yet.

2.3.2 Antioxidant Effects of Ginger Components

A common mechanism offered to explain the actions and health benefits of ginger and its various components is related to potent antioxidant properties [207,208]. Several hundred dietary plants were systematically analyzed to reveal that ginger

root contained a very high concentration (3.85 mmol/100 g) of total antioxidants [209]. Comparatively, only some wild berries (e.g., blackberries, blueberries, and strawberries) and pomegranate exhibited a higher total antioxidant concentration [209]. Several research studies have reported that ginger is an effective antioxidant *in vivo*. For example, ginger compounds were shown to inhibit lipid peroxidation [210,211], production of superoxide [212,213], and aging-associated oxidative stress markers [214]. Ginger was also reported to effectively lower experimentally-induced lipid peroxidation and maintain glutathione levels [215,216].

Nitric oxide (NO) and peroxynitrite (ONOO-) are reactive nitrogen species that have been suggested to modulate signal transduction and cause DNA damage, thus contributing to carcinogenesis. [6]-Gingerol was shown to substantially suppress iNOS activity and nitric oxide production in LPS-activated J774.1 macrophages [217]. This ginger component also protected against peroxynitrite-mediated DNA and protein damage [217]. In contrast, ginger (100 μg/ml) has been shown to stimulate macrophage-inducible nitric oxide synthase (macNOS) mRNA expression and nitric oxide production in RAW264.7 cells, a murine macrophage cell line [218]. Imanishi et al. [218] concluded that the induction of the macNOS-mediated activation of biodefense mechanisms was at least partially responsible for the beneficial effects of ginger.

Zerumbone is a compound found in subtropical ginger *Zingiber zerumbet* Smith and was recently reported to induce phase II detoxification enzymes such as glutathione *S*-transferase in RL34 cells [219]. The mechanism was suggested to be associated with a ginger-induced nuclear localization of the transcription factor Nrf2 that binds to antioxidant response element (ARE) of the phase II enzyme genes [219].

Ginger extracts have also been reported to have radioprotective effects in mice that are apparently attributable to antioxidant activities [220,221]. In these studies, mice were given 10 mg/kg ginger extract intraperitoneally before exposure to 6–12 Gy of gamma radiation. Treatment with ginger had a protective effect, preventing radiation sickness and reducing mortality. The protective effect was associated with decreased lipid peroxidation and maintenance of glutathione levels [220]. Similar results were recently reported by the same group but in this case, mice were administered 250 mg/kg of ginger extract by oral gavage prior to exposure to gamma radiation [221]. Results indicated that feeding ginger extracts protected against both gastrointestinal-related and bone marrow-related deaths and the effects were associated with the ability of ginger to scavenge hydroxyl and superoxide radicals [221].

2.3.3 Chemopreventive Effects of Ginger and Its Components

2.3.3.1 TPA-Induced Carcinogenesis

Tumor promoters, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), may act by promoting oxidative stress through the activation of cellular free radical-generating

systems, such as the NADPH oxidase system and/or the xanthine oxidase system. Several ginger components have been shown to have good antitumor promoter activity based on their ability to inhibit TPA-induced Epstein-Barr virus early antigen (EBV-EA) in Raji cells [222,223]. The potent antioxidant activity of various components of ginger may be at least partially responsible for preventing 7,12-dimethylbenz[a]anthracene (DMBA)/TPA-induced skin carcinogenesis in the two-stage mouse model. This model of skin carcinogenesis is probably the most common model used to study the effectiveness of potential anti-skin tumor agents such as ginger *in vivo*. In this paradigm, tumors are initiated by one application of DMBA followed by repeated topical applications of TPA beginning a few days later. Ginger and its constituents have also been reported to inhibit tumor promotion in SENCAR mice [224] and in female ICR mice [225]. More recently, Chung et al. [226] reported that in the DMBA/TPA skin tumor model, topical application of [6]-paradol and [6]-dehydroparadol prior to the application of TPA significantly reduced both the number of tumors per mouse and the fraction of mice with tumors [226].

Many edible Japanese plants, including mioga ginger, were reported to be very effective in suppressing TPA-induced superoxide generation from NADPH oxidase and xanthine oxidase in HL-60 cells and AS52 cells, respectively [213]. Kim et al. [227] recently reported that a constituent of the mioga ginger, aframodial (20 μM), markedly suppressed TPA-induced superoxide generation in HL-60 cells and LPS/interferon-gamma-induced nitric oxide generation in RAW264.7 cells. Aframodial also suppressed LPS-induced expression of nitric oxide synthase, IL-1beta, IL-6, and granulocyte-macrophage colony-stimulating factor, and prevented the degradation of IκB, the primary negative regulator of NF-κB [227].

2.3.3.2 Colon Carcinogenesis

Earlier studies suggested that gingerol was an effective inhibitor of azoxymethane-induced intestinal carcinogenesis in rats [228]. Ginger extracts have been reported to inhibit the growth of *Helicobacter pylori*, which is the primary etiological agent associated with the development of gastric and colon cancer, which may contribute to its chemopreventive effects [229]. Recently, Manju and Nalini [230] reported the effects of ginger on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in male Wistar rats. DMH (20 mg/kg) was administered by a weekly subcutaneous injection for 15 weeks and ginger (50 mg/kg) was given orally everyday. Results indicated that the number and incidence of tumors was significantly decreased in rats treated with ginger. The decrease in colon carcinogenesis was associated with substantially reduced circulating lipid peroxidation and enhanced antioxidant levels compared to untreated rats [230]. These results substantiated results from another study in which topical application of zerumbone (16 μmol) was shown to suppress tumor development in the DMBA/TPA 2-stage carcinogenesis model [231]. Zerumbone (2 μmol) effects were related to increased expression of several free radical scavenging proteins, including superoxide

dismutase and glutathione peroxidase-1. TPA-induced COX-2 protein expression and ERKs phosphorylation were also decreased by zerumbone treatment [231]. In contrast to these reports, Dias et al. [232] recently reported that dietary consumption of 0.5% or 1% dose levels had no effect on aberrant crypt foci formation induced by DMH in male Wistar rats.

2.3.3.3 Antiproliferative Effects-Induction of Apoptosis and Inhibition of Cell Cycle Progression

Besides potent antioxidant activity, evidence also suggests that ginger and its constituents may act as chemopreventive agents by inducing programmed cell death or apoptosis [233]. Earlier studies suggested that ginger compounds suppress proliferation of human cancer cells through the induction of apoptosis [202,203]. [6]-Gingerol has been shown to induce cell death in promyelocytic leukemia HL-60 cells, an effect that was associated with increased DNA fragmentation and inhibition of antiapoptotic Bcl-2 expression [234]. A synthetic gingerdione derivative, 1-(3,4-dimethoxyphenyl)-3,5-dodecenedione, has recently been demonstrated to also be an effective antitumor agent in human promyelocytic leukemia HL-60 cells [235]. In this case, gingerdione was shown to suppress cell proliferation apparently by inducing G1 arrest and apoptosis. The G1 arrest corresponded with increased mRNA expression of p53 target proteins, p15 and p27, and decreased mRNA expression of cell cycle proteins, cyclin D2, cyclin E, and cdc25A. Apoptosis induced by gingerdione was accompanied by an apparent upregulation of caspase-3 and downregulation of Bcl-2 [235].

The effect of two structurally related compounds of the ginger family, [6]-gingerol and [6]-paradol, on EGF-induced cell transformation and AP-1 activation was investigated [204]. The results indicated that both compounds blocked EGF-induced cell transformation and although [6]-gingerol inhibited AP-1 activation, both could act by inducing apoptosis [204]. Others showed that [6]-paradol and structurally related derivatives, [10]-paradol, [3]-dehydroparadol, [6]-dehydroparadol, and [10]-dehydroparadol, inhibited proliferation of KB oral squamous carcinoma cells [236]. [6]-Dehydroparadol induced apoptosis through a caspase-3-dependent mechanism [236]. Exposure of Jurkat human T-cell leukemia cells to galanals A and B, isolated from the flower buds of a Japanese ginger, myoga (*Zingiber mioga* Roscoe), resulted in apoptosis mediated through the mitochondrial pathway [237]. Apoptosis was accompanied by caspase-3 activation and a downregulation of antiapoptotic Bcl-2 protein together with an enhancement of proapoptotic Bax expression [237].

2.3.4 Effects of Ginger on NF-κB and COX-2

Gingerols have been shown to be effective inhibitors of arachidonic acid-induced platelet release and aggregation, an effect that has been attributed to inhibition of COX-2 [238]. Ginger components were also shown to inhibit COX-2 in cultured human airway epithelial A549 cells [239]. COX-2 is abnormally upregulated in

many premalignant and malignant tissues and cells. Topical application of [6]-gingerol has been reported to inhibit PMA-induced COX-2 expression and NF-κB DNA-binding activity in mouse skin [240]. These effects were associated with an inhibition of phosphorylation of p38 mitogen-activated protein kinase [240], which is an upstream effector of NF-κB and COX-2 [21,241]. Kim et al. [242] found that topical application of [6]-gingerol inhibited TPA-induced COX-2 expression in mouse skin. As before, the decreased COX-2 expression was also associated with a suppression of TPA-induced NF-κB DNA binding and transcriptional activation and inhibition of p38 phosphorylation. However, the decreased COX-2 expression also corresponded with an inhibition of IκBα degradation, p65 translocation to the nucleus, and its interaction with cAMP response element binding (CREB) protein, a transcriptional coactivator of NF-κB [242]. In contrast, [8]-gingerol, but not [6]-gingerol, was earlier shown to inhibit COX-2 expression [243].

Zerumbone has also been shown to suppress NF-κB activation induced by tumor necrosis factor (TNF), okadaic acid, cigarette smoke condensate, PMA, and H_2O_2 [244]. The inhibition of NF-κB corresponded with a suppression of IκBα kinase activity, phosphorylation, and degradation and also p65 phosphorylation and nuclear translocation. Numerous NF-κB transcriptional targets were also downregulated by zerumbone resulting in an increased sensitivity to apoptosis induced by chemotherapeutic agents [244].

2.3.5 Inhibition of Angiogenesis by Ginger

At least one group [245] reported that [6]-gingerol could inhibit the vascular endothelial growth factor (VEGF)-induced proliferation of human endothelial cells apparently by causing G1 cell cycle arrest. Intraperitoneal injection of [6]-gingerol to mice receiving intravenous injection of B16F10 melanoma cells also inhibited the formation of lung metastases with no cytotoxicity [245]. Overall these studies suggest that ginger and its components have great potential as anticancer agents. However, specific mechanisms and interacting gene or protein targets are still to be delineated.

2.4 CONCLUSION

The major focus in our laboratory is the study of the molecular mechanisms of cancer development and the action of anticancer agents in cancer prevention. We address fundamental questions concerning the response of animal and/or human cells to carcinogens and tumor promoters such as UV light, arsenic, TPA, and growth factors. This research has provided the basis for the carcinogenic process caused by environmental carcinogens and molecular mechanisms for cancer prevention. Further, such discoveries have identified key molecular targets for screening novel natural anticancer drugs with fewer side effects. Nutritional or dietary factors have attracted a great deal of interest because of their perceived ability to act as highly effective chemopreventive agents. They are perceived as being generally

safe and may have efficacy as chemopreventive agents by preventing or reversing premalignant lesions and/or reducing second primary tumor incidence. Many of these compounds appear to act on multiple tumor promoter-stimulated cellular pathways. Some of the most interesting and well documented are components of tea, such as EGCG, theaflavins, and caffeine, and other potentially effective dietary compounds including ginger. A continuing emphasis on obtaining rigorous research data and critical analysis of those data regarding these and other food factors is vital to determining the molecular basis and long-term effectiveness and safety of these compounds as chemopreventive agents. Large-scale animal and molecular biology studies are needed to address the bioavailability, toxicity, molecular target, signal transduction pathways, and side effects of dietary factors. Clinical trials based on clear mechanistic studies are also needed to assess the effectiveness of these dietary factors in the human population.

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3 Modulation of MAPK Pathways by Food Phytochemicals: Risks and Benefits

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CONTENTS

3.1 SEARCH FOR CANCER PREVENTIVE FOOD PHYTOCHEMICALS

Rapid and reliable methods of identifying cancer preventive compounds include screening of a wide range of natural or synthetic compounds that have biological and biochemical relevance to human carcinogenesis using *in vitro* assay systems. An alternative natural product chemistry approach that directs bioactive crude extracts involves activity-guiding separation, and then isolation and identification

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of the active principle, which is a powerful and efficient technique to achieve the goal of compound identification. Phorbol ester tumor promoters have been used in a great number of studies that attempted to identify novel candidates for cancer prevention, of which 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was shown to activate oncogenic Epstein-Barr virus in several B-lymphoblastoid cell lines [1,2]. Our laboratory has conducted extensive screening tests of not only domestic vegetables and fruits [3], but also those from Southeast Asian countries, such as Thailand [4], Indonesia [5], and Malaysia [6]. Interestingly, the *in vitro* antitumor promoting activities found in those subtropical plants were markedly higher than in plants typically found in Japan. Scientific reasons for the pronounced potentials of those plants remain to be elucidated. However, it is important to note that all plants have the potential to biosynthesize certain chemicals in response to environmental stress stimuli, such as ultraviolet (UV) exposure, invading insects, bacteria, and viruses (Figure 3.1). For example, plants biosynthesize polyphenols in order to scavenge free radicals generated from UV light exposure. Otherwise the proteins and DNA, which are critical for their survival, would become substantially damaged. Thus, it is not surprising that subtropical plants exposed to harsher types of stress produce greater numbers of biologically active phytochemicals of higher quality. As a result, we consider that vegetables and fruits commonly found in Southeast Asian countries are an attractive and promising source of cancer preventive compounds.

Activity-guided fractionation and purification processes using repeated column chromatography and spectroscopic analyses have resulted in identification of active phytochemicals that have potential cancer preventive activities. However, most of those compounds have not been used in animal experiments because of sample size limitations (see Figures 3.2 and 3.3). In contrast, 1′-acetoxychavicol

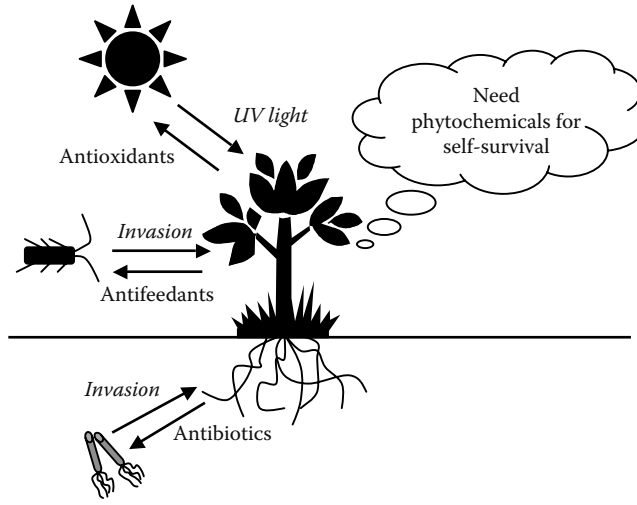

FIGURE 3.1 Possible roles of phytochemicals in protecting plants from environmental stresses.

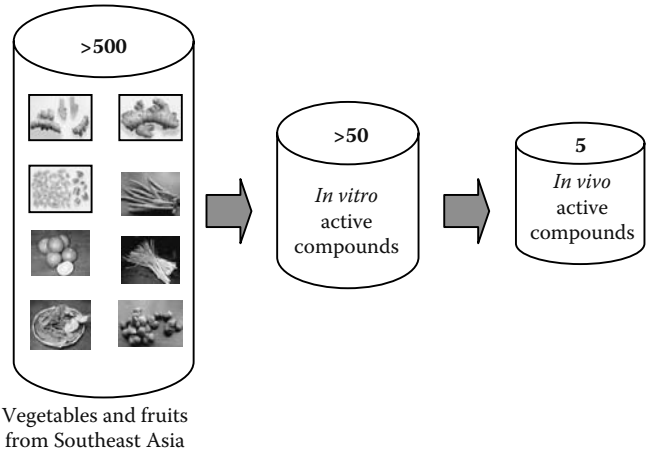

FIGURE 3.2 (See color insert following page 74.) Screening for cancer preventive compounds derived from vegetables and fruits.

acetate (ACA, phenylpropanoid) [7] and zerumbone (sesquiterpene) [8] are abundantly present in the essential oils obtained from the rhizomes of zingiberaceous plants (*Alpinia galanga* Stuntz and *Zingiber zerumbet* Smith, respectively). In addition, a geranyloxyl coumarin, auraptene [9], and the polymethoxyflavonoid nobiletin [10] occur in certain citrus fruits, while (±)-13-hydroxy-10-oxo-*trans*-11-octadecenoic acid (HOA) and its derivatives are components of corn germ and rice bran [11]. Although the biological activities of these compounds have already

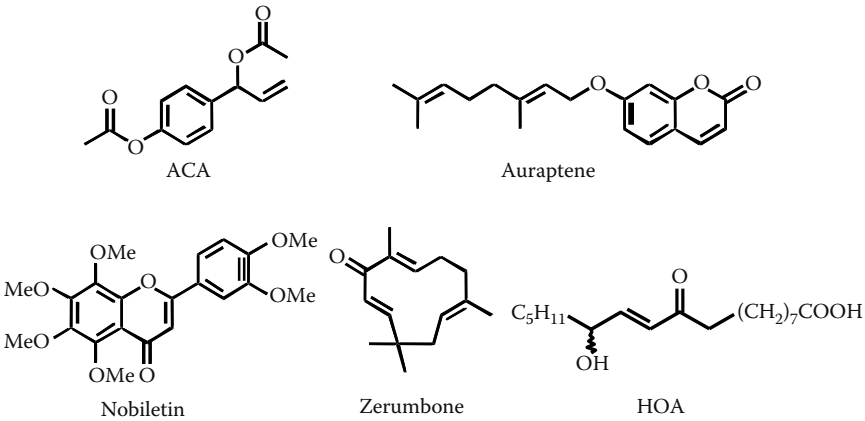

FIGURE 3.3 Structures of cancer preventive food phytochemicals derived from some subtropical plant. Both ACA and auraptene can be chemically synthesized, whereas the other three compounds are able to be prepared by purification of each plant extract for use in rodent experiments.

been described by other groups [12–16], we present herein for the first time findings regarding their chemopreventive efficacy.

In a study published in 1996, we found that ACA at a very low-dose suppressed phorbol ester-induced tumor promotion in mouse skin [17]. Also, 4-nitroquinoline 1-oxide-induced oral carcinogenesis was dramatically suppressed by this compound in other experiments [18]. Thereafter, ACA was shown to inhibit azoxymethane-induced aberrant crypt foci formation [19] and colon carcinogenesis [20], the formation of glutathione *S*-transferase (GST) placental form-positive focal lesions in rat livers [21], *N*-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis [22], and *N*-nitrosobis(2-oxopropyl)-amine-induced initiation of cholangiocarcinogenesis in Syrian hamsters [23]. In addition, auraptene has also exhibited a wide range of promising cancer preventive activities in experiments using mouse skin [9], rat colon [24–26], rat oral cavity [27], rat esophagus [28], and rat liver [29] samples. On the other hand, chemopreventive data for nobiletin, zerumbone, and HOA are limited [10,11,30–33].

3.2 POSSIBLE ACTION MECHANISMS

Several *in vitro* and *in vivo* biological systems that mimic the processes of carcinogenesis have been used in our and other laboratories for dissecting the action mechanisms underlying the chemopreventive effects of the aforementioned compounds. It is well established that endogenous and exogenous procarcinogens are potentially susceptible to exclusion from the body by xenobiotic-metabolizing enzymes, such as phase I (cytochrome P450, etc.) and phase II (GST, etc.) enzymes. Elevation of phase I enzyme levels leads to oxidation and hydroxylation of hydrophobic xenobiotics, after which phase II enzymes conjugate them with glucuronic acid and/or sulfuric acid, thereby converting them into hydrophilic metabolites that circulate in the bloodstream. Many procarcinogens such as polyaromatic hydrocarbons are also chemically modulated by phase I enzymes and the resultant ultimate carcinogens are potentially genotoxic if the activities of phase II enzymes are not potent enough to deactivate the harmful mutagens. Thus, selective elevation of phase II, but not phase I, enzymes are beneficial for reducing the risk of tumor initiation [34]. We previously showed that oral administration of auraptene to F344 rats led to significant and selective elevation of phase II enzyme activity in their livers without affecting that of phase I enzymes [35]. Similarly, topical application of zerumbone to mouse skin increased the mRNA expression of manganese superoxide dismutase, glutathione peroxidase-1, GST-P1, and NAD(P)H quinone oxidoreductase, but not that of cytochrome P450 1A1 or 1B1 [32].

There is compelling evidence that chronic inflammation plays a crucial role in tumor development [36,37]. For example, certain chronic inflammatory diseases overlap with the onset and development of cancer, such as ulcerative colitis and Crohn's disease (colorectal cancer), reflux esophagitis, Barrett's esophagus (esophageal carcinoma), and hepatitis (hepatocellular carcinoma) [38]. In the process of nonpathologic inflammation (e.g., wound healing), platelets are known to release

several types of mediators that tightly regulate vascular permeability and recruit fibrinogen, leading to the formation of fibrin clots. These activities also induce and produce chemotactic factors, which lead to the activation of stromal cells that are responsible for the release of a cocktail of proteases, such as the matrix metalloproteinase (MMP) superfamily that can virtually degrade the extracellular matrix. Concurrently, some immune cells, including neutrophils and monocytes, are matured and recruited, after which they infiltrate into inflamed tissue as part of the innate immune machinery. These are well known as biological sources of reactive oxygen and nitrogen species (RONS), prostaglandins (PGs), inflammatory cytokines, and chemokines, as well as others. RONS, including free radicals, are chemically unstable molecules that are able to modify, denature, and decompose biological components such as lipid membranes, proteins, and DNA. Furthermore, PGE_2 has been demonstrated to induce angiogenesis and is known to play some notable roles in the growth of fibroblasts, as well as endothelial and epithelial cells. On the other hand, cytokines and chemokines are systematically activated and released into neighboring tissues in a coordinated manner, after which they circulate in the bloodstream for further activation of the immune system. All of these biological phenomena progress in a concerted fashion toward re-epithelialization and healing resolution. In pathogenic conditions, most of these processes are shared, though they are sustained and exaggerated in a dysregulated manner.

Using cellular and animal models, we have presented experimental evidence suggesting that the aforementioned cancer preventive compounds share a common mechanistic characteristic, that is, attenuation of leukocytic activation [39]. Watanabe et al. provided the first report showing that ACA is an inhibitor of phagocytosis [40], after which ACA [17,41], auraptene [9,42], nobiletin [10,43], zerumbone [44], and HOA [11] were shown to suppress phorbol ester- or endotoxin-induced free radical generation, as well as the production of proinflammatory cytokines in inflammatory cells. We reported that topical applications of those cancer preventive agents decreased phorbol ester-induced leukocytic activation in mouse epidermis samples [10,11,42,44,45].

3.3 MOLECULAR MECHANISMS UNDERLYING COX-2 SUPPRESSION BY PHYTOCHEMICALS

Cyclooxygenase (PGH_2 synthase, COX) donates two oxygen molecules to arachidonic acid to form PGG_2 by peroxidation, which in turn is reduced to PGH_2. To date, there are at least two known COX isoforms, COX-1 and COX-2. In contrast to COX-1, COX-2 mRNA and protein are only slightly expressed in most normal mammalian tissues, though they are highly induced in response to physical, chemical, and biological stimuli. In recent years, COX-2 has received the attention of numerous researchers because of the implication of its expression in the pathogenesis of cancer, including neoplasm development. Of note, zerumbone was particularly highlighted by us because of its distinct ability to suppress COX-2 in macrophages [44], as well as in rat colon [33] and mouse skin [32] specimens, though the underlying molecular mechanism is not fully understood. In macrophages,

the endotoxin LPS is known to bind to Toll-like receptor (TLR)-4 [46]. TLR-4 has several associated proteins, such as MD2 and Myd88, needed for full activation, after which it stimulates some protein kinases, including phosphatidylinositol 3-kinase and phosphoinositide-dependent kinase. The phosphorylated protein kinase B (Akt) in turn activates IkappaB (IκB) kinase (IKK), which is composed of the α, β, and γ subunits, and phosphorylates IκB protein [47], leading to degradation of this protein. Since IκB is a suppressive partner that binds to the nuclear factor κB (NFκB) transcription factor in a normal state, LPS-induced IκB degradation results in the activation of this particular transcription factor for COX-2 mRNA induction. On the other hand, many reports have indicated that MAPK activation is associated with the induction of COX-2. MAPKs control many cellular events, including differentiation, proliferation, and cell death, as well as short-term changes required for homeostasis and acute hormonal responses [48]. To date, at least three major MAPK cascades have been described that involve the activation of ERK1/2, JNK1/2, and p38 MAPKα/β/γ. ERK activation has been shown to be involved in the phosphorylation of p65, thereby activating NFκB [49], and a similar observation was reported in a study that used RAW264.7 cells [50]. Further, the importance of Ser^{536} as the phosphorylation site of p65, an NFκB component, has been proposed [51]. Chen et al. reported that ERK activation led to NFκB activation that was related to COX-2 expression [52]. In addition, there is ample evidence that activation of the JNK pathway leads to activator protein (AP)-1 transcriptional activation, because phosphorylated c-Jun, one of the direct targets of JNK, is a component of the AP-1 complex [53].

Transcription coactivators, including cAMP-responsive element binding protein (CREB)-binding protein (CBP) and its homologue p300, are known to play major roles in various stimuli-induced or -repressed intracellular signaling pathways [54], and was shown to potentiate the transcriptional activities of AP-1 [55], NFκB [56], and CREB [57]. In addition, ASC-2 has emerged as a novel coactivator participating in the transcriptional activation of NFκB and AP-1 [58]. Further, COX-2 mRNA contains the AU-rich element (ARE) in its 3′-untranslated region (UTR), which plays some critical roles in the stability of its mRNA. Several reports using different cell types have shown that activation of p38 MAPK leads to stabilization of COX-2 mRNA. Subsequently, a substrate for p38 MAPK, MAPK-activated protein kinase 2 (MAPKAPK-2) [59], was found to phosphorylate certain candidate proteins such as HSP27 [60], heterogeneous nuclear ribonucleoprotein (hnRNP) A0 [61], and Hu antigen R (HuR) [62], which bind to AREs, thereby contributing to a rapid synthesis of COX-2 protein. In addition, Sully et al. identified several proteins, including AU-rich element/poly(U)-binding/degradation factor-1 (AUF-1), AUF-2, tristetraprolin, HuR, and far-upstream-sequence-element-binding protein 1 (FBP1), which target conserved region 1 (CR 1) located at 3′-UTR [63].

Of great interest, molecular targets of food phytochemicals appear to differ [64] (Figure 3.4). For example, HOA abrogated LPS-induced MAPKs and their upstream protein kinases in RAW264.7 mouse macrophages [11], suggesting that it targets the TLR complex, while ACA abolished LPS-induced both ERK1/2 and JNK1/2

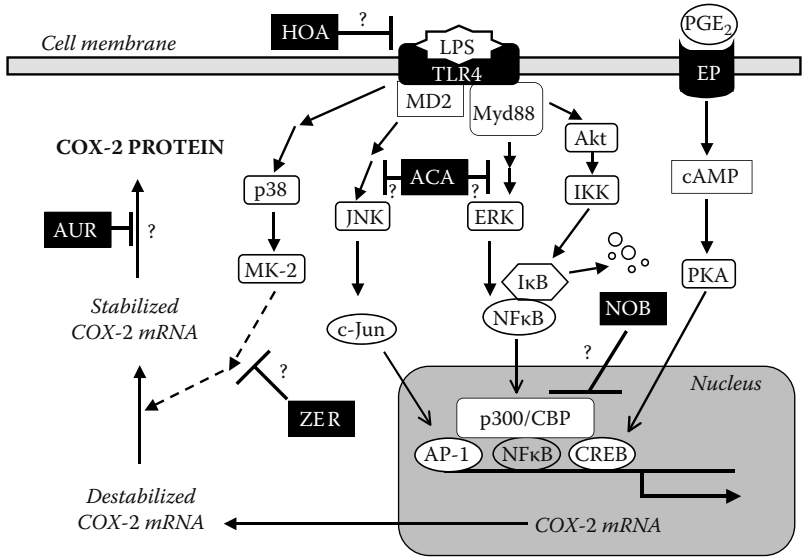

FIGURE 3.4 Possible molecular mechanisms by which food phytochemicals suppress COX-2 induction and production in macrophages. HOA, (±)-13-hydroxy-10-oxo-*trans*-11-octadecenoic acid; NOB, nobiletin; ZER, zerumbone; AUR, auraptene; LPS, lipopolysaccharide; TLR, Toll-like receptor; ACA, 1′-acetoxychavicol acetate; IKK, IκB kinase; NFκB, nuclear factor κB; CREB, cAMP-responsive element binding protein; CBP, CREB-binding protein; AP-1, activator protein-1; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase 2; PGE_2, prostaglandin E_2; PKA, protein kinase A.

activation as well as IκB degradation [64]. Citrus nobiletin did not attenuate MAPK activation, whereas it caused significant decreases in the transcription activities of NFκB, AP-1, and CREB, implicating that it disrupts the function of the coactivator CBP/p300 [64], though such a mechanism was not seen in the suppression of phorbol ester-triggered scavenger receptor expression in THP-1 human monocytes [65]. The mode of action of zerumbone is unique, as it did not disrupt LPS-induced MAPK and IKK activation or the transcriptional activation of NFκB, AP-1, and CREB, whereas it potently inhibited COX-2 mRNA expression [64]. These results show that zerumbone may target the stabilization process of COX-2 mRNA. Finally, citrus auraptene may interfere with the protein translational step of COX-2, because it did not attenuate the expression of mRNA [64], while it attenuated that of COX-2 protein [44].

3.4 ZERUMBONE ACTIVATES MAPKs FOR CYTOKINE RELEASE

As described earlier, the anti-inflammatory property of zerumbone has been demonstrated in macrophages [44], as well as mouse skin [32] and rat colon [33] specimens. It should also be noted that those results were generated in experimental

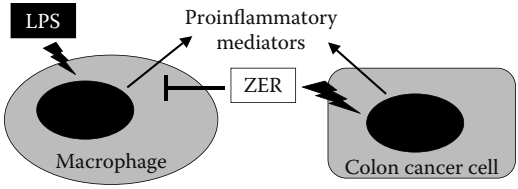

FIGURE 3.5 Possible anti- and proinflammatory mechanisms of zerumbone. LPS, lipopolysaccharide; ZER, zerumbone.

conditions in which chemical stimuli (LPS, phorbol ester, and carcinogens, respectively) were used. Thus, it was considered worthwhile to examine the outcomes of added food factors including zerumbone in nontreated cells. We treated Caco-2 human colon adenocarcinoma cells with zerumbone, L-ascorbic acid, quercetin, benzyl isothiocyanate, α-tocopherol, nobiletin, sodium butyrate, auraptene, and ACA, and then semiquantified the changes in mRNA expression levels of COX-1, COX-2, IL-1α, IL-1β, IL-6, inducible nitric oxide synthase (iNOS), p53, vascular endothelial growth factor, and tumor necrosis factor (TNF)-α. Although the expression levels of most of the target genes were not significantly changed following treatment with those food factors, zerumbone markedly increased those of IL-1α, IL-1β, IL-6, and TNF-α [66]. Furthermore, those effects were reproduced in colo320DM and HT-29 human colon cancer cells [66]. Results of pharmacological blockade of MAPK kinase (MEK)1/2 (U0126 and PD98059), JNK1/2 (SP600125), and p38 MAPK (SB203580) suggested that zerumbone-induced IL-1β expression was mediated via the activation of both ERK1/2 and JNK1/2, but not p38 MAPK, pathways [66]. This notion may be supported by the findings of Owuor and Kong, who reported that some electrophiles have the potential to activate MAPKs [67], since zerumbone carries an electrophilic α,β-unsaturated carbonyl group. In any case, it is intriguing that the pro and anti-inflammatory properties of zerumbone may be dependent upon the presence or absence of stimuli and cell types (macrophage and colon cancer cell) used (Figure 3.5).

3.5 URSOLIC ACID TRIGGERS ERK2 ACTIVATION FOR MIF RELEASE

Trumbull et al. published one of the earliest reports suggesting the anti-inflammatory and anticarcinogenic activities of a natural triterpenoid, ursolic acid (UA) [68]. Thereafter, both *in vitro* and *in vivo* reports have been presented confirming those properties, for example, the suppression or inhibition of phorbol ester-induced EB virus activation [69], skin tumor promotion [70,71], lipoxygenase activities [72], mutagenesis [73], and tumor invasive protease expression [74]. In addition, Suh et al. documented that natural and synthetic triterpenoids have significant abilities to attenuate LPS-induced iNOS and COX-2 expression in mouse macrophages [75], which may be mediated via the regulation of NFκB [75]. Conversely, Shishodia et al. showed that

UA inhibits TNF-α-, phorbol ester-, okadaic acid-, hydrogen peroxide- and cigarette smoke-induced NFκB activation in various cell lines [76]. In contrast, You et al. reported interesting findings showing that UA activates NFκB for inducing iNOS and TNF-α in nontreated RAW264.7 macrophages [77], raising the possibility that UA is a double-edged sword, whose pro and anti-inflammatory activities are dependent upon the biological status of macrophages, namely nontreated or stimulated.

To extend our knowledge of the pro and anti-inflammatory aspects of UA, we investigated the effects of UA on the production of macrophage migration inhibitory factor (MIF) in RAW264.7 cells [78]. MIF is a cytokine that plays a critical role in several inflammatory conditions by regulating the activation of macrophages and T cells [79]. This cytokine shows a variety of biological functions, including induction of TNF-α, COX-2, and TLR4 [80–82], and is a key molecule in the convergence of inflammatory processes with those of carcinogenesis, as shown by its ability to functionally inactivate the tumor suppressor protein p53 [83,84]. RAW264.7 cells express MIF mRNA and protein in constitutive manners, while they scarcely secrete MIF protein into media. Treatment of macrophages with UA led to a marked increase in the concentration of MIF protein in culture media, whereas the levels of MIF mRNA did not change significantly. It was notable that the amount of intracellular MIF protein decreased when the cells were stimulated with UA, which strongly suggested that MIF protein stored in the intracellular compartment is transported and released from cells upon stimulation with this triterpenoid. UA also induced the activation of ERK1/2, but not JNK1/2 or p38 MAPK, while the involvement of ERK2, and to a lesser extent ERK1, following activation caused by the release of MIF was revealed in experiments using siRNAs [78] (Figure 3.6). It is conceivable

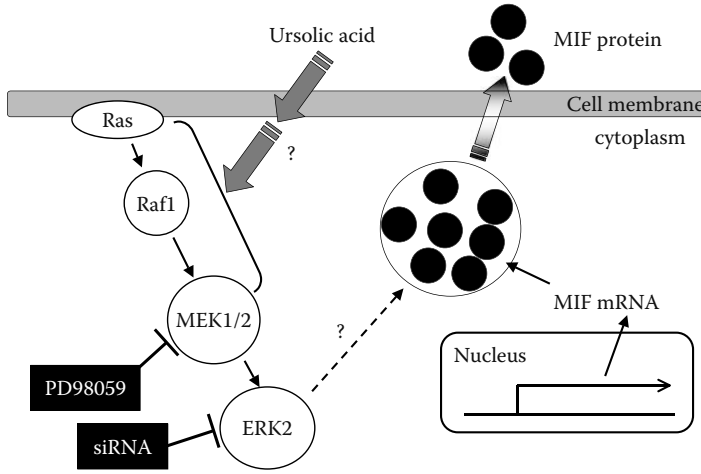

FIGURE 3.6 Ursolic acid activates ERK2 for promoting the release of MIF in macrophages. UA, ursolic acid; MIF, macrophage migration inhibitory factor; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA. PD98059 is a MEK1/2 inhibitor.

that the UA-triggered activation of ERK2 may also be associated with the activation of NFκB, and the resultant induction of iNOS and TNF-α [77], based on related results [49–51]. In addition, we recently reported the *in vivo* proinflammatory effects of UA following topical applications to non- and TPA-treated mouse skin for 2 weeks, which led to a significant increase in mRNA expression of COX-1, COX-2, and TNF-α [85].

3.6 CONCLUSION

The effects of food factors on MAPK pathways are complex, and occasionally depend on cell types and stimuli. Therefore, it is essential not to focus on a single experimental method, but rather to use a wide range of bioassay systems that have biological and biochemical relevance to tumorigenesis for more precise elucidation of molecular mechanisms. If not, unexpected and unwanted side effects, which may be latent under limited experimental conditions, could emerge in human intervention studies, in which the genetic and epigenetic factors are much more diverse. In addition, it should be kept in mind that MAPK activation itself is a double-edged sword, as green tea catechin-induced MAPK activation has been shown to be concurrently involved in both apoptosis [86] and pro-MMP-7 production [87] in HT-29 human colon cancer cells.

3.7 PERSPECTIVES

In principle, plants produce secondary metabolites for their own use and not for human consumption, and those chemicals are biosynthesized to maintain homeostasis and protect the plant from environmental stresses. It may be a coincidence that certain food phytochemicals have notable potential for cancer prevention, however, it is considered worthwhile to note that MAPK pathways are signal transducing machineries common between plants and mammals. Plant MAPK pathways play some central roles in regulating various types of biotic and abiotic stress, such as infections caused by wounds and pathogens, and temperature stress and drought [88], as well as in activating their immune systems [89]. Thus, it is tempting to hypothesize that a portion of plant secondary metabolites are synthesized *de novo* to modulate endogenous MAPK activity. If that speculation is correct, it would not be surprising to find that phytochemicals that modulate endogenous MAPK activities also have effects on those in mammalian cells through similar mechanisms, and thereby exhibit cancer preventive actions. Although accumulated evidence shows that phytochemicals modulate mammalian MAPK activities, there is limited information on those activities in plants. A future direction of study should be toward understanding the effects of phytochemicals on plant MAPKs to increase knowledge of their roles in plants, as well as their effects on mammalian MAPK activities.

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ABBREVIATIONS

ACA	1′-acetoxychavicol acetate
AP-1	activator protein-1
ARE	AU-rich element
AUF-1	AU-rich element/poly(U)-binding/degradation factor-1
CBP	CREB-binding protein
COX	cyclooxygenase
CR 1	conserved region 1
CREB	cAMP-responsive element binding protein
ERK	extracellular signal-regulated kinase
FBP1	far-upstream-sequence-element-binding protein 1
GST	glutathione *S*-transferase
hnRNP	heterogeneous nuclear ribonucleoprotein
HOA	(±)-13-Hydroxy-10-oxo-*trans*-11-octadecenoic acid
HuR	Hu antigen R
IKK	IkappaB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun NH_2-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAPKAPK-2	MAPK-activated protein kinase 2
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinase
NFκB	nuclear factor κB
PG	prostaglandin
RNOS	reactive oxygen and nitrogen species
TLR	Toll-like receptor
TNF	tumor necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UA	ursolic acid
UTR	3′-untranslated region
UV	ultraviolet

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4 Turmeric as Cure-Cumin: Promises, Problems, and Solutions

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CONTENTS

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4.1 INTRODUCTION

The turmeric plant (*Curcuma longa*) is a perennial herb belonging to the ginger family that is cultivated extensively in India and Southeast Asia. The rhizome (a modified stem) is the most useful part of the plant. It is used as a dietary spice,

as a coloring agent in foods and textiles, and as medicine for numerous diseases. The rhizome is rich in curcuminoids. Curcumin is the most biologically active curcuminoid of turmeric and makes up 2% to 5% of the spice. The characteristic yellow color of turmeric is due to curcumin.

For centuries, curcumin has been consumed as a dietary spice at doses up to 100 mg/day. Extensive investigation over the last five decades has indicated that curcumin reduces blood cholesterol [1–7]; prevents low-density lipoprotein (LDL) oxidation [8–10]; inhibits platelet aggregation [11,12]; suppresses thrombosis [13] and myocardial infarction [14–17]; suppresses symptoms associated with type 2 diabetes [18–22], rheumatoid arthritis [23], multiple sclerosis [24], and Alzheimer's disease [25,26]; inhibits human immunodeficiency virus (HIV) replication [27–31]; enhances wound healing [32–34]; protects from liver injury [35]; increases bile secretion [1]; protects from cataract formation [36], pulmonary toxicity, and fibrosis [37–40]; and has anti-leishmaniasis [41–43] and anti-atherosclerotic activities [44,45]. Moreover, extensive literature suggests that curcumin has potential in the prevention and treatment of a variety of other diseases.

Although curcumin has been reported to possess a wide range of therapeutic utilities in traditional Indian (Ayurveda), Unani, and Siddha systems of medicine, it was first isolated in 1815 and obtained in crystalline form in 1870 [46,47]. With the explosion in the last decade in the number of patents and publications concerning curcumin and its analogs, the time has come when we must develop our understanding of the mechanisms of action of curcumin at cellular and genetic levels, its delivery to target cells and tissues, the enzymes involved in its degradation and metabolism, and the safety of using it in larger doses so that we can identify its wider therapeutic applications.

4.2 DISEASE TARGETS OF CURCUMIN

Ayurveda (*Ayur* = long life; *veda* = knowledge), the ancient texts of Indian medicine, describe the use of curcumin for a wide variety of inflammatory diseases including sprains and swellings caused by injury, wound healing, and abdominal problems [48]. Texts on traditional medicine in China describe administration of curcumin for the treatment of abdominal pain. Perhaps most of the effects associated with curcumin are based on its ability to suppress inflammation. Curcumin has been shown to be effective in a number of diseases (Figure 4.1).

4.2.1 Anti-Inflammatory and Antioxidant Properties

Several studies have shown that curcumin is a potent antioxidant. In fact, curcumin has been found to be at least 10 times more active as an antioxidant than even vitamin E [49]. Curcumin prevents oxidation of hemoglobin and inhibits lipid peroxidation (for references, see Ref. [50]). The antioxidant activity of curcumin could be mediated through antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Curcumin has been shown to serve as a Michael acceptor, reacting with glutathione and thioredoxin 1 [51]. Reaction

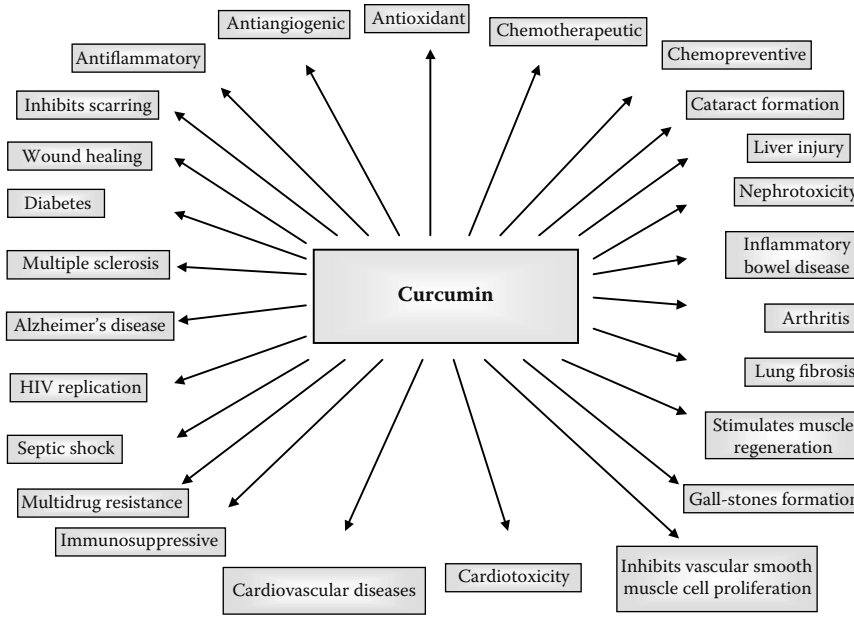


FIGURE 4.1 Disease targets of curcumin.

of curcumin with these agents reduces intracellular glutathione in cells. The suppression of lipid peroxidation by curcumin could lead to attenuation of inflammation.

4.2.2 Anticancer Properties

The anticancer potential of curcumin in various systems was reviewed recently [50]. Curcumin has been shown to block transformation, tumor initiation, tumor promotion, invasion, angiogenesis, and metastasis. In *in vivo* studies, curcumin suppressed carcinogenesis of the skin, forestomach, colon, and liver in mice. Curcumin has been shown to inhibit proliferation of a wide variety of tumor cells, including B-cell and T-cell leukemias [52–55], colon carcinoma [56], epidermoid carcinoma [57], head and neck squamous cell carcinoma [58], multiple myeloma [59], and mantle cell lymphoma [60]. It has also been shown to suppress proliferation of various breast carcinoma cell lines in culture [61–63].

Mehta et al. examined the antiproliferative effects of curcumin against several breast tumor cell lines, including hormone-dependent and -independent and multidrug-resistant cell lines [61]. All the cell lines tested, including the multidrug-resistant ones, were highly sensitive to curcumin. The growth-inhibitory effect of curcumin was time and dose dependent, and correlated with its inhibition of ornithine decarboxylase activity. Curcumin preferentially arrested cells in the G_2/S phase of the cell cycle.

Fang et al. reported that rat thioredoxin reductase activity in thioredoxin-dependent disulfide reduction was inhibited by curcumin [64]. By using mass spectrometry and blotting analysis, they showed that this irreversible inhibition by curcumin was caused by alkylation of both residues (Cys^{496}/Seleno-Cys^{497}) in the catalytically active site of the enzyme. Kang et al. reported that exposure of human hepatoma cells to curcumin led to a significant decrease of histone acetylation [65]. Curcumin can selectively downregulate transcription of human papillomavirus type 18, which is etiologically associated with development of cancer of the uterine cervix in women, as well as activator protein 1 (AP-1) binding activity in HeLa cells. Most interestingly, curcumin can reverse the expression dynamics of c-fos and fra-1 in this tumorigenic cell line [66].

Curcumin had synergic activity with chemotherapeutic agent vinorelbine in suppressing the growth of human squamous cell lung carcinoma H520 cells [67]. It significantly inhibited the growth of human gastric carcinoma AGS cells in a dose- and time-dependent manner [68]. Using time-lapse video and immunofluorescence labeling methods, Holy demonstrated that curcumin significantly altered microfilament organization and cell motility in PC-3 and LNCaP human prostate cancer cells *in vitro* [69]. Chemoresistance is a major problem in the treatment of patients with multiple myeloma due to constitutive expression of nuclear factor-kappaβ (NF-κB) and signal transducer and activator of transcription (STAT) 3. Bharti et al. showed that suppression of NF-κB and STAT3 activation in multiple myeloma cells by *ex vivo* treatment with curcumin resulted in decreases in adhesion to bone marrow stromal cells, secretion of cytokines, and viability of cells [59,70].

Helicobacter pylori is a Group 1 carcinogen that is associated with the development of gastric and colon cancers. Curcumin inhibited the growth of all strains of *H. pylori in vitro* with a minimum inhibitory concentration range of 6.25–50 μg/ml [71]. Chen et al. used microarray analysis of gene expression profiles to characterize the anti-invasive mechanisms of curcumin in highly invasive lung adenocarcinoma cells (CL1-5) [72]. In these studies, curcumin significantly reduced the invasive capacity of CL1-5 cells in a concentration range far below its levels of cytotoxicity (20 μM), and this anti-invasive effect was concentration dependent. Kim et al. evaluated the antiangiogenic activity of demethoxycurcumin, a structural analog of curcumin, and found that nine angiogenesis-related genes were downregulated by at least five-fold in response to this agent [73].

Numerous studies have evaluated the cancer-chemopreventive properties of curcumin. The anticancer potential of curcumin was examined *in vivo* in mice using Dalton's lymphoma cells grown as ascites [74]. When curcumin was administered in liposomal formulations at a concentration of 1 mg/animal, all animals survived 30 days and only two of the animals developed tumors and died before 60 days. Similarly, Busquets et al. showed that systemic administration of curcumin for 6 consecutive days to rats bearing the highly cachectic Yoshida AH-130 ascites hepatoma resulted in a significant inhibition of tumor growth [75]. Interestingly, curcumin was able to reduce

in vitro tumor cell content by 24% at concentrations as low as 0.5 μM without promoting any apoptotic events.

Menon et al. reported curcumin-induced inhibition of B16F-10 melanoma lung metastasis in mice [76]. Oral administration of curcumin at concentrations of 200 nmol/kg body weight reduced the number of lung tumor nodules by 80%. The life span of the animals treated with curcumin was increased by 143.85% [76]. Curcumin treatment (10 μg/ml) significantly inhibited the invasion of B16F-10 melanoma cells by inhibition of matrix metalloproteinases (MMP), thereby inhibiting lung metastasis.

Curcumin decreases the proliferative potential and increases the apoptotic potential of both androgen-dependent and -independent prostate cancer cells *in vitro*, largely by modulating the apoptosis suppressor proteins and by interfering with the growth factor receptor signaling pathways as exemplified by the epidermal growth factor receptor (EGFR) [77]. The chemopreventive activity of curcumin was observed when it was administered prior to, during, and after carcinogen treatment as well as when it was given only during the promotion/progression phase of colon carcinogenesis [78]. The chemopreventive effect of curcumin was also examined on the development of adenomas in the intestinal tract of a mouse model of human familial adenomatous polyposis coli [79]. Curcumin at 0.2% and 0.5% of diet reduced adenoma multiplicity by 39% and 40%, respectively.

Odot et al. showed that curcumin was cytotoxic to B16-R melanoma cells resistant to doxorubicin [80]. Treatment with a prophylactic immune preparation of soluble proteins from B16-R cells, in combination with curcumin, resulted in substantial inhibition of growth of B16-R melanoma and a significant increase in the median survival time of the animals.

4.2.3 Cardioprotective Effects

Curcumin has been shown to have activity against atherosclerosis and myocardial infarction (for references, see Ref. [81]). Dikshit et al. examined the prevention of ischemia-induced biochemical changes by curcumin in the cat heart [14]. Myocardial ischemia was induced by ligation of the left descending coronary artery. Curcumin (100 mg/kg intraperitoneally) was given 30 min before ligation. Curcumin protected the animals against decreases in heart rate and blood pressure following this induced ischemia.

Proliferation of peripheral blood mononuclear cells and vascular smooth muscle cells, which are hallmarks of atherosclerosis, is inhibited by curcumin. Curcumin prevents oxidation of LDL, inhibits platelet aggregation, and reduces the incidence of myocardial infarction [1–6,82]. Soudamini et al. investigated the effects of oral administration of curcumin on serum cholesterol levels and on lipid peroxidation in the liver, lung, kidney, and brain of mice treated with carbon tetrachloride, paraquat, and cyclophosphamide [4]. These chemicals increase peroxidation of lipids in these tissues, but oral administration of curcumin significantly reversed this peroxidation.

4.2.4 Skin Diseases

Curcumin has been shown to be effective against various skin conditions, including skin carcinogenesis, psoriasis [83], scleroderma [84], and dermatitis. Numerous reports suggest that curcumin accelerates wound healing. Sidhu et al. examined the wound-healing capacity of curcumin in rats and guinea pigs [32]. Punch wounds closed faster in curcumin-treated animals than in untreated animals. Biopsies of the wounds showed re-epithelialization of the epidermis and increased migration of various cells, including myofibroblasts, fibroblasts, and macrophages, in the beds of curcumin-treated wounds. Curcumin-treated animals showed extensive neovascularization in multiple areas within the dermis and greater collagen deposition in the wounds. Curcumin seemed to have potent effects in inhibiting proliferation and contraction of excessive scar-derived fibroblasts [85]. Curcumin also plays a role in muscle regeneration following trauma (for references, see Ref. [81]).

4.2.5 Diabetes

Administration of curcumin significantly reduced the blood sugar and glycosylated hemoglobin levels in an alloxan-induced rat model of type 2 diabetes. Diabetic rats maintained on a curcumin diet for 8 weeks excreted less albumin, urea, creatinine, and inorganic phosphorus than rats not fed curcumin. Dietary curcumin also partially reversed abnormalities in plasma albumin, urea, creatinine, and inorganic phosphorus in diabetic animals (for references, see Ref. [81]).

4.2.6 Rheumatoid Arthritis

Curcumin has been shown to possess antirheumatic and antiarthritic effects, most likely through downregulation of cyclooxygenase 2 (COX-2), tumor necrosis factor (TNF), and other inflammatory cytokines. Deodhar et al. were the first to report on the antirheumatic activity of curcumin in human subjects [23]. They performed a short-term double-blind crossover study in 18 patients with rheumatoid arthritis to compare the antirheumatic activity of curcumin (1200 mg/day) with that of phenylbutazone (300 mg/day). Subjective and objective assessment in patients who were taking corticosteroids just prior to the study showed significant improvements in morning stiffness, walking time, and joint swelling following 2 weeks of curcumin therapy.

Liacini et al. examined the effects of curcumin in articular chondrocytes. Interleukin (IL)-1, the main cytokine instigator of cartilage degeneration in arthritis, induces MMP-3 and MMP-13 RNA and protein in chondrocytes through activation of mitogen-activated protein kinase (MAPK), AP-1, and NF-κB transcription factors [86]. Curcumin achieved 48%–99% suppression of MMP-3 and 45%–97% suppression of MMP-13 in human chondrocytes and 8%–100% (MMP-3) and 32%–100% (MMP-13) suppression in bovine chondrocytes. Inhibition of IL-1 signal transduction by these agents could be useful for reducing cartilage resorption by MMPs in arthritis.

4.2.7 Multiple Sclerosis

Multiple sclerosis is characterized by the destruction of oligodendrocytes and myelin sheath in the central nervous system (CNS). Curcumin inhibits experimental allergic encephalomyelitis (EAE), a model for multiple sclerosis, by blocking IL-12 signaling in T cells, suggesting that it would be effective in the treatment of multiple sclerosis. Natarajan and Bright investigated the effect of curcumin on the pathogenesis of CNS demyelination in EAE [24]. *In vivo* treatment of SJL/J mice with curcumin significantly reduced the duration and clinical severity of active immunization and adoptive transfer in EAE [24]. Curcumin inhibited EAE in association with a decrease in IL-12 production from macrophage/microglial cells and differentiation of neural antigen-specific Th1 cells. *In vitro* treatment of activated T cells with curcumin inhibited IL-12–induced tyrosine phosphorylation of Janus kinase 2, tyrosine kinase 2, and STAT3 and STAT4 transcription factors. Inhibition of the Janus kinase-STAT pathway by curcumin resulted in a decrease in IL-12–induced T-cell proliferation and Th1 differentiation. These findings show that curcumin inhibits EAE by blocking IL-12 signaling in T cells and suggest a rationale for its use in the treatment of multiple sclerosis and other Th1 cell–mediated inflammatory diseases.

Verbeek and coworkers examined the effects of curcumin on autoimmune T-cell reactivity in mice and on the course of EAE. Continuous oral administration of curcumin significantly affected antigen-specific proliferation and interferon-γ production by lymph node–derived T cells following immunization with an EAE-inducing peptide [87]. The overall effects of oral curcumin were mild but beneficial.

4.2.8 Alzheimer's Disease

Brain inflammation in Alzheimer's disease is characterized by increased cytokines and activated microglia. Curcumin can suppress oxidative damage, inflammation, cognitive deficits, and amyloid accumulation in Alzheimer's disease [88]. Lim et al. found that curcumin reduces oxidative damage and amyloid pathology in an Alzheimer's transgenic mouse model [25].

4.2.9 Inflammatory Bowel Disease

Inflammmatory bowel disease is characterized by oxidative and nitrosative stress, leukocyte infiltration, and upregulation of proinflammatory cytokines. Ukil et al. recently investigated the protective effects of curcumin on inflammatory bowel disease induced in a mouse model. Pretreatment of mice with curcumin for 10 days significantly ameliorated the appearance of diarrhea and the disruption of colonic architecture [89]. Curcumin is able to attenuate colitis in the dinitrobenzene sulfonic acid-induced murine model of colitis [90]. When given before the induction of colitis, it reduced macroscopic damage scores and NF-κB activation, reduced myeloperoxidase activity, and attenuated the dinitrobenzene

sulfonic acid-induced message for IL-1β. Thus, curcumin attenuates experimental colitis through a mechanism that also inhibits activation of NF-κB and effects a reduction in the activity of p38 MAPK. The authors proposed that this agent might have therapeutic implications for human inflammatory bowel disease.

4.2.10 Cystic Fibrosis

Cystic fibrosis, the most common lethal hereditary disease in the white population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. *CFTR* mutation disrupts the surface localization and/or gating of the CFTR chloride channel. The most common cystic fibrosis mutant is deltaF508-CFTR, which inefficiently traffics to the surfaces of most cells. In a recent report, Egan et al. demonstrated that curcumin corrected the cystic fibrosis defects in deltaF508 cystic fibrosis mice [91]. Most likely, curcumin exerts these effects by directly stimulating the CFTR chloride channels [92].

4.2.11 Others

Curcumin has been shown to have other activities that suggest potential clinical applications:

- Curcumin was found to be a potent and selective inhibitor of HIV-1 long terminal repeat–directed gene expression, which governs the transcription of HIV-1 provirus.
- Curcumin was shown to prevent cataractogenesis in an *in vitro* rat model.
- Treatment with curcumin prevented experimental alcoholic liver disease.
- Curcumin had a protective effect on cyclophosphamide-induced early lung injury.
- Chemotherapy-induced nephrotoxicity were prevented with curcumin (for references, see Ref. [81]).

4.3 MOLECULAR TARGETS OF CURCUMIN

Various studies have shown that curcumin modulates numerous targets (see Figure 4.2). These include the growth factors, growth factor receptors, transcription factors, cytokines, enzymes, and genes regulating apoptosis.

4.3.1 Cytokines and Growth Factors

Numerous growth factors have been implicated in the growth and promotion of tumors. Curcumin has been shown to downregulate expression of several cytokines, including TNF, IL-6, IL-8, IL-12, and fibroblast growth factor 2 (for references, see Ref. [81]).

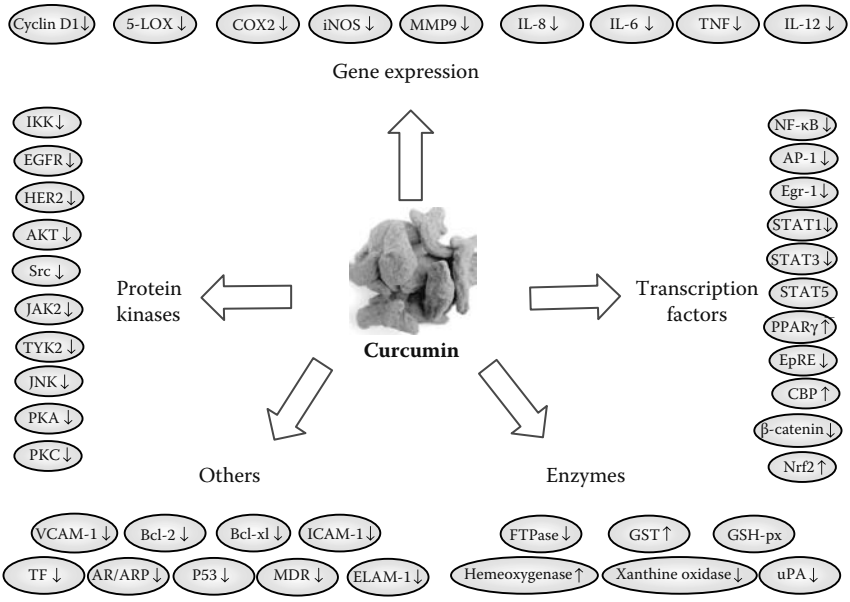


FIGURE 4.2 (See color insert following page 74.) Molecular targets of curcumin.

4.3.2 Receptors

HER2/neu and EGFR activity represent one possible mechanism by which curcumin suppresses the growth of breast cancer cells. Almost 30% of breast cancer cases overexpress the *HER2/neu* proto-oncogene [93], and both HER2 and EGF receptors stimulate proliferation of breast cancer cells. Overexpression of these two proteins correlates with progression of human breast cancer and poor patient prognosis (for references, see Ref. [93]). Curcumin has been shown to downregulate the activity of EGFR [57,94] and HER2/neu [57,94] and to deplete the cells of HER2/neu protein [95].

Prostate cancer cell lines LNCaP and PC-3 were treated with curcumin, and its effects on signal transduction and expression of androgen receptor (AR) and AR-related cofactors were analyzed. The results showed that curcumin downregulated transactivation and expression of AR and cAMP response element-binding protein (CBP)–binding protein. It also inhibited the transforming activities of both cell lines as evinced by reduced colony-forming ability in soft agar. These findings suggest that curcumin has a potential therapeutic effect on prostate cancer cells through downregulation of AR and AR-related cofactors [96].

4.3.3 Transcription Factors

Curcumin may operate through suppression of various transcription factors, including NF-κB, STAT3, early growth response protein 1, AP-1, peroxisome

proliferators–associated receptor gamma (PPAR-γ), and beta-catenin (for references, see Ref. [81]). These transcription factors play essential roles in various diseases. The constitutively active form of NF-κB has been reported in a wide variety of cancers. NF-κB is required for the expression of genes involved in cell proliferation, cell invasion, metastasis, angiogenesis, and resistance to chemotherapy. Bharti et al. demonstrated that curcumin inhibited IL-6–induced STAT3 phosphorylation and consequent STAT3 nuclear translocation [59]. Activation of PPAR-γ inhibits the proliferation of nonadipocytes. Xu et al. demonstrated that curcumin dramatically induced expression of the *PPAR-γ* gene and activated PPAR-γ [97]. AP-1, another transcription factor that has been closely linked with proliferation and transformation of tumor cells, has been shown to be suppressed by curcumin.

4.3.4 Proinflammatory Enzymes

COX-2 and lipoxygenase (LOX) are important enzymes that mediate inflammation through production of prostaglandins and leukotrienes, respectively. Curcumin has been shown to suppress the expression of both COX-2 and LOX proteins as well as their enzymatic activities, most likely through the downregulation of NF-κB, which is needed for COX-2 expression. Several groups have shown that curcumin downregulates the expression of COX-2 protein in various tumor cells [98,99]. Chun et al. reported that curcumin inhibited phorbol ester–induced expression of COX-2 in mouse skin through suppression of extracellular signal-regulated kinase activity and NF-κB activation [100]. Plummer et al. measured COX-2 protein induction and prostaglandin E_2 production in human blood after incubation with lipopolysaccharide. When 1 μM curcumin was added *in vitro* to blood from healthy volunteers, lipopolysaccharide-induced COX-2 protein levels and concomitant prostaglandin E_2 production were reduced by 24% and 41%, respectively [101].

4.3.5 Protein Kinases

Curcumin suppresses a number of protein kinases, including MAPK, c-Jun N-terminal kinase, protein kinase A (PKA), protein kinase C (PKC), src tyrosine kinase, phosphorylase kinase, IκBα kinase, JAK kinase, and the growth factor receptor protein tyrosine kinases. Treatment with curcumin inhibited highly purified PKA, PKC, protamine kinase, phosphorylase kinase, autophosphorylation-activated protein kinase, and pp60c-src tyrosine kinase [102]. Phorbol myristate acetate–induced activation of cellular PKC is suppressed by curcumin [103]. Treatment of cells with curcumin inhibited tetradecanoylphorbol acetate–induced PKC activity without affecting the level of PKC. Curcumin inhibited the PKC activity *in vitro*, as well as in the cells [104].

4.3.6 Cell Cycle

Curcumin modulates cell cycle–related gene expression. Specifically, curcumin-induced G_0/G_1 and/or G_2/M phase cell cycle arrest, upregulated cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and p53, and slightly downregulated cyclin B1 and cdc2. We found that curcumin can downregulate cyclin D1 expression at the transcriptional and posttranscriptional levels [102–104].

4.3.7 Adhesion Molecules

The expression of various cell surface adhesion molecules such as intercellular cell adhesion molecule 1, vascular cell adhesion molecule 1, and endothelial leukocyte adhesion molecule 1 on endothelial cells is absolutely critical for tumor metastasis [105]. Curcumin inhibits inflammation by blocking the adhesion of monocytes to endothelial cells by inhibiting activation of these cell adhesion molecules. The expression of these molecules is in part regulated by NF-κB [106]. We have shown that treatment of endothelial cells with curcumin blocks the cell surface expression of adhesion molecules, and this accompanies the suppression of tumor cell adhesion to endothelial cells [107]. We also have demonstrated that downregulation of these adhesion molecules is mediated through downregulation of NF-κB activation [107]. Gupta and Ghosh reported that curcumin inhibits TNF-induced expression of adhesion molecules on human umbilical vein endothelial cells [108]. Jaiswal et al. showed that curcumin treatment caused p53- and p21-independent G_2/M phase arrest and apoptosis in colon cancer cell lines [109]. Their results suggest that curcumin treatment impairs both Wnt signaling and cell–cell adhesion pathways, resulting in G_2/M phase arrest and apoptosis in HCT-116 cells.

4.3.8 Antiapoptotic Proteins

Curcumin is known to downregulate expression of apoptosis suppressor proteins such as Bcl-2 and Bcl-X_L in several cancer cell lines. We found that curcumin induces apoptosis through a mitochondrial pathway involving caspase-8, Bid cleavage, cytochrome *c* release, and caspase-3 activation. Our results also suggest that Bcl-2 and Bcl-X_L are critical negative regulators of curcumin-induced apoptosis [110]. Curcumin suppresses the constitutive expression of Bcl-2 and Bcl-X_L in mantle cell lymphoma [60] and multiple myeloma [111] cell lines. It also activates caspase-7 and caspase-9 and induces polyadenosine-5′-diphosphate-ribose polymerase cleavage in both cell lines. Thus, curcumin induces apoptosis by targeting several apoptotic pathways, inducing cytochrome *c* release, Bid cleavage, and caspase-9 and -3 activation, and by downregulating the antiapoptotic proteins Bcl-2 and BclX_L.

4.3.9 Multidrug Resistance

Multidrug resistance is a phenomenon that is often associated with enhanced drug efflux and thus decreased intracellular drug accumulation in tumor cells. It often

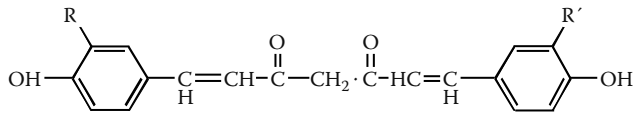
is related to overexpression of P-glycoprotein on the surface of tumor cells, which reduces drug cytotoxicity. Curcumin has been shown to suppress the overexpression of P-glycoprotein in the multidrug-resistant human cervical carcinoma cell lines [112]. Curcumin also downregulates drug resistance by inhibiting expression of the *mdr* gene, which is responsible for this phenomenon [113].

4.4 METABOLISM OF CURCUMIN

Turmeric contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%), and moisture (13.1%), along with the yellow pigment, curcumin. The essential oil (5.8%) obtained by steam distillation of turmeric rhizomes includes α-phellandrene (1%), sabinene (0.6%), cineol (1%), borneol (0.5%), zingiberene (25%), and sesquiterpenes (53%) [114]. The yellow pigment, forming 3%–4% by weight of turmeric, is a mixture of curcumin (I, 94%) and curcuminoids demethoxy curcumin (Ia 6%) and *bis*-demethoxy curcumin (Ib 0.3%) [115,116] (Figure 4.3).

Curcumin, whose full chemical name is 1,7-*bis*-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione/diferuloyl methane, has a flexible C−C chain and is stable in *trans* position, with two phenyl rings at both ends, and belongs to the family of diarylheptanoids, which form excellent lead compounds for a varied class of drugs.

Turmeric is considered a nutraceutical, that is, a functional food ingredient [117]. As a food additive, curcumin is considered a nontoxic herbal product as evidenced by its safe consumption in doses as high as 100 mg/day in humans and 5 g/day in rats [118]. Despite daily intake of curcumin (approximately 1–2 mg/day/kg body weight) by humans all over the world, no toxic effect has ever been reported. The bioavailability of curcumin *in vivo* is low after oral ingestion [48] and is reported to be enhanced dramatically by co-ingestion of piperine (an alkaloid present in black pepper) in both rats and humans [119]. Interactions of curcumin with other food additives are also under study [120]. Thus, synergic effects are possible when curcumin is combined with other dietary constituents as well as drugs. Some examples are curcumin's enhancement of the efficacy of the anticancer drug cisplatin against fibrosarcoma [121], its induction of cellular differentiation when mixed with all-*trans* retinoic acid or 1α,25-dihydroxy vitamin D_3 [122], and the chemopreventive synergism between curcumin and epigallocatechin-3-gallate in normal, realigned, and malignant human oral epithelial cells [123]. Curcumin associates with serum albumin through hydrophilic interactions [124]. We found that some



I R = R′ = –OCH_3; Ia R = –OCH_3, R′ = H; Ib R = R′ = H

FIGURE 4.3 Structure of curcumin.

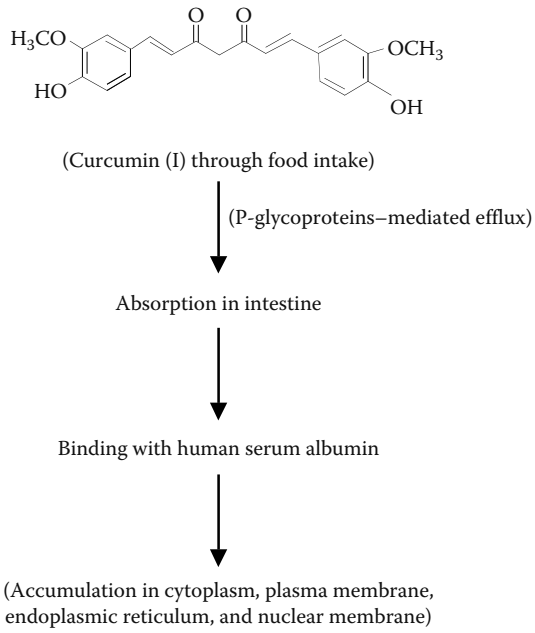


FIGURE 4.4 Absorption of curcumin after oral intake.

conjugates of curcumin, such as 4,4′-O-*bis*-diglycinoyl and 4,4′-O-*bis*-tetraglycinoyl derivatives, bind much more strongly with bovine serum albumin than curcumin itself (unpublished results). Therefore, it is evident that curcumin and its conjugates are transported to target cells through binding with serum albumin. These may penetrate into cytoplasm and also may accumulate in cellular membranes, such as plasma membranes, endoplasmic reticulum, and nuclear envelopes [125] (Figure 4.4).

In red blood cell membranes exposed to curcumin, changes in cell shape were accompanied by transient exposure to phosphatidyl serine. The enzyme aminophospholipid translocase, responsible for maintaining asymmetry and fluidity of these membranes, remained active in the presence of curcumin [126]. After intraperitoneal administration of curcumin (0.1 g/kg) in mice, approximately 2.25 μg/ml of curcumin appeared in the plasma within the first 15 min. After 1 h, the levels of curcumin in intestines, spleen, liver, kidneys, and brain were 177.04, 26.06, 26.90, 7.51, and 0.41 μg/g, respectively [127]. Curcumin gets biotransformed to dihydrocurcumin (III) and tetrahydrocurcumin (V), which subsequently get converted to monoglucuronide conjugates (Figure 4.2). Thus, curcumin-glucuronide (II), dihydrocurcumin-glucuronide (IV), tetrahydrocurcumin-glucuronide (VI), and tetrahydrocurcumin (V) are the major metabolites of curcumin *in vivo* [127,128] (Figure 4.5). Reduction of carbonyl groups to hydroxy functions also can occur to some extent. Ferulic and dihydroferulic acids have been identified as metabolites using ionization mass spectrometry [129]. According to the evidence obtained by

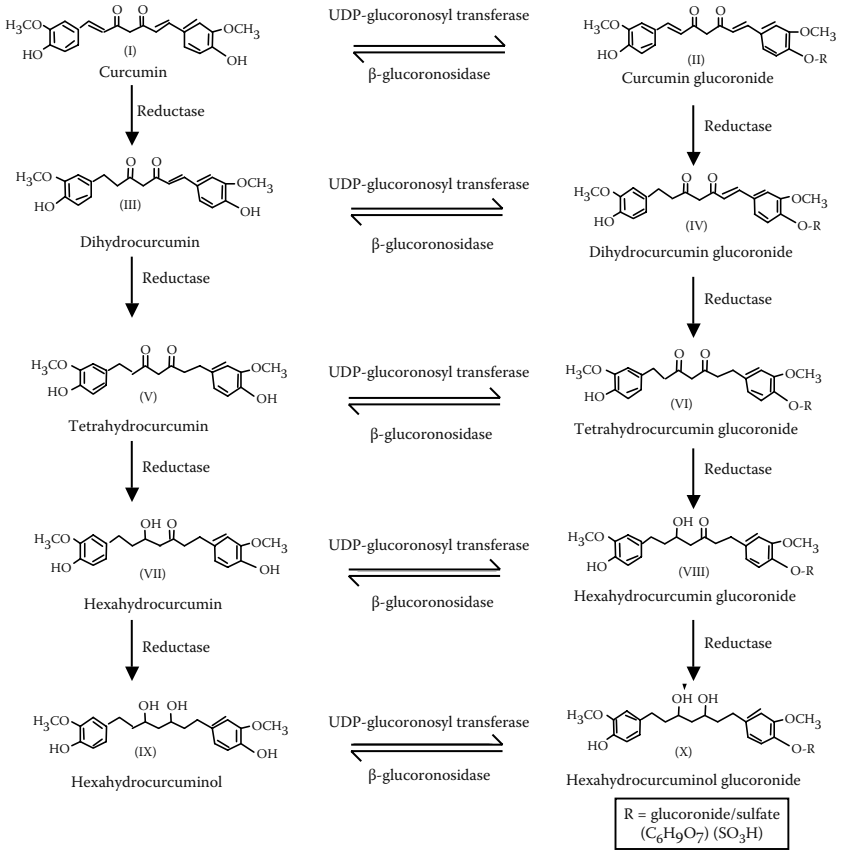


FIGURE 4.5 Absorption, biotransformation, and metabolism of curcumin.

Ireson et al. [130] in their study of the inhibitory effect of curcumin on prostaglandin E_2 formation, di-, tetra-, and hexahydrocurcumins are less bioactive than curcumin, while hexahydrocurcuminol (IX) is totally bioinactive (Figure 4.5).

From our results, it can be concluded that curcumin with both its phenolic functions esterified with piperic acid, amino acids, acetic acid, or other molecules that are recognized by their respective carrier proteins, gets transported through intestinal wall and also may bind more strongly to human serum albumin (unpublished results) and reach the target tissue (i.e., cytoplasm or cellular membranes) in a much higher concentration and thus be more effective than curcumin [129–137]. Moreover, the time taken by the natural esterases to hydrolyze the ester bonds and release curcumin (dihydro/tetrahydro curcumin) on the target site may delay glucoronide/sulfate formation and thus excretion of the drug. However, such conclusions need more experimental evidence for final verification.

4.5 LIMITATIONS TO THE USE OF CURCUMIN

4.5.1 Solubility

One of the major limitations of curcumin's clinical utility is its poor solubility in water. While curcumin is practically insoluble in water, it is soluble in ethanol or dimethylsulfoxide. The degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices have been established [131]. When curcumin was incubated in 0.1 M phosphate buffer and serum-free medium (pH 7.2 at 37°C), about 90% decomposed within 30 min. A series of pH conditions ranging from 3 to 10 were tested, and the results showed that decomposition was pH dependent and occurred faster at neutral-basic conditions. Curcumin is more stable in cell culture medium containing 10% fetal calf serum and in human blood. In these media, less than 20% of curcumin decomposed within 1 h, and after incubation for 8 h, about 50% of curcumin still remained. In a separate report, Souza and coworkers studied the influence of water activity on the stability of curcuminoid pigments in curcumin- and turmeric oleoresin-microcrystalline-cellulose model systems during storage at 21 ± 1°C [132]. The authors did not observe any influence of water activity on the stability of curcuminoid pigments in these systems.

4.5.2 Color

Curcumin under normal conditions has a brilliant yellow color and is used as a coloring agent for foods and textiles. Several studies have shown that curcumin may be beneficial in a variety of skin disorders such as psoriasis [83], wounds [32], and keloid and hypertrophic scars [85]. Curcumin, by lowering phosphorylase kinase levels in psoriatic epidermis, has been shown to resolve psoriasis, most likely through decreasing the population of Ki-67 cells within the epidermis. Unfortunately, curcumin stains the skin a bright yellow color upon application. This poses a limitation to the use of curcumin in skin formulations. There are, however, analogs of curcumin that are colorless yet have even greater activity than curcumin [51].

4.5.3 Dosing

Curcumin has been consumed as turmeric in human diets for several centuries without any known toxic effects. A diet containing 1.5 g of turmeric per day translates to a probable maximum curcumin intake of 150 mg per day [140]. The question that remains is how much curcumin is sufficient to induce therapeutic effect in human beings. Various studies have been performed to determine the optimal dosage and its biological safety in human beings. No significant adverse events were observed in patients with preinvasive malignant or high-risk premalignant conditions receiving up to 8 g of oral curcumin daily for 3 months [133]. Doses greater than 8 g per day were not tolerated by patients because of the large number of capsules that had to be consumed. Administration of 1.2 to 2.1 g of oral curcumin

daily to patients with rheumatoid arthritis for 2 to 6 weeks resulted in no adverse effects [23]. Similarly, 10 patients with inflammatory bowel disease received pure curcumin daily at doses between 0.55 and 1.65 g for up to 2 months without clinical manifestations of toxicity [134]. Despite the high doses that can be well tolerated in human beings, the bioavailability of curcumin is very low. Healthy volunteers who ingested 2 g pure curcumin powder after fasting showed less than 10 ng/ml curcumin in their plasma 1 h later [119]. Thus, it becomes important to establish a well-tolerated dose and make it bioavailable.

4.5.4 Bioavailability

Several studies have been performed to determine the bioavailability of curcumin. In one study, patients with preinvasive malignant or high-risk premalignant conditions of the bladder, skin, cervix, stomach, or oral mucosa received 0.5–8 g curcumin by mouth daily for 3 months [133], and their plasma curcumin concentrations were found to peak 1 to 2 h after intake and gradually decline within 12 h. The 8 g/day dose resulted in a peak serum concentration of 1.75 ± 0.80 μM. When curcumin in micronized form was administered orally with orange juice at doses of 50–200 mg to 18 healthy volunteers, the average level of curcumin in the plasma was approximately 0.63 ng/ml.

When curcumin in the form of "Curcuminoids C3" (Sabinsa Corp., 90% curcumin) was consumed orally for up to 4 months at daily doses of curcumin between 0.45 and 3.6 g by 15 patients with advanced colorectal cancer, the average level of drug and glucuronide/sulfate conjugates detected in plasma was only 5 pmol/ml [135]. When curcumin at 0.45, 1.8, or 3.6 g per diem was given to patients with colorectal cancer for 7 days prior to surgery, the mean concentrations of curcumin in normal and malignant colorectal tissue of patients who had ingested 3.6 g curcumin daily were 12.7 and 7.7 nmol/g tissue, respectively [136]. These preliminary results in humans suggest that a daily dose of 3.6 g curcumin achieves measurable levels in colorectal tissue with negligible distribution outside the gut.

The data from these studies suggest that curcumin has low systemic bioavailability and rapid elimination from the body following oral consumption. Efficient intestinal metabolism of curcumin, particularly glucuronidation and sulfation, may explain its poor systemic availability when administered via the oral route. Thus, the challenge now is to present curcumin in dosage forms that will allow greater bioavailability. Some of the promising alternatives for overcoming the limitations of curcumin are described in the next section.

4.6 POTENTIAL RESOLUTION OF THE PROBLEMS

4.6.1 Piperine Enhances Bioavailability

Piperine is an alkaloid commonly found in black pepper (*Piper nigrum*). The use of black pepper in combination with long pepper (*Piper longa*) and ginger *(Zingiber officinale*), commonly called Trikatu, has been described in Ayurveda to

enhance drug delivery. Piperine has been shown to inhibit the metabolism of several drugs, including propanolol, theophylline, phenytoin, sulfadiazine, rifampicin, isoniazid, ethambutol, pyrazinamide, dapsone, coenzyme Q_{10}, and beta-carotene [137,138], and to increase their bioavailability. Shoba et al. found that co-ingestion of curcumin with 20 mg of piperine appeared to increase the bioavailability of curcumin by 2000% [119]. Studies on human liver microsomes have shown that dietary piperine could affect plasma concentrations of P-glycoprotein and CYP3A4 substrates in humans, particularly if these drugs are administered orally [139]. In animal studies, piperine also inhibited other enzymes important in drug metabolism [138,140]. Thus, administration with piperine is one potential means of enhancing the bioavailability of curcumin.

4.6.2 Liposomal Curcumin

Liposomes are biodegradable and nontoxic and can be formulated and processed to differ in size, composition, charge, and lamellarity. Hence, a wide range of compounds may be incorporated into either the lipid or trapped aqueous space. As a result, liposomes are used frequently as drug-delivery vehicles. Further, liposomes can serve as a depot system for the sustained release of an associated compound. Liposomes can alter the biodistribution of entrapped substances and protect the enclosed materials from inactivation by host enzymes. Therefore, liposomes can be used as vehicles to achieve specific delivery of therapeutic drugs to target organs.

Kuttan et al. examined the anticancer potential of curcumin in mice bearing Dalton's lymphoma cells grown as ascites [74]. They encapsulated curcumin (5 mg/ml) into neutral and unilamellar liposomes prepared by sonication of phosphatidylcholine and cholesterol. An aliquot of liposomal curcumin (50 mg/kg) was administered intraperitoneally to mice the day after injection of the Dalton's lymphoma cells and repeated for 10 days. Surviving animals were counted after 30 and 60 days. Treatment with the liposomal formulation at a concentration of 1 mg/animal allowed all animals to survive at least 30 days and only two of the animals developed tumors and died before 60 days.

The *in vitro* and *in vivo* effects of liposome-encapsulated curcumin on proliferation, apoptosis, signaling, and angiogenesis in human pancreatic carcinoma cells have been investigated [141]. Intravenous injection of liposome-encapsulated curcumin consistently suppressed NF-κB binding and decreased expression of NF-κB–regulated gene products COX-2 and IL-8. The activity of liposomal curcumin was equal to or better than that of free curcumin at equimolar concentrations. These experiments provide a biological rationale for treatment of pancreatic carcinoma with curcumin-encapsulated liposomes for systemic delivery, although none of these data suggest that this formulation increases the bioavailability of curcumin.

Marczylo et al. explored whether formulation of curcumin with phosphatidylcholine (Meriva) increases the oral bioavailability or affects the metabolic profile of curcumin [142]. Curcumin and the accompanying curcuminoids were

identified in plasma, intestinal mucosa, and liver of the rats that had received Meriva. Peak plasma levels of parent curcumin were five-fold higher in the Meriva-treated rats than in those treated with the equivalent values of unformulated curcumin.

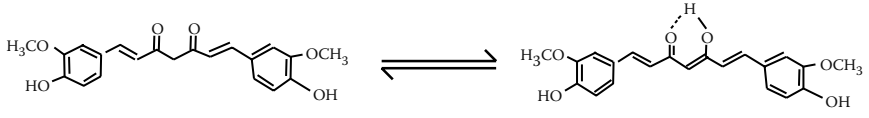
4.6.3 Nanotechnology

Nanotechnology refers to the interactions of cellular and molecular components and nanoparticles ranging from 1 to 100 nm. Nanoparticles have novel optical, electronic, and structural properties that are not shared by individual molecules. Nanotechnology has led to the development of biodegradable self-assembled nanoparticles, which are being engineered for the targeted delivery of anticancer drugs and imaging contrast agents.

The use of nanoparticles for targeted oral drug delivery to inflamed gut tissue in inflammatory bowel disease was investigated by Lamprecht et al. [143]. Rolipram, an anti-inflammatory model drug, was incorporated within poly(lactic-coglycolic acid) nanoparticles, which were administered once a day orally for 5 consecutive days to male Wistar rats with trinitrobenzenesulfonic acid–induced experimental colitis. The new delivery system enabled the drug to accumulate in the inflamed tissue with higher efficiency than a solution of the drug. Similarly, Bala et al. [144] developed a sustained-release nanoparticulate formulation containing antioxidant-ellagic acid as a potential prophylaxis system for oral administration. Ellagic acid–loaded nanoparticles showed better free radical scavenging effect in a yeast cell culture model and in a cell-free system. Thus, curcumin-conjugated nanoparticles could serve as customizable, targeted drug-delivery vehicles to improve the bioavailability of curcumin.

4.6.4 Structural Analogs

To overcome the low potency and poor absorption characteristics of curcumin, several analogs of curcumin have been designed and tested. The novel curcumin analogs synthesized at Emory University are symmetrical alpha- or beta-unsaturated and -saturated ketones [145]. The majority of the analogs demonstrated a moderate degree of anticancer activity. The novel synthetic curcumin analog EF24 induced cell cycle arrest and apoptosis by means of a redox-dependent mechanism in MDA-MB-231 human breast cancer cells and DU-145 human prostate cancer cells [51]. EF24 induced G_2/M arrest in both cell lines, which was followed by induction of apoptosis. EF24 induced depolarization of the mitochondrial membrane potential, suggesting that the compound also may induce apoptosis by altering mitochondrial function. EF24, like curcumin, serves as a Michael acceptor reacting with glutathione and thioredoxin 1. Reaction of EF24 with these agents *in vivo* significantly reduced intracellular glutathione as well as oxidized glutathione in both wild-type and Bcl-x_L–overexpressing HT29 human colon cancer cells. Although some of the analogs were more effective than curcumin, whether they had greater bioavailability is not known.



Keto-form Enol-form

FIGURE 4.6 Keto–enol tautomerism in curcumin.

4.7 STRUCTURE–ACTIVITY RELATIONSHIP OF CURCUMIN ANALOGS

To relate chemical structure with biological activity, the curcumin analogs or bioconjugates so far reported can be classified as follows.

4.7.1 Curcumin Analogs with Retention of Basic Skeleton and Change in Functional Groups

1,7-Diarylheptane is the basic skeleton of curcuminoids. The following functional groups are present on this skeleton:

- Two phenolic groups (at 4,4′-positions on aromatic rings)
- Two methoxy groups (at 3,3′-positions on aromatic rings)
- Two double bonds in the 7-C chain (linker)
- β–Diketone
- Active methylene group at C-4

The double bonds and ketonic functions are conjugated, accounting for the yellow color of curcumin. The structure explains the keto–enol tautomerism present in curcumin, although the amount of the enol form is much less than the stable keto form (Figure 4.6).

For changes in functional groups, there can be four broad classifications with subclasses as follows:

- Substitution at phenolic groups
  - Esters
  - Ethers
  - Salts
  - Glycosides
- Substitution in linker and phenolic functions
- Substitution in linker and various substituents on phenyl rings
- Analogs with various substituents on one phenyl ring

4.7.1.1 Substitution at Phenolic Groups (Table 4.1)

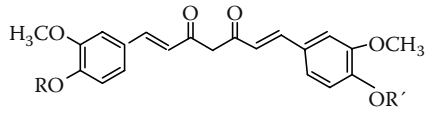
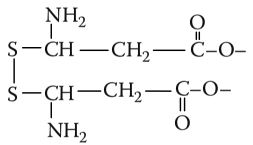
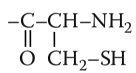
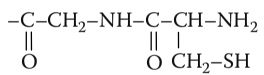
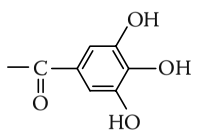
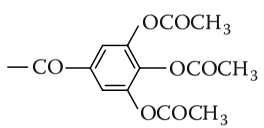


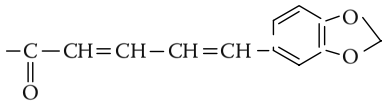
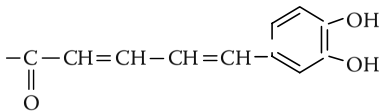
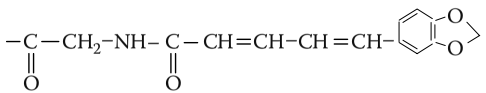
TABLE 4.1
Curcumin Analogs with Retention of Basic Skeleton and Change of Functional Groups-Substitution at Phenolic Groups

No.	R = R′	R	R′	Name of Compound	Reference	Activity[a]
Esters						
1	$-\overset{\overset{O}{\|\|}}{C}-CH_2-NH_2$			Di-O-glycinoyl curcumin	[146–154]	Ab, Af, Ao, Ap, Aap, Ac
2		$-\overset{\overset{O}{\|\|}}{C}-CH_2-NH_2$	H	Mono-O-glycinoyl curcumin	Unpublished	Ab, Af
3	$-\overset{\overset{O}{\|\|}}{C}-CH_2-NH-CO-CH_2-NH_2$			Tetra-O-glycylglycinoyl curcumin	[155,156]	Ab, Af, Ao, Ap, Ac
4	$-\overset{\overset{O}{\|\|}}{C}-\underset{\underset{CH_3}{\|}}{CH}-NH_2$ (D and L)			Di-O-(D & L)-alaninoyl curcumin	[146,149]	Ab
5	$-\overset{\overset{O}{\|\|}}{C}-\underset{\underset{CH(CH_3)_2}{\|}}{CH}-NH_2$			Di-O-valinoyl curcumin	Unpublished	Ab, Af
6		$-\overset{\overset{O}{\|\|}}{C}-\underset{\underset{CH(CH_3)_2}{\|}}{CH}-NH_2$	H	Mono-O-valinoyl curcumin	Unpublished	Ab, Af
7	$-CO-\underset{\underset{(CH_2)_2}{\|}}{\underset{\underset{COOH}{\|}}{CH}}-NH_2$			Di-O-glutamoyl curcumin	Unpublished	Ab, Af

(*continued*)

TABLE 4.1 (continued)
Curcumin Analogs with Retention of Basic Skeleton and Change of Functional Groups-Substitution at Phenolic Groups

No.	R = R′	R	R′	Name of Compound	Reference	Activity[a]
8				Cystinyl curcumin (cyclic structure)	[150,154,156]	Ab, Af
9				Di-O-cysteinyl curcumin	[150,154,156]	Ab, Af
10				Di-O-cysteinyl-glycinoyl curcumin	[150,156]	Ab, Af
11				Curcumin diacetate	[150,154,156]	Ab, Af, Ap, Ao, Ai
12				Di-O-galloyl curcumin	[154,156]	Ab
13				Di-O-(3,4,5-O-acetyl)galloyl curcumin	[154,156]	Ab

14		Di-O-piperoyl curcumin	[150,154,156]	Ab, Af
15		Di-O-demethylenated piperoyl curcumin	Unpublished	Ab, Af, Ac
16		Di-O-glycyl-piperoyl curcumin	[150,154,156]	Ab, Af
Ethers				
17	-CH3	Dimethyl curcumin	[157–159]	Ac (colon/prostate) Antiprotozoan
18	-C_2H5	Diethyl curcumin	[160]	Ai
Salts				
19	Na	Disodium curcumin	[159–161]	Ab, Ai
Glycosides				
20	D-glucose	Curcumin-di-glucoside	[149]	Ab

[a] Ab = antibacterial, Af = antifungal, Ao = antioxidant, Ac = anticancer, Ap = apoptotic, Ai = anti-inflammatory, Aap = antiapoptotic

4.7.1.2 Substitution in Linker and Phenolic Functions (Table 4.2)

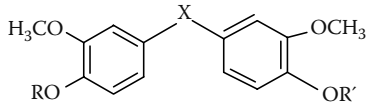


TABLE 4.2
Curcumin Analogs with Retention of Basic Skeleton and Change of Functional Groups-Substitution in Linker and Phenolic Functions

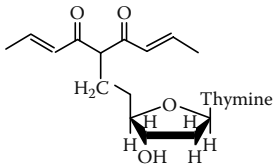
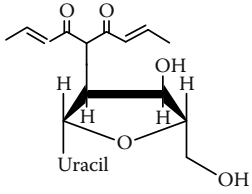
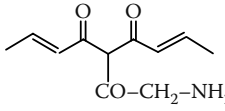
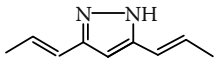
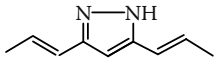
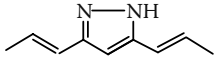
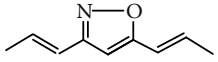
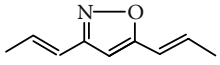
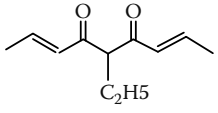
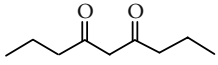
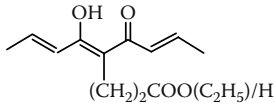
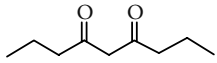
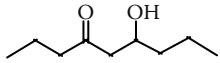
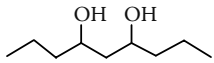
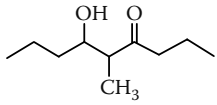
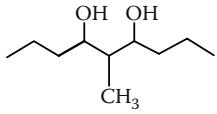
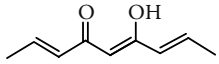
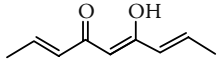
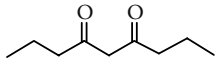
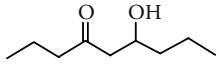
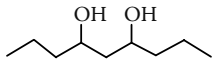
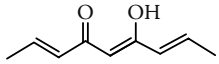
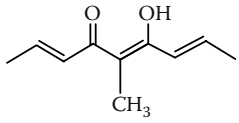
No.	X	R	R′	Reference	Activity[a]
21		H	H	[146,162]	Ab
22		H	H	[146,162]	Ab
23		−CO−CH_2−NH_2	−CO−CH_2−NH_2	[146,147, 162]	Ab
24		H	H	[158]	Ai, Ao
25		−CH_3	−CH_3	[158]	Ai, Ao
26		H	−CH_3	[158]	Ai, Ao
27		−H	−H	[163]	Ai, Ao
28		−CH_3	−CH_3	[163]	Ai
29		−C_2H_5	−C_2H_5	[160]	Ai
30		H	H	[164]	Ao, Ai, Ac

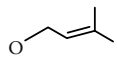
TABLE 4.2 (continued)
Curcumin Analogs with Retention of Basic Skeleton and Change of Functional Groups-Substitution in Linker and Phenolic Functions

No.	X	R	R′	Reference	Activity[a]
31		H	H	[158]	Ac (prostate)
32		CH_3	CH_3	[164]	Ai, Ao (weak), Ac (colon)
33		CH_3	CH_3	[164]	Ai, Ao, Ac (colon)
34		CH_3	CH_3	[164]	Inactive
35		CH_3	CH_3	[158]	Ac (prostate) activity reduced
36		CH_3	CH_3	[158]	Ac (prostate) activity reduced
37		CH_2COOCH_3	CH_2COOCH_3	[158]	Ac (prostate)
38		CH_2COOCH_3	H	[158]	Ac (prostate)
39		H	H	[160]	Ai
40		H	H	[158]	Ac (prostate)
41		H	H	[164]	Ao
42		OTHP	OTHP	[165]	Ac (prostate)
43		CH_3	CH_3	[158]	Ac (prostate) activity reduced

[a] Ab = antibacterial, Af = antifungal, Ao = antioxidant, Ac = anticancer, Ap = apoptotic, Aag = antiangiogenic, Ai = anti-inflammatory, Aap = antiapoptotic

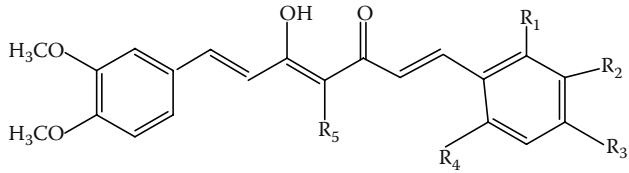
4.7.1.3 Substitution in Linker and Various Substituents on Phenyl Ring (Table 4.3)

TABLE 4.3
Curcumin Analogs with Retention of Basic Skeleton and Change of Functional Groups-in Linker and Various Substituents on Phenyl Rings

No.	R_1	R_2	R_3	R_4	R_5	R_6	Reference	Activity[a]
44	H	OH	OCH_3	H	H	H	[165]	Ac (activity reduced)
45	OCH_3	H	OCH_3	H	H	H	[165]	Ac (prostate)
46	OCH_3	OCH_3	OCH_3	H	H	H	[165]	Ac (prostate)
47	H	OCH_3	OCH_3	H	OCH_3	H	[165]	Ac (prostate)
48	H	OCH_3	OTHP	H	H	$(CH_2)_2COOC_2H_5$	[165]	Ac (prostate)
49	H	OCH_3	OTHP	H	H	H	[165]	Not determined
50	H	OCH_3	OC_2H_5	H	H	H	[165]	Ac (prostate)
51	H	OCH_3		H	H	H	[165]	Ac (prostate)
52	H	OCH_3	OH	OCH_3	H	H	[165]	Ac (prostate)
53	H	CH_3	OH	H	H	H	[165]	Ac (prostate)
54	H	CH_3	OCH_3	H	H	H	[165]	Ac (prostate) activity reduced
55	H	OCH_3	CH_2COOCH_3	H	H	H	[165]	Ac (prostate) activity reduced

[a] Ac = anticancer

4.7.1.4 Analogs with Various Substituents on One Phenyl Ring (Table 4.4)



4.7.2 Curcumin Analogs with Change in Basic Skeleton and Functional Groups

This group can be classified as follows:

- Aryl rings removed or substituted with other rings
  - Analogs with one phenyl ring
  - Analogs with heterocyclic rings

TABLE 4.4
Curcumin Analogs with Retention of Basic Skeleton and Change of Functional Groups-Analogs with Various Substituents on One Phenyl Ring

No.	R_1	R_2	R_3	R_4	R_5	Reference	Activity[a]
56	H	OCH_3	OH	H	H	[165]	Ac (prostate)
57	H	OH	OCH_3	H	H	[165]	Ac (prostate)
58	OCH_3	OCH_3	OCH_3	H	H	[165]	Ac (prostate)
59	H	OCH_3	OCH_3	OCH_3	H	[165]	Ac (prostate)
60	H	OCH_3	OH	H	$(CH_2)_2COOC_2H_5$	[165]	Ac (prostate)

[a] Ac = anticancer

- Variation in linker length or other modifications along with changes in aromatic substitution
- Analogs with different substitution in aryl rings

4.7.2.1 Analogs with One Phenyl Ring (Table 4.5)

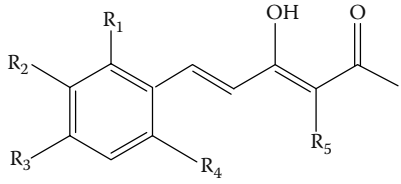


TABLE 4.5
Curcumin Analogs with Change in Basic Skeleton and Functional Groups-Analogs with One Phenyl Ring

No.	R_1	R_2	R_3	R_4	R_5	Reference	Activity[a]
61	H	OCH_3	OH	H	H	[165]	Ac cytotoxicity (prostate) reduced
62	H	OCH_3	OCH_3	H	H	[165]	Ac cytotoxicity (prostate) reduced
63	H	OH	OCH_3	H	H	[165]	Ac cytotoxicity (prostate) reduced
64	OCH_3	H	OCH_3	H	H	[165]	Ac cytotoxicity (prostate) reduced
65	OCH_3	OCH_3	OCH_3	H	H	[165]	Ac cytotoxicity (prostate) reduced
66	H	OCH_3	OCH_3	OCH_3	H	[165]	Ac cytotoxicity (prostate) reduced
67	H	OCH_3	OH	H	$(CH_2)_2COOC_2H_5$	[165]	Ac cytotoxicity (prostate) reduced

[a] Ac = anticancer

4.7.2.2 Analogs with Heterocyclic Rings (Table 4.6)

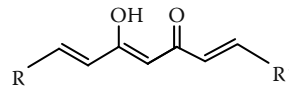
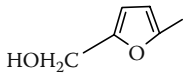
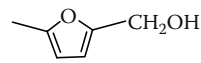
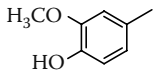
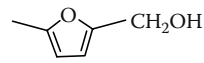
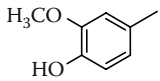
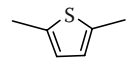
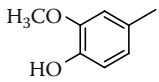
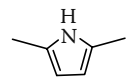
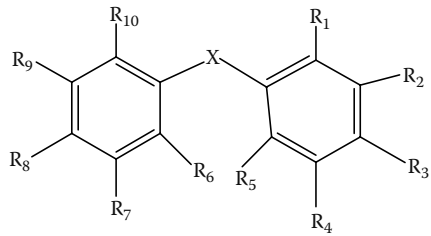


TABLE 4.6
Curcumin Analogs with Change in Basic Skeleton and Functional Groups-Analogs with Heterocyclic Rings

No.	R	R′	Reference	Activity[a]
68			[165]	Ac cytotoxicity (prostate) reduced
69			[165]	Ac cytotoxicity (prostate) reduced
70			[165]	Ac cytotoxicity (prostate) reduced
71			[165]	Ac cytotoxicity (prostate) reduced

[a] Ac = anticancer

4.7.2.3 Variation in Linker Length or Other Modifications along with Changes in Aromatic Substitution (Table 4.7)



4.7.2.4 Analogs with Different Substitutions in Aryl Rings (Table 4.8)

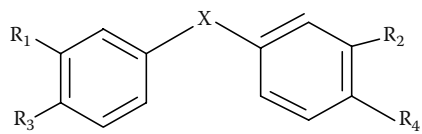
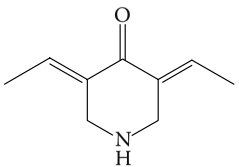
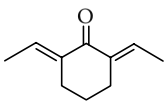
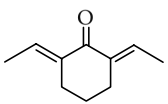
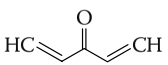
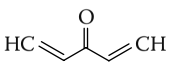
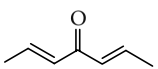
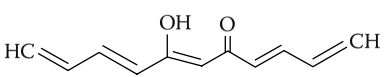
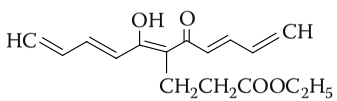
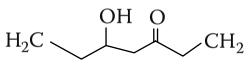
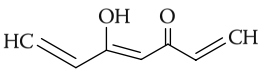
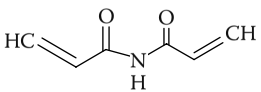
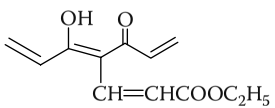
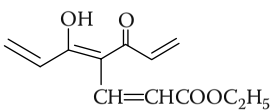


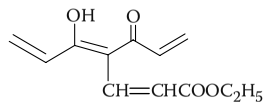
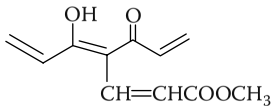
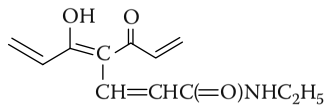
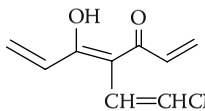
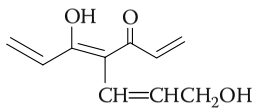
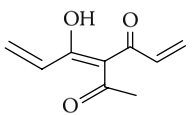
TABLE 4.7
Curcumin Analogs with Change in Basic Skeleton and Functional Groups-Variation in Linker Length or Other Modifications along with Changes in Aromatic Substitution

No.	X	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	Reference	Activity[a]
72		F	H	H	H	H	H	H	H	H	F	[166]	Ac (breast/ melanoma)
73		H	OCH_3	OH	H	H	H	H	OH	OCH_3	H	[167]	Aag, Ao, Ap
74		H	F	OH	H	H	H	H	OH	F	H	[167]	Ao, Ap
75		H	OCH_3	OH	H	H	H	H	OH	OCH_3	H	[165]	Ac (prostate)
76		H	OCH_3	OH	H	H	H	H	OH	OCH_3	H	[165]	Ac (prostate)
77		OCH_3	H	OCH_3	H	H	H	H	OCH_3	H	OCH_3	[165]	Inactive for prostate
78		H	OCH_3	OH	H	H	H	H	OH	OCH_3	H	[165]	Inactive for prostate

(*continued*)

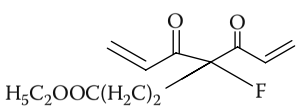
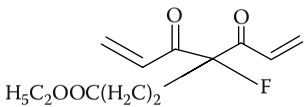
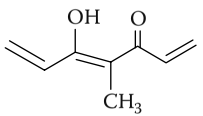
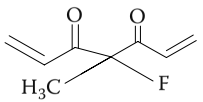
TABLE 4.7 (continued)
Curcumin Analogs with Change in Basic Skeleton and Functional Groups-Variation in Linker Length or Other Modifications along with Changes in Aromatic Substitution

No.	X	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	Reference	Activity[a]
79		H	OCH_3	OH	H	H	H	H	OH	OCH_3	H	[165]	Ac (prostate) activity reduced
80		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Inactive for prostate
81		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Inactive for prostate
82		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Inactive for PC-3 cells, active for LNCaP (prostate) Ac
83		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Ac (prostate)
84		H	OCH_3	OTHP	H	H	H	H	OTHP	OCH_3	H	[165]	Ac (prostate)

85		H	OCH_3	OH	H	H	H	H	OH	OCH_3		[165]	Ac (prostate)
86		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3		[165]	Ac (prostate)
87		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	OCH_3	[165]	Ac (prostate)
88		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Ac (prostate)
89		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Ac (prostate)
90		H	OCH_3	OH	H	H	H	H	OH	OCH_3	H	[165]	Ac (prostate)

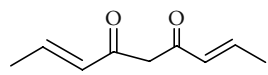
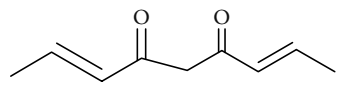
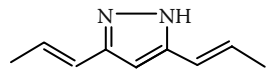
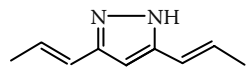
(*continued*)

TABLE 4.7 (continued)
Curcumin Analogs with Change in Basic Skeleton and Functional Groups-Variation in Linker Length or Other Modifications along with Changes in Aromatic Substitution

No.	X	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	Reference	Activity[a]
91		H	OCH_3	OTHP	H	H	H	H	OTHP	OCH_3	H	[165]	Inactive
92		H	OCH_3	OH	H	H	H	H	OH	OCH_3	H	[165]	Inactive
93		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Ac (prostate)
94		H	OCH_3	OCH_3	H	H	H	H	OCH_3	OCH_3	H	[165]	Inactive

[a] Ao = antioxidant, Ac = anticancer, Ap = apoptotic, Aag = antiangiogenic

TABLE 4.8
Curcumin Analogs with Change in Basic Skeleton and Functional Groups-Analogs with Different Substitution in Aryl Ring

No.	R_1	R_2	R_3	R_4	X	Reference	Activity[a]
95	OCH_3	OH	OH	OH		[159,168]	Ao
96	OH	OH	OH	OH		[159,168]	Ao
97	OCH_3	OCH_3	OH	OH		[163]	Ao
98	OH	OH	OH	OH		[163]	Ao

[a] Ao = antioxidant

4.7.3 Curcumin Analogs Conjugated with Bioactive Species (Proteins and Oligonucleotides)

These analogs are conjugated or linked with either protein or oligonucleotide (DNA/RNA) sequences, which can target the desired cells or tissues.

4.7.3.1 Analogs Conjugated with Oligonucleotides (DNA/RNA)

- The 4,4′-O-diglycinoyl curcumin attached through a linker to a telomerase repeat sequence of DNA and tested on DU-145 prostate cancer cell lines [151]
- The 4,4′-O-tetraglycinoyl curcumin attached through a two-carbon linker to a telomerase repeat sequence of DNA, tested on KB and HeLa cell lines [156]
- The 4,4′-O-demethylenated piperoyl curcumin attached to a telomerase RNA template through phosphate group and tested on KB and HeLa cell lines (unpublished results)

The complexes in these three cases were effective in picomolar amounts, while the corresponding curcumin analogs under identical conditions were effective only in micromolar amounts.

4.7.3.2 Analogs Conjugated with Proteins [166]

Cytotoxic factor EF24 is a biomimic of curcumin that, when linked with phenylalanine-phenylalanine-arginine through a methyl ketone linker and conjugated with the complex of the soluble protein factor VIIa and tissue factor (f VIIa/TF protein–protein complex), a step occurring during the blood coagulation cascade,

yields the drug EF24-FFR-mk-f VIIa, which can arrest breast cancer MDA-MB-231 cells and human melanoma RPMI-7951 cells to a much greater extent than EF24 alone (nonspecific binding).

Structure–activity relationship studies of the curcumin molecule with respect to its multiple biological activities have indicated that the presence of two phenyl rings with a 7-C linker having β-diketo function (C=O groups as hydrogen acceptors and C-4 [active methylene] as hydrogen donor) is the basic necessity. Unsaturation in the linker is important for curcumin's antitumor/anticancer activity, but not for its redox regulatory or apoptotic activities. Tetrahydrocurcumin has even greater antioxidant properties than curcumin.

For antioxidant and apoptotic activity, substitution in the ortho position is necessary to the freeing of phenolics by methoxy groups, fluorine, or other activating groups. Thus, free phenolics (or those bonded with biodegradable bonds) activated by groups present in the ortho position, along with the β-diketo function, appear to be responsible for induction of apoptosis taking place through mitochondrial uncoupling. In the case of dimethyl curcumin, the mechanism of induction of apoptosis may be generation of reactive oxygen species induced by some other free radical, possibly a tertiary radical.

The most important factor in the utility of any compound as a drug is its propensity for building up a therapeutic concentration in the target cells or tissues. Since curcumin metabolizes very fast, esterification of one or both of its free phenolics with amino acids, piperic acid, acetic acid, or any other acid can result in its facile uptake by the carrier proteins, which recognize the acids and thereafter delay its metabolism in the cell by ester hydrolysis. We have found that monoglycinoyl, diglycinoyl, tetraglycinoyl, dipiperoyl, diglutamoyl, and di and mono valinoyl esters are much better antibacterials than curcumin, and monoglycinoyl and tetraglycinoyl derivatives are highly active against Candida species (i.e., antifungal) [149].

In our work on the effects of curcumin, its diacetate, and the dipiperoyl, 4,4′-di-O-glycinoyl-piperoyl, diglycinoyl, and 4,4′-O monocystinoyl esters on AK-5 rat histocytoma cells, we found that the dipiperoyl and diglycinoyl esters were most potent in inducing apoptosis, and at much lower concentrations than curcumin itself [134]. The diacetate had moderate activity, while the cystinoyl esters had almost lost apoptotic activity, although the apoptotic activity with these analogs was closely associated with generation of reactive oxygen species. Targeting the analogs by conjugating them with proteins or oligonucleotides that can target cancer cells has shown promising results [131,135,136,155–158]. These designed molecules were effective in picomolar concentrations, whereas the corresponding unconjugated analogs were effective in micromolar concentrations.

For antiandrogenic activity, the presence of two phenyl rings having 3′,4′ substitution and a conjugated β-diketone moiety is reported to be essential [158,165]. Substitution of aryl rings with heterocyclic rings or removal of one phenyl ring eliminated the activity. However, since curcumin is not active in the cell lines used in these studies, while dimethyl curcumin is highly active, it can be concluded that the size of the group at 4,4′ positions in the phenyl rings is more important than

the hydrogen donor substituent, the phenolic-hydroxide. The presence of hydrogen donor groups in the linker is favorable. Regarding the cytotoxic activity of curcumin analogs, the following can be concluded in general:

- The 4,4′ positions in the two aryl rings should be substituted with two methoxy groups, two hydroxy groups, or one methoxy and one hydroxy. Bulkier groups are not favorable, and symmetry is not important.
- The 2/2′ positions should be unsubstituted.
- The 3/3′ positions should be substituted. Either methoxy or methyl groups are favorable, but hydroxyl groups are not favorable in these positions.
- The elongation of the linker between the two phenyl rings does not enhance activity.
- The unsaturation and conjugation in the linker are necessary for cytotoxicity.
- Substitution of an active methylene group with –NH (imide) or any heteroatom is unfavorable.
- Substitution at an active methylene group with an unsaturated group is favorable and with saturated and bulky groups unfavorable.

Since the targets involved vary in different cancers, studies of proper docking of curcumin analogs at active sites are essential if effective drugs are to be developed in the future.

4.8 CONCLUSION

The medicinal properties of curcumin have been known for centuries, although the scientific basis of its actions has been investigated only over the last couple of decades. Most of the folklore claims associated with curcumin have been evaluated scientifically and found to be true. Curcumin has been shown to target numerous molecules inside the cell that are known to modulate several signaling pathways. Curcumin is a potent antioxidant, anti-inflammatory, and anticancer agent and has therapeutic efficacy in numerous diseases. Despite its proven safety over the ages, its efficacy has been limited by its poor solubility, vivid color, and poor bioavailability. Several analogs of curcumin have been designed to overcome these limitations. Some of these analogs have yielded promising results in *in vitro* and animal studies, but whether these more bioavailable forms are equally safe is still unknown. Therefore, it is important to evaluate the curcumin analogs for bioavailability and clinical efficacy.

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5 Flavanols and NF-κB Activation: Relevance for Inflammation and Associated Diseases

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CONTENTS

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5.1 INTRODUCTION

Increasing epidemiological evidence demonstrates that diets rich in fruits and vegetables promote health, and attenuate, or delay, the onset of various diseases, including cardiovascular disease (CVD), diabetes, certain cancers, and several other age-related degenerative disorders [1–3]. The plant components and the physiological and molecular mechanisms by which fruits and vegetables reduce the risk of these diseases are matters of intense investigation. One group of phytochemicals, the flavonoids, is gaining acceptance, playing a major role in the health benefits offered by fruits and vegetables, as it was proposed by Reanud and de Lergeril [4].

Originally described as a transcription factor present in B cells, nuclear factor-kappaB (NF-κB) is now known to be ubiquitously present in cells [5]. Multiple stimuli can trigger NF-κB activation, including proinflammatory cytokines, bacteria, viruses and their products, oxidants, drugs, environmental pollutants, and other stressors [6]. The nature of the stimuli and the genes regulated by NF-κB revealed that this transcription factor is involved in the cell response to stress and consequently can be a key player in several diseases. The identification of compounds that can modulate NF-κB activation is a current and relevant pharmaceutical [7] and nutritional goal [8]. In this review, we present information on the potential effects of a particular type of flavonoids, flavanols and related procyanidins, on the modulation of NF-κB, and the implications for relevant human diseases.

5.2 FLAVONOIDS, FLAVANOLS, AND PROCYANIDINS

5.2.1 Chemical Structures of Flavonoids, Flavanols, and Procyanidins

Flavonoids are polyphenols that are widely distributed in plants and include several thousand compounds. The flavonoids share a common basic chemical structure (Figure 5.1), and depending on the substitution pattern in the B- and C-rings, can be classified into several subgroups: flavanols, flavanones, flavones, isoflavones,

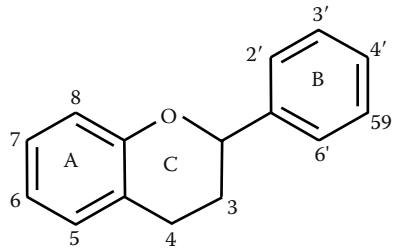

FIGURE 5.1 Generic structure of flavonoids.

flavonols, and anthocyanidins, the most studied with regard to human diet and health [9].

Flavanols are distinct from other flavonoid classes because in plants they do not appear as glycosides but instead are present in the aglycone form as: (1) monomeric units, such as (−)-epicatechin (epicatechin, EC) and (+)-catechin (catechin, CT; Figure 5.2); (2) gallate derivatives of the monomeric flavanols, mainly of EC, including (−)-epigallocatechin (EG), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG; Figure 5.2); or (3) oligomers of the monomeric flavanols. EC and CT oligomers are denominated procyanidins (also known as condensed tannins or proanthocyanidins), of which the chemical structure is defined not only by the structure of the monomer but also by the way that monomers are

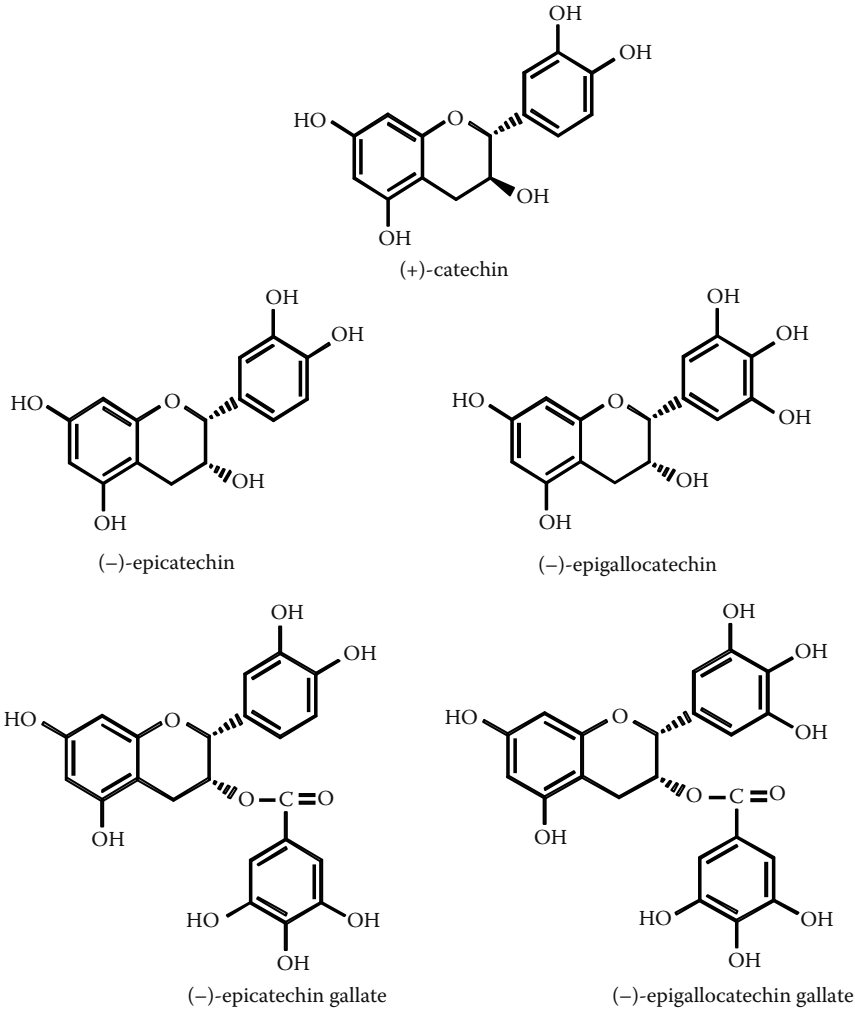

FIGURE 5.2 Chemical structures of monomeric flavanols.

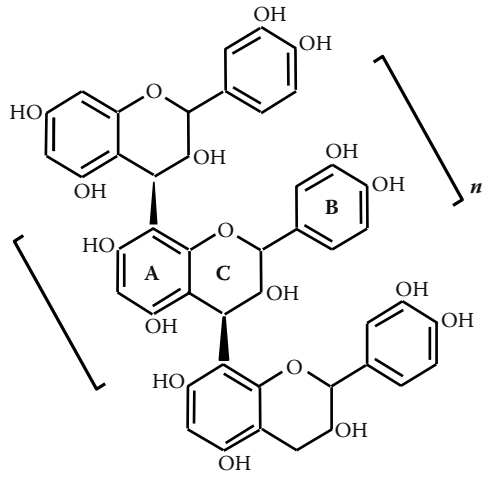

FIGURE 5.3 Chemical structure of procyanidins. Procyanidins have (n + 2) monomer units.

linked. Figure 5.3 shows the structure of a procyanidin whose monomeric units are linked through 4 → 8 carbon–carbon bonds (e.g., B-type dimer, C-type trimer). Other possible oligomerization patterns are through 4 → 6 carbon–carbon, 2 → 7 ether bonds, or combining different types of linkages [10].

5.2.2 Flavanols and Procyanidins in the Human Diet

Examples of edible plants containing a high amount of flavanols include cacao, grapes, tea, apples, nuts, and berries. The qualitative and quantitative content of flavanols is different among these plants [10]. In tea, flavanols are mostly present as gallate derivatives; while in grapes, the main flavanol present is CT, which polymerizes in tannins when grapes are converted to wine. In cocoa, and when cocoa is processed into chocolate, flavanols are mainly present as EC, CT, and B-type procyanidins [11]. In peanuts, monomers oligomerize as A-type dimers and trimers. In plants commonly used as foods, dimers to dodecamers are generally the procyanidins present in biologically relevant concentrations, being larger oligomers present in very minute concentrations [9].

The intake of flavonoids, as well as flavanols and procyanidins, varies considerably depending on the region and the dietary and cultural habits of the population being considered. Typical flavonoid intakes in Europe were first estimated to be about 1 g per day [12]. However, recent studies in several European countries limited flavonoid intakes under 50 mg per day [10]. This uncertainty is related to the difficulty in assessing both flavonoid-rich food consumption and the amount of flavonoids present in the consumed plants [13].

Although the determination of polyphenols or flavonoids consumed by a population is of certain value, from a health perspective and based on increasing

experimental evidence, the determination of the consumption levels of the individual compounds is becoming more relevant. This consideration is based on the fact that individual molecules perform defined biological actions, and that such actions go beyond the broad chemical characteristic of a group, for example, polyphenols and flavonoids.

5.2.3 Bioavailability of Flavanols and Procyanidins

When discussing the biological activity of flavanols and procyanidins, an important factor to be considered is their bioavailability. Monomers and dimers can pass through membranes and accumulate into cells. They can be absorbed in the digestive tract, reach blood, and target different organs [14]. That is the case for the flavanols EC, CT, ECG, EGC, and EGCG, as well as EC dimers. Once in the mesenteric circulation, monomeric flavanols predominately exist as both methylated and glucuronidated derivatives [14–17]. Studies have confirmed the presence of these conjugates in the plasma and urine of animal and human subjects [16,18–20], as well as in the bile [16], liver, and kidney of rats [14]. Conflicting results have being presented on the presence of these compounds in the brain of rats [21,22]. It has been reported that colonic microflora can break the flavan structure of flavonoids to form simple phenolics and ring-fission metabolites that may be physiologically relevant [23,24]. In human subjects, increased urinary excretion of such phenolic acids has been found 9–48 h after cocoa consumption [25]. Concerning larger procyanidins, although it was initially thought that they could not pass the acidic conditions of the stomach [26], data from human subjects show that procyanidins are stable during gastric transit [27]. Thus, large procyanidins could have beneficial health effects at the level of the gastrointestinal tract before being excreted. In summary, depending essentially on metabolism and transport, nonmetabolized flavanols or metabolites of flavanols can be present in different tissues, and their presence would define their capacity to exert a biological effect.

In humans, total plasma concentrations of epicatechin plus epicatechin-metabolites can be found in the micromolar range as soon as 1 h after cocoa consumption [19,28–30], with the major metabolite being 4′-*O*-methyl-epicatechin-7-β-D-glucuronide [30]. Concerning procyanidin bioavailability, some dimers have been detected in the plasma of humans and rats in modest concentrations relative to the monomers [20,28,29,31].

5.3 MODULATION OF THE NF-κB SIGNALING PATHWAY BY FLAVANOLS AND PROCYANIDINS

5.3.1 NF-κB Signaling Pathway

The Rel/NF-κB family of proteins in eukaryotic cells include c-Rel, RelB, p65 (RelA), p50/p105, and p52/p100 [5]. In the classical pathway of NF-κB activation, the activity of the Rel/NF-κB homo and heterodimers (mainly p50-p65 heterodimer)

is regulated by their interaction with inhibitory IκB proteins which anchor the transcription factor to the cytosol by masking its nuclear localization signal [32]. In general, the activation of NF-κB is triggered by the degradation of IκB, which starts with the phosphorylation of two serine residues present in IκB by a specific IκB kinase complex (IKK) [33]. After phosphorylation, the ubiquitination of the IκB protein leads to its degradation by the 26S proteasome. The unmasking of the nuclear localization signal sequence in NF-κB directs the NF-κB protein to the nuclear pore complex where translocation takes place [34–36]. In the nucleus, NF-κB binds to its DNA consensus sequence and modulates the transcription of numerous genes involved in inflammation (e.g., TNFα and chemokines), cell survival (e.g., Bcl-xl, Bcl-2, XIAP, and c-FLIP), proliferation (e.g., cyclin D1, c-Myc, and IL-1), tumor promotion (iNOS, MMP-9, and COX-2), and angiogenesis (e.g., MCP-1, VEGF, TNFα, and IL-8) [37–41].

5.3.2 Flavanols and Procyanidins and NF-κB

Flavan-3-ols and procyanidins have been shown to inhibit NF-κB in different experimental models. EGCG, the major polyphenol in tea, prevented the activation of NF-κB in several cell types, being triggered by different stimuli, and by acting at different steps in the activation cascade. For example, EGCG and other tea polyphenols inhibited lipopolysaccharide (LPS)-induced TNFα secretion *in vitro* and *in vivo* [42]. EGCG prevented LPS-induced increases in NF-κB nuclear binding activity in RAW 264.7 macrophages and interleukin-1β-induced NF-κB activation in A549 lung epithelial cells [43]. In human epidermal keratinocytes, EGCG also inhibited NF-κB activation triggered by UVB exposure, which was proposed to be relevant for the photochemopreventive activity of EGCG [44]. Different molecular mechanisms could explain the capacity of EGCG to inhibit NF-κB, including the inactivation of IKK [45,46], the activation of caspases involved in the degradation of the NF-κB protein Rel A [47], and the control of the cellular levels of reactive oxygen and nitrogen species.

A series of papers have demonstrated that in some cell types, EC, CT, and related procyanidins can modulate the expression of NF-κB-regulated cytokines, for example, IL-2, IL-1β, and IL-4 [48–53]. EC, CT, and type B-dimers (dimer B1 and dimer B2) inhibited the interferon γ-induced expression of NF-κB-dependent genes in RAW 264.7 macrophages [51]. All of these compounds markedly inhibited the secretion of TNFα, while the monomers, and to a lesser extent the dimers, inhibited the expression of a NF-κB-driven luciferase reporter gene [51]. In Hodgkin's lymphoma cells, characterized by a marked constitutive activation of NF-κB and secretion of NF-κB-regulated cytokines, EC caused a modest decrease of RANTES and no effect on TNFα secretion [53]. However, in Jurkat T cells, EC, CT, and a dimer fraction isolated from cocoa, inhibited PMA-induced IL-2 production and NF-κB activation [52].

We have presented evidence that the modulation of NF-κB by flavanols and procyanidins can be ascribed to events that occur at different steps in the NF-κB

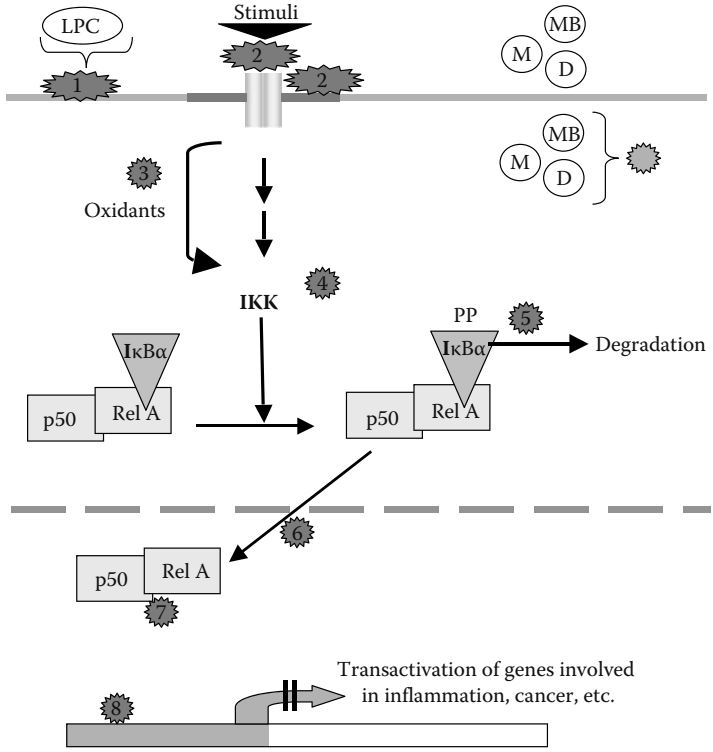

FIGURE 5.4 Potential events in which flavanols and procyanidins can inhibit NF-κB activation. Procyanidins of three or more monomer units (LPC) that are not transported inside the cells could affect the binding of the ligand (stimuli) to its receptor by (1) changing the biophysical characteristics of the membrane and modifying the receptor environment; and (2) interacting directly with the receptor. Monomers (M), dimers (D), and related metabolites (MB) that are transported into the cells could also act as (3) antioxidants, attenuating the increase of oxidants associated with the activation of NF-κB by several stimuli; (4) inhibitors of events upstream of IKK or directly inhibiting IKK; (5) inhibitors of the phosphorylation and/or degradation of the inhibitory protein IκBα; (6) affecting the transport of the active NF-κB from the cytosol into the nucleus; and inhibiting the binding of NF-κB to κB DNA sites either through a direct interaction with NF-κB proteins (7) or at the level of the DNA (8).

activation cascade [52]. Figure 5.4 schematizes possible events mediated by flavanols and procyanidins. Large procyanidins (with three or more units) that are not transported inside the cells could modulate NF-κB activation at the membrane level. It has been reported that procyanidins can interact with synthetic and cell membranes affecting membrane physical properties [54–58]. Thus, flavanols and procyanidins could act affecting the binding of the ligand (stimuli) to its receptor by (1) changing the biophysical characteristics of the membrane and modifying the receptor environment; or (2) interacting directly with the receptor. Monomers

and dimers (and related metabolites) can be transported into the cells and then could act by (1) attenuating the increase of oxidants associated with selected stimuli and the subsequent activation of NF-κB (antioxidant effect); (2) inhibiting events upstream of IKK or directly inhibiting IKK; (3) inhibiting the phosphorylation and/or degradation of the inhibitory protein IκBα; (4) affecting the transport of the active NF-κB from the cytosol into the nucleus; and (5) inhibiting the binding of NF-κB to κB DNA sites either through a direct interaction of monomers and dimers with NF-κB proteins or by interacting with DNA.

5.4 NF-κB MODULATION BY FLAVANOLS AND PROCYANIDINS: RELEVANCE FOR HUMAN DISEASES

NF-κB modulates the activation of numerous genes involved in inflammation, immunity, cell growth, and death. All of these events are directly or indirectly involved in cardiovascular disease, cancer, diabetes, neurodegeneration, and other pathologies. Therefore, NF-κB modulation can be a central event in the control of a number of conditions that either initiate the disease or are secondary effects of the disease. In the following section we will review some of the most relevant literature on the documented effects of flavanols and procyanidins on NF-κB activation in cancer, cardiovascular disease (CVD), and diabetes. In addition, we will finally discuss the potential influence of NF-κB modulation by flavanols and procyanidins on inflammatory bowel disease (IBD).

5.4.1 Flavanols and Procyanidins and NF-κB in Cancer

Through the regulation of genes that promote proliferation, prevent apoptosis, and modulate angiogenesis and metastasis, NF-κB plays a key role in oncogenesis [39,40]. Furthermore, many different human cancer types are characterized by a constitutive activation of NF-κB. Thus, the development of new therapeutic agents that could inhibit NF-κB activation has gained particular interest [7], as well as the investigation of the anticancer activity of numerous phytochemicals with NF-κB inhibitory capacity [8].

In a large number of *in vitro* studies, EGCG has been shown to affect several signaling cascades, including NF-κB, leading to the inhibition of the cell cycle and the induction of apoptosis in cancer cells (reviewed in Refs. [44,59]). In animal models, numerous studies have consistently shown that EGCG and green tea supplementation exert antitumor activity in different cancers, such us gastrointestinal, lung, prostate, breast, and skin cancer (reviewed in Refs. [60–62]). However, in humans, data obtained through epidemiological studies or clinical trials with EGCG or green tea supplementation are largely inconsistent and conflicting (reviewed in Refs. [60–62]).

Although these compounds can inhibit NF-κB, the potential anticancer activity of EC, CT, and procyanidins is less well characterized. Most of the research has been done using extracts (apple, grape, cocoa, etc.) or plant extracts containing

multiple components. Thus, the observed effects cannot be ascribed to selected molecules present in the extracts. For example, it was reported that a flavonoid-containing grape seed extract induced apoptosis in breast cancer cells (4T1) both *in vitro* and when the 4T1 cells were implanted in mice that received the extract as part of the diet [63]. In both experimental models a downregulation of the NF-κB-regulated antiapoptotic protein Bcl-2 was observed [63]. A different study identified gallic acid as the component of a grape seed extract with the highest capacity to promote apoptosis and inhibit growth in DU145 human prostate carcinoma cells [64]. However, dimers isolated from the same grape extract also had proapoptotic effects but at a relative lower extent. We recently found that in Hodgkin's lymphoma cell lines, EC inhibited constitutive activation of NF-κB mostly through the proposed mechanism of a direct interaction of EC with NF-κB [53].

Given their relatively poor intestinal absorption [65], large procyanidins may have their main anticarcinogenic action limited to the gastrointestinal tract. In this regard, the consumption of diets rich in flavonoids was associated with a 70% reduction of risk to suffer cancers of the aerodigestive tract [66]. In an animal model of dimethylhydrazine-induced colon cancer, the oral supplementation with cloudy apple juice decreased the number of aberrant crypt foci development in the distal colon [67]. Although the juice was enriched in EC, and dimers B1 and B2, the observed effects could not be unequivocally ascribed to the presence of EC, dimer B1 or dimer B2, but to the presence of other flavonoids or related compounds present in the apple juice.

5.4.2 Flavanols and Procyanidins and NF-κB in Cardiovascular Disease and Diabetes

Epidemiological data have related the consumption of flavanol-containing foods to the incidence of CVD [68,69]. In line with the epidemiological data, extensive research has been developed addressing *in vivo* effects of purified flavanols and flavanol-containing foods on markers of CVD [70–75]. Essentially, flavanols and related procyanidins can interfere with CVD through their antioxidant, antiproliferative, antithrombotic, and antihyperlipidemic effects [76]. The modulation of enzyme activities involved in cardiovascular processes by flavanols and procyanidins has provided alternative mechanisms for the action of these phytochemicals. For example, the inhibitory effects of flavanols and procyanidins on 15-lipooxygenase and angiotensin converting enzyme activities have been reported in *in vitro* systems [77–79]. Furthermore, increases in tissue NO production were reported to be associated with nitric oxide synthase activation in experiments done in humans [30,72,75].

The participation of NF-κB in the etiology and progression of CVD is well documented. It includes NF-κB-associated preservation of both endothelial and smooth muscle cell function, and in controlling the generation of inflammatory molecules by immune cells [80–82]. Several of the steps leading to the development

of the atherosclerotic plaque are influenced by NF-κB-dependent cytokine production, which implies that a downregulation of NF-κB could prevent plaque progression. It has also been reported that in rats, EGCG inhibits both NF-κB activation associated to ischemia-reperfusion damage [83], and smooth muscle cell proliferation [84]. A recent report showed that an apple extract added in culture downregulated NF-κB signaling in HUVEC cells [85]. However, no identification of the compounds present in the apple extract that could be responsible for the observed effects was reported.

Concerning diabetes, it is accepted that NF-κB is involved in the etiology of insulin resistance and type 2 diabetes, which are also risk factors for CVD [86–88]. The consumption of tea has been linked to a better glucose tolerance, but did not reverse the diabetes-induced modifications of serum proteins in mice [89]. Further studies on the effects of flavanols and procyanidins on NF-κB modulation in animal models of type 2 diabetes or obesity and in humans are necessary to assess the real relevance of those observations.

5.4.3 Flavanols and Procyanidins and NF-κB in Inflammatory Bowel Disease

NF-κB activation is a consistent finding in different conditions of human IBD, including ulcerative colitis, and colorectal cancer [90,91]. Experimental evidence indicates that, when the integrity of the epithelial barrier is lost, the activation of NF-κB in native immune cells at the lamina propria leads to the release of proinflammatory mediators that are involved in tissue damage and in the subsequent activation of NF-κB in epithelial cells [92–94]. The involvement of NF-κB regulation in IBD pathogenesis is further supported by the evidence that drugs widely used in the treatment of patients with IBD (i.e., sulfasalazine and steroids) have the capacity to inhibit NF-κB activation [95]. We recently published experimental evidences supporting the hypothesis that dietary nonabsorbable procyanidins (large procyanidins) could have potential health benefits at the level of the gastrointestinal tract [96]. Using Caco-2 cell monolayers as a model of intestinal epithelium, we observed that hexamers isolated from cocoa, although not incorporated into the cells, protected them from damage induced by bile acids and oxidants. Treatment of Caco-2 cell monolayers with deoxycholic acid or a lipophilic oxidant induced an increase in cell oxidation, alterations in the paracellular transport, and redistribution of the tight function protein ZO-1 into the cytoplasm. Hexamers partially inhibited all these events at concentrations ranging from 2.5 to 20 μM by interacting with the cell membranes. Extending these observations, we recently found that in Caco-2 cells, hexamers inhibited NF-κB activation induced by different proinflammatory stimuli [97]. The above results and the fact that the used concentrations of procyanidins can be achieved in the gastrointestinal tract after the consumption of a flavonoid-rich diet [27] supports the hypothesis that procyanidins can have a beneficial effect in IBD.

5.5 CONCLUSION

Inhibition of NF-κB activation could represent a therapeutic strategy to control inflammation, and consequently, a series of inflammation-driven diseases including CVD, cancer, and IBD. An extensive body of research has developed in the last years regarding to flavanol components of foods, mainly EGCG, as regulators of NF-κB activation. Recent research has extended those investigations to the effects of procyanidins present in certain fruits and vegetables, for example, grapes, cocoa, and apples. Interestingly, flavanols and procyanidins can modulate different steps of the NF-κB activation cascade, exerting their effects from extracellular membrane to interference with DNA binding. The final identification of the mechanisms that can reconcile the bioavailability of flavanols and procyanidins with the proposed biochemical functions will allow the assessment of the final relevance of these compounds for human health.

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6 Regulation of Inflammation, Redox, and Glucocorticoid Signaling by Dietary Polyphenols

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CONTENTS

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6.1 POLYPHENOLS: GENERAL CONSIDERATIONS

A wide variety of dietary plants, including grains, legumes, fruits, vegetables, tea, and grapes, have been reported to be beneficial against various diseases and such effects have been attributed to the antioxidants and polyphenols present in these dietary sources [1,2]. Although polyphenols are a large group of complex compounds and fall into several classes, not all of these compounds have been reported to have beneficial health effects. This is especially in view of the fact that polyphenols are known to undergo various biochemical transformations that affect their bioavailability as well as bioefficacy. In this chapter, we will discuss the various aspects of polyphenol absorption, pharmacokinetics, metabolism, bioavailability, and the roles of polyphenols as antioxidants and in modulation of cellular signalling and inflammation.

6.1.1 Chemistry of Polyphenols

Polyphenols are secondary metabolites of plants with over 8000 structural variants and comprise of a wide range of substances having aromatic ring(s) bearing one or more hydroxyl moieties (Table 6.1). Chemically, polyphenols may vary from a simple molecule, such as phenolic acid, to highly polymerized compounds, such as tannins [3]. The most widely distributed group of plant phenolics are flavonoids having a general structure as that of diphenylpropanes, consisting of two aromatic rings linked through three carbon atoms that usually form an oxygenated heterocycle [3]. Figure 6.1 shows the basic structure and the system used for the carbon numbering of a flavonoid nucleus and the structures of some important polyphenols and their structural relationship with steroids. The flavonoids subclasses include

TABLE 6.1
Major Classes of Phenolic Compounds in Plants

Class	Number of Atoms	Carbon Skeleton	Examples
Simple phenols, benzoquinone	6	C_6	Catechol, hydroquinone 2,6-Dimethoxy-benzoquinone
Phenolic acids	7	C_6–C_1	Gallic, salicylic
Acetophenones	8	C_6–C_2	3-Acetyl-6-methoxybenzaldehyde
Tyrosine derivatives			
Phenylacetic acids			Tyrosol p-hydroxyphenylacetic
Hydroxycinnamic acids	9	C_6–C_3	Caffeic, ferulic Myristicin, eugenol
Phenylpropenes	10		
Coumarins			Umbelliferone, aesculetin
Isocoumarins			Bergenon
Chromones			Eugenin
Naphthoquinones	10	C_6–C_4	Juglone, plumbagin
Xanthones	13	C_6–C_1–C_6	Mangiferin
Stilbenes	14	C_6–C_2–C_6	Resveratrol
Anthraquinones			Emodin
Flavonoids	15	C_6–C_3–C_6	Quercetin, cyanidin
Isoflavonoids			Genistein
Lignans	18	$(C_6$–$C_3)_2$	Pinoresinol
Neolignans			Eusiderin
Biflavonoids	30	$(C_6$–C_3–$C_6)_2$	
Lignins	n	$(C_6$–$C_3)n$	
Catechol, melanins		$(C_6)n$	
Flavolans (condensed tannins)		$(C_6$–C_3–$C_6)n$	

Source: Adapted from Harborne, J.B., in *Encyclopedia of Plant Physiology, Secondary Plant Products,* Springer-Verlag, Berlin, 1980, 329.

flavonols, flavones, flavanols, isoflavones, anthocyanins, and flavanones (Table 6.2). When glycosylated, flavonoids become more water-soluble and lose their reactivity toward free radicals. Considering the large number of polyphenolic compounds present in dietary sources, this chapter will focus on the better known and most studied polyphenols such as resveratrol, curcumin, and catechins. Minor sections on the absorption, metabolism, and bioavailability of polyphenols have also been considered as these aspects of polyphenols are still to be fully deciphered and proper understanding of these phenomena will provide new insights for designing new strategies to develop polyphenol-based therapies for chronic inflammation.

6.1.1.1 Resveratrol

Resveratrol (3,5,4′-trihydroxystilbene) was discovered in 1976 by Langcake and Pryce as a phytoalexin in grapes [4] and is composed of two phenolic rings

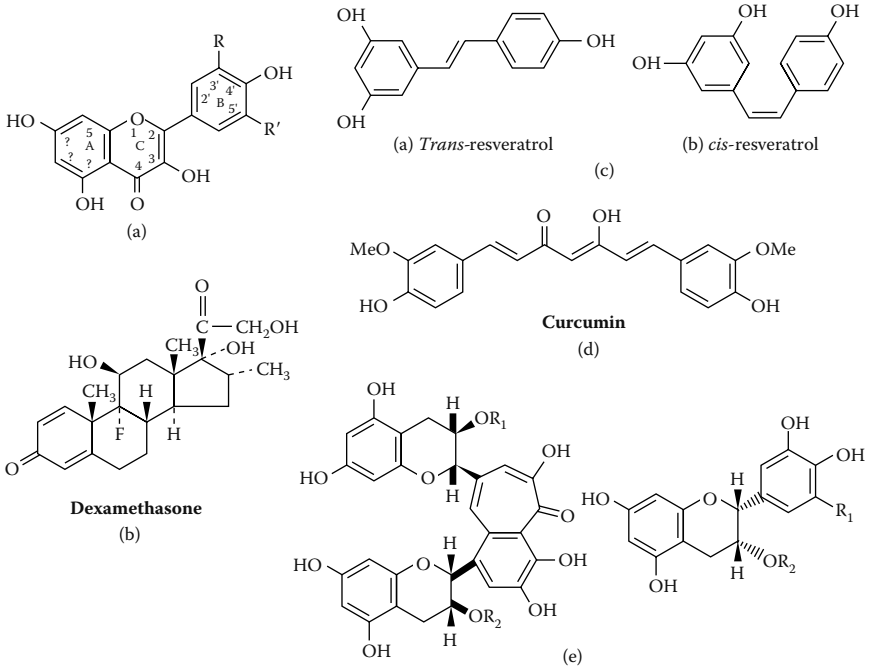

FIGURE 6.1 Basic structure of polyphenols. Flavonoids (C_6–C_3–C_6), (a) basic structure and system used for carbon numbering of the flavonoid nucleus, (b) polyphenols structural relationship with steroids (dexamethasone), and the structures of some important polyphenols: (c) resveratrol, (d) curcumin, and (e) catechins.

connected by a double bond (Figure 6.1). Two isoforms of resveratrol, *trans*-resveratrol and *cis*-resveratrol, are known, with the *trans* isomer being more stable than the *cis* form [5]. While *trans* to *cis* isomerization is facilitated by ultraviolet light and high pH, the *cis* to *trans* conversion is facilitated by visible light, high temperature, or low pH [5]. Resveratrol has generated a great scientific interest over the years mainly due to a spate of evidence from both *in vitro* and *in vivo* studies suggesting its cancer preventive and cardio-protective properties, the so-called French paradox [6].

6.1.1.2 Curcumin

Curcumin, a yellow phenolic pigment, is a member of the curcuminoid family of compounds obtained from powdered rhizome of *Curcuma longa* Linn. (Family: Zingiberaceae). The hydroxy and the methoxy groups of curcumin have been

TABLE 6.2
Subclasses, Sources, and Dietary Flavonoids

Subclass of Flavonoids	Sources	Dietary Forms
Anthocyanins	Red, blue and purple berries, red and purple grapes, red wine	Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin
Flavanols	Catechins: teas (particularly green and white), chocolate, grapes, berries, apples Theaflavins Thearubigins: teas (particularly black and oolong) Proanthocyanidins: chocolate, apples, berries, red grapes, red wine	Monomers (Catechins): Catechin, Epicatechin, Epigallocatechin Epicatechin gallate, Epigallocatechin gallate Dimers and polymers: Theaflavins, Thearubigins, Proanthocyanidins
Flavanones	Citrus fruits and juices (e.g., oranges, grapefruits, lemons)	Hesperedin, Naringenin, Eriodictyol
Flavonols	Widely distributed: yellow onions, scallions, kale, broccoli, apples, berries, teas	Quercetin, Kaempferol, Myricetin, Isorhamnetin
Flavones	Parsley, thyme, celery, hot peppers	Apigenin, Luteolin
Isoflavones	Soybeans, soy foods, legumes	Daidzein, Genistein, Glycitein

Source: Adapted from http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids/catechins.html.

found to impart antioxidant and anticarcinogenic activities to this compound (Figure 6.1). Typically, crude extract of rhizomes of *C. longa* contain 70%–76% curcumin, 16% demethoxycurcumin, and 8% bisdemethoxycurcumin. Various therapeutic effects such as anti-inflammatory, antibacterial, antiviral, antifungal, antitumor, antispasmodic, and hepatoprotective agent have been attributed to curcumin [7].

6.1.1.3 Catechins

Catechins are monomeric forms of flavanols, which include related compounds such as catechin, epicatechin (EC), epigallocatechin, epicatechin gallate (EGC), and epigallocatechin gallate (EGCG) [8]. Green tea contains mainly catechins whereas black tea contains theaflavins and thearubigins. Oolong tea differs from green and black tea in its flavanol content and the concentration is somewhere intermediate compared to the later two varieties.

6.1.2 Absorption, Pharmacokinetics, Tissue Distribution, and Metabolism of Polyphenols

The chemically complex structure of polyphenols is reflected in their absorption, biotransformation, and bioavailability characteristics. It is therefore apt to consider

these aspects before we embark on the investigation of the molecular mechanisms and therapeutic applications of these versatile compounds. Proper understanding of pre- and post-absorption transformation of polyphenols will provide new insights into the understanding of the molecular mechanism(s) that underpin the effects of polyphenols and will aid in designing appropriate pharmacotherapeutic strategies involving these compounds.

6.1.2.1 Resveratrol

Approximately 20% of the available resveratrol is absorbed, mainly in the duodenum as evidenced from studies in rat intestines. Resveratrol glucuronide is the major form absorbed when compared to very minute amounts of unconjugated resveratrol and resveratrol sulfate [9]. Studies with radio-labeled resveratrol in mice have revealed that resveratrol was first distributed in duodenum 1.5 h post-administration and also in liver and kidney around the same time period [10] and was detectable until 6 h. In the lung, spleen, heart, brain, and testis, resveratrol-induced radioactivity could be detected only after 3 h. In general, resveratrol radioactivity was found to be distributed in all organs. Resveratrol is glucuronated in the human liver and sulfated in both the liver as well as in the duodenum. The human liver and duodenum are the sites for glucuronidation of resveratrol, the major derivatives being *trans*-resveratrol-3-O-glucuronide, *trans*-resveratrol-4-O-glucuronide, and *trans*-resveratrol-3-O-sulfate [11]. Kinetic analysis of resveratrol transformation suggests that in the liver, glucuronidation is favored over sulfation with almost similar rates of reaction. The metabolic modifications of resveratrol can be inhibited by quercetin, a polyphenol also found in wine. Clinical and *in vivo* studies have indicated that free *trans*-resveratrol in plasma is very sparse and short lived.

6.1.2.2 Curcumin

Pharmacokinetic studies have shown that about 40%–85% of ingested curcumin remains unaltered in the gastrointestinal tract. Most of the absorbed curcumin is metabolized in the intestinal mucosa and liver [12]. Curcumin undergoes *O*-conjugation to curcumin glucuronide and curcumin sulfate and reduced to tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol in rats and mice *in vivo* and in human hepatic cell suspension [13]. Products of curcumin reduction are also subject to glucuronidation. Certain curcumin metabolites, such as tetrahydrocurcumin, possess anti-inflammatory and antioxidant activities similar to those of their metabolic progenitor. However, conflicting reports are available regarding the anti-inflammatory property being lost when curcumin is transformed to tetrahydrocurcumin, with the antioxidant property still intact. It has been suggested that the intestinal tract plays an important role in the metabolic disposition of curcumin; a notion that is based predominantly on experiments with [^{3}H] labeled curcumin [14]. While metabolites of curcumin, such as curcumin glucuronide, were identified in intestinal and hepatic microsomes,

curcumin sulfate, tetrahydrocurcumin, and hexahydrocurcumin were found as curcumin metabolites in intestinal and hepatic cytosol from humans and rats. Curcumin is unstable at neutral and basic pH values and is degraded to ferulic acid (4-hydroxy-3-methoxycinnamic acid) and feruloylmethane (4-hydroxy-3-methoxycinnamoylmethane) [15]. Although the exact mechanism of degradation is still to be fully understood, the idea that oxidative mechanism may be involved gains credence from observations that the presence of antioxidants, such as ascorbic acid, *N*-acetylcysteine, or glutathione, can completely block the degradation of curcumin at pH 7.4. Curcumin appears to be stable in the stomach and small intestines because the pH is between 1 and 6, and degradation of curcumin is extremely slow in these conditions. Therefore, although the pharmacokinetic properties of curcumin are fairly well understood, many questions remain pertaining to its fate after ingestion.

6.1.2.3 Catechins

Tea polyphenols EGCG, EGC, and (2)-epicatechin (EC) are believed to be responsible for the beneficial effects of tea. In a study using decaffeinated green tea in rats [16], it was found that β-elimination half-life was greatest for EGCG followed by EGC and then EC. An intravenous administration of decaffeinated green tea recorded the highest levels of EGCG in the intestinal samples, and the levels declined with a half-life of 173 min. The highest levels of EGC and EC were recorded in the kidney, and the levels declined rapidly with half-lives of 29 and 28 min, respectively. The liver and lung levels of EGCG, EGC, and EC were generally lower than those in the intestine and the kidney. The results from distribution studies suggested that EGCG is mainly excreted through bile, and that EGC and EC are excreted through both the bile and urine.

6.1.3 Bioavailability of Polyphenols

The actual mechanism as to how polyphenols are absorbed, made bioavailable, and distributed and metabolized is still to be fully decrypted. However, it appears that some polyphenols are bioactive and are absorbed through the intestine in their native or modified form. The absorbed forms are then metabolized and the end products may be detected in plasma in nanomolar ranges. The plasma forms of polyphenols may retain at least part of the antioxidant capacity and then excreted [16]. In general, the bioavailability of flavonoids is limited due to low absorption. Of all the flavonoid glycosides amenable to the small intestine, only flavonoid aglycones and flavonoid glucosides are absorbed in the small intestine, where they are rapidly transformed into methylated, glucuronidated, or sulfated equivalents [17]. Since colonic microflora play an important role in flavonoid metabolism and absorption, the derivatives formed, near the intestine especially, do not possess the same biological activity as that of the parent flavonoids [18]. Therefore, one has to be careful in extrapolating results obtained from flavonoid studies *in vitro* using purified compounds and the dosage used.

Pharmacologically, curcumin has been reported to be safe and human clinical trials indicated no toxicity even when administered at doses up to 10 g/day [19]. Curcumin has low oral toxicity in man but has low oral bioavailability (500–1000 nM after 8 g/day oral dose). However, consumption of curcumin along with piperin, an active ingredient in peppers and a known inhibitor of intestinal and hepatic β-glucuronidation of curcumin, may improve the oral bioavailability to twenty-fold but with a short half-life of 40 min [20]. Dosage studies with resveratrol in humans have shown that a person of 70 kg can safely consume a minimum of 14 mg of resveratrol per day. It is also calculated that daily consumption of pure resveratrol and its analog piceatannol with a dose of 25–50 mg daily leads to nM concentration (which is beneficial) of circulatory levels of resveratrol. It is estimated that 20 glasses of red wine provide 25 mg of resveratrol.

Bioavailability differs markedly among catechins. EGCG is the only known polyphenol present in plasma in large portion (77%–90%) in a free form [16]. The other catechins are highly conjugated with glucuronic acid and/or sulfate groups. In a study using pure catechins, van Amelsvoort et al. [21] demonstrated that galloylation of catechins reduces their absorption. They found that only epigallocatechin was methylated and that 4-*O*-methyl-epigallocatechin accounted for 30%–40% of the total metabolites of epigallocatechin. This observation was supported by a parallel study by Meng et al. who showed that the 3′ position of EGCG was preferentially methylated [22]. Therefore, the mean bioavailability parameters calculated may be underestimated due to diverse derivatization of the catechins *in vivo* [23]. Several microbial metabolites of catechins mostly in conjugated forms were also identified in plasma and urine of volunteers after ingestion of green tea [22]. Since polyphenols are poorly absorbed and undergo extensive biotransformation, clinical studies have recently demonstrated that it is safe to consume EGCG or polyphenol E (a defined, decaffeinated green tea–polyphenol mixture) in amounts equivalent to the EGCG content in 8–16 cups of green tea once a day, or in divided doses twice a day for 4 weeks.

6.2 ANTIOXIDANT NATURE OF POLYPHENOLS

A recent increase in the interest of the role of dietary molecules in the modulation of the cellular redox system in pathophysiology has revealed the beneficial role of polyphenols during oxidative stress. Further investigations have led to the identification of flavonoids and other dietary polyphenol antioxidants present in plant foods as bioactive molecules. Supporting data are available that health benefits associated with fruits, vegetables, and red wine in the diet are probably linked to the high amounts of polyphenol antioxidants they contain. Since most beneficial properties of polyphenols were initially attributed to their free radical scavenging ability, the following sections will be devoted to the antioxidant properties of curcumin, resveratrol, and catechins.

As early as 1976 [24], the antioxidant properties of curcumin were identified wherein curcumin was reported to be an effective oxygen-free radical scavenger. Curcumin (in micro- to millimolar range) was found to have a broad spectrum

of antioxidant property as evidenced from its ability to scavenge reactive oxygen species (ROS), like superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO), both *in vitro* and *in vivo* [25]. The effect was more pronounced against H_2O_2-induced damage in human keratinocytes, fibroblasts, and in NG 108-15 cells (a mouse neuroblastoma–rat glioma hybrid cell line) [26]. A recent observation from our laboratory has revealed that curcumin in concentration of 1 to 50 μM can scavenge ROS in 1 to 4 h as determined by electron paramagnetic resonance technique [27,28]. Curcumin was much faster in terms of scavenging ROS compared to resveratrol and quercetin. Further evidence of antioxidant properties of curcumin was obtained from the reports of curcumin's ability to lower lipid peroxidation and activate antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [29]. Since ROS have been implicated in the pathogenesis of various chronic and inflammatory conditions [30], curcumin may therefore have a potential therapeutic application in the control of inflammatory diseases. However, contradictory effects of curcumin have also come to the fore in view of its failure to prevent single-strand DNA breaks by H_2O_2, a damage that was prevented by vitamin E [31]. Such a pro-oxidantproperty may be due to the generation of phenoxyl radical of curcumin by peroxidase–H_2O_2 system, which co-oxidizes cellular glutathione or NADH, accompanied by O_2 uptake to form ROS [32]. Therefore, it appears that curcumin may not always be a preferred antioxidant under all situations of oxidative stress. The antioxidant mechanism of curcumin is attributed to its unique conjugated structure, which includes two methoxylated phenols and an enol form of β-diketone [33]. The net mechanism of action of curcumin appears to be mediated through increased levels of glutathione (GSH), which lead to lowered ROS production [34].

Catechins have been found to be very effective scavengers of free radicals *in vitro* [35]. Since, even with very high intakes, plasma and intracellular flavonoid concentrations in humans are likely to be 100 to 1000 times lower than concentrations of other antioxidants, such as vitamin C or GSH, it is possible that catechins may not be as effective as antioxidants compared to other polyphenols. The low antioxidant activity of catechins may also be due to the fact that most circulating flavonoids are transformed metabolites, some of which have lower antioxidant activity than the parent flavonoid. For these reasons, the relative contribution of dietary flavonoids to the plasma and tissue antioxidant pool is therefore likely to be relatively minor [18]. It has been suggested that catechins may exert their cellular protective functions by way of modulating cellular signalling rather than as an antioxidant [18].

The di- or trihydroxyl groups on the B-ring and the *meta*-5,7-dihydroxyl groups on the A-ring of catechin molecules have been identified as centers imparting antioxidant properties on catechins. A trihydroxyl structure on the D-ring (gallate) in EGCG and ECG is known to further enhance the antioxidant potential of these compounds [36]. Among tea catechins, EGCG is most effective in scavenging most ROS. Another feature that adds to the antioxidant repertoire of catechins is their metal ion chelating capacity as evidenced by their ability to inhibit

Cu_2^+ catalyzed oxidation of lipoproteins *in vitro* in macrophages [37]. This property of catechins is thought to be due to their vicinal dihydroxyl and trihydroxyl structures, which can prevent the generation of free radicals. Although the effect of catechins on biomarkers of oxidative stress has been reported, more elaborate studies are required in humans. This is due to the observation in humans that there is only a very transient and modest increase in the total plasma antioxidant levels post tea consumption [38]. It appears that the *in vivo* biological activity of polyphenols is limited by their bioavailability [39]. The relatively short half-life of EGCG (0.5–2 h) in *in vitro* conditions could be prolonged by the addition of SOD [40] suggesting that superoxide radical may be responsible for the short half-life. Since the pO_2 in a cell culture system is much higher (160 mm Hg) than that in the blood or tissues (<40 mm Hg) [41], it is not yet clear whether the pro-oxidant properties of EGCG can also be envisaged at relatively lower pO_2 conditions *in vivo*. Under appropriate conditions, EGCG and other catechins can be oxidized to form phenoxyl radical, superoxide radical, and hydrogen peroxide, and thus create signalling anomalies and contribute to cell apoptosis. Thus, like other antioxidants, EGCG and other tea polyphenols may also act as pro-oxidants.

Resveratrol, also an antioxidant, is known to exert a global influence on oxidative stress situations. Resveratrol is a potent inhibitor of lipid peroxidation, which plays a major role in atherosclerosis [42] and also protects against metal catalyzed oxidation, the later effect being more pronounced than flavonoids [43]. *In vivo* data, however, do not corroborate these findings, since hyperlipidemic rabbits fed resveratrol showed no respite from increased serum cholesterol levels or atherosclerotic lesions [44]. Resveratrol also exhibits pro-oxidant properties and the anti- and pro-oxidant activities of resveratrol are believed to be concentration and cell-type dependent. For instance, while resveratrol was found to induce formation of ROS in human leukemia cells, in prostate cancer cells, resveratrol decreased intracellular ROS (in particular, O_2^-) levels in a dose-dependent manner. The reported cardioprotective ability of resveratrol has been attributed to its ability to modulate the production of NO from vascular endothelium [45]. Similarly, resveratrol has also been shown to inhibit platelet aggregation, another major contributor in the process of atherosclerosis and hence cardiovascular complications [46]. Human *in vivo* studies demonstrating increased antioxidant activity in blood of moderate red wine consumers further support the above observations. Whether resveratrol is responsible for the said cardiovascular, neurological, and hepatoprotective actions still needs in-depth studies.

6.3 ROLE OF POLYPHENOLS IN CELLULAR SIGNALING AND INFLAMMATION

In the earlier phases of studies with polyphenols much emphasis was laid as to their antioxidant properties, which was attributed to the availability of hydroxyl (–OH) and the system of conjugated double bonds present in these molecules.

The possibility that polyphenols may also modulate cellular signaling arose from the failure to explain the anti-inflammatory, antitumor, and antiatherogenic abilities of polyphenols solely on the basis of their antioxidant properties. Investigations as to how polyphenolic compounds exerted their actions shed light on the fact that polyphenols merely do not act as free radical scavengers, but may also modulate cellular signaling processes during inflammation or may themselves serve as signaling agents [47]. In the following sections, an overview of the anti-inflammatory properties of various polyphenols with respect to nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling are outlined to obtain a general idea of the wide variety of cellular signaling processes the polyphenolic compounds can modulate. Before that it would be worth considering in brief NF-κB and MAPK, since most inflammatory processes revolve around these two important signaling components in a cell.

6.3.1 Nuclear Factor-Kappa B (NF-κB)

NF-κB was first identified as a regulator of the expression of the kappa light-chain gene in murine B lymphocytes [48] but has subsequently been found in many different cells. Several different NF-κB proteins have been characterized [49,50]. NF-κB is comprised of a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory response. In addition, NF-κB is also involved in protecting cells from undergoing apoptosis in response to DNA damage or cytokine treatment. Stimulation of the NF-κB pathway is mediated by diverse signal transduction cascades. These signals activate the IκB kinases, IKKα and IKKβ, which phosphorylate inhibitory proteins known as IκB to result in their ubiquitination and degradation by the proteasome. The degradation of IκB results in the translocation of NF-κB from the cytoplasm to the nucleus where it activates the expression of specific cellular genes (Figure 6.2). Agents that inhibit this pathway, such as glucocorticoids and aspirin, can reduce the inflammatory response, while other agents, such as dominant negative IκB proteins, potentiate the effects of chemotherapy and radiation therapy in the treatment of cancer.

NF-κB regulates host inflammatory and immune responses [51–54] and cellular growth properties [55] by increasing the expression of specific cellular genes. These include genes encoding at least 27 different cytokines and chemokines, receptors involved in immune recognition, such as members of the major histocompatibility complex (MHC), proteins involved in antigen presentation, and receptors required for neutrophil adhesion and migration [52]. Cytokines that are stimulated by NF-κB, such as interleukin 1β (IL-1β) and tumor necrotic factor-α (TNF-α), can also directly activate the NF-κB pathway, thus establishing a positive auto regulatory loop that can amplify the inflammatory response and increase the duration of chronic inflammation (Figure 6.2). NF-κB also stimulates the expression of enzymes whose products contribute to the pathogenesis of the inflammatory process, including the inducible form of nitric oxide synthase (iNOS), which generates NO, and the inducible cyclooxygenase (COX-2), which

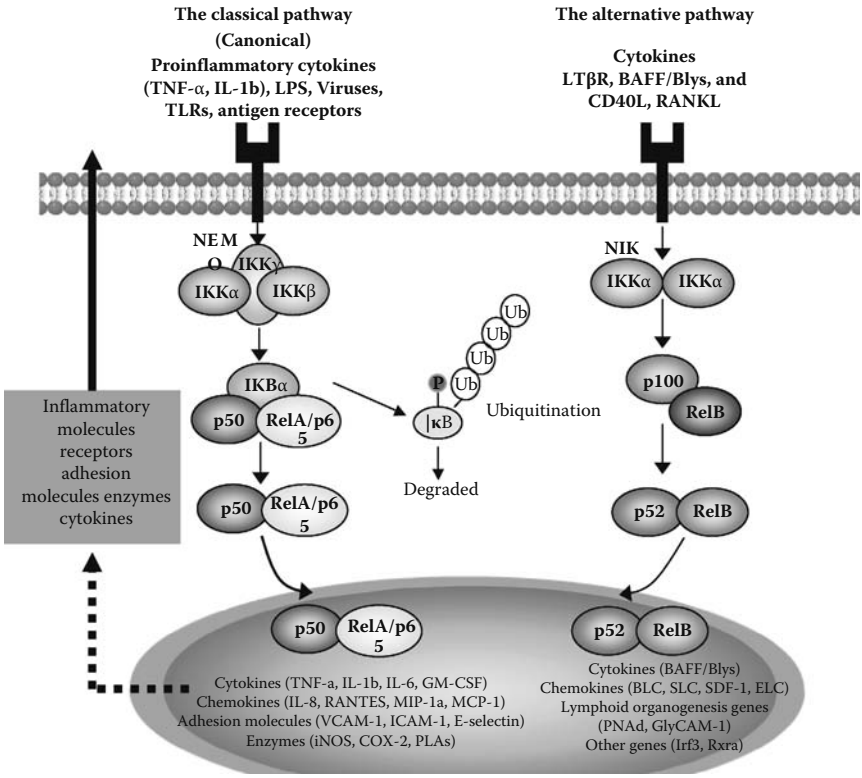

FIGURE 6.2 NF-κB activation pathways and the mechanism of amplification loop. The classical and alternate pathways of NF-κB activation lead to induction of proinflammatory mediators. Activation of NF-κB involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein IκB by specific IκB kinases. The free NF-κB (a heterodimer of p50 and p65) then passes into the nucleus, where it binds to κB sites in the promoter regions of genes for inflammatory proteins such as cytokines, enzymes, and adhesion molecules. P denotes protein. NF-κB may be activated by a variety of inflammatory signals, resulting in the coordinated expression of the genes for several cytokines, chemokines, enzymes, and adhesion molecules. The cytokines interleukin-1β and tumor necrosis factor α (TNF-α) both activate and are amplified by NF-κB.

generates prostanoids [52]. The NF-κB pathway is likewise important in the control of the immune response. It modulates B-lymphocyte survival, mitogen-dependent cell proliferation, and isotype switching, which lead to the differentiation of B-lymphocytes into plasma cells [53]. In addition, NF-κB regulates IL-2 production, which increases the proliferation and differentiation of T lymphocytes [52,53]. Thus, activation of NF-κB leads to the induction of multiple genes that regulate the immune and the inflammatory responses.

6.3.2 Mitogen-Activated Protein Kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are proline-directed serine/threonine kinases [56] and play an important role in processing various extracellular signals into intracellular responses through serial phosphorylation cascades [57]. Three distinct but parallel MAPK cascades have been identified in mammalian cells [58]. Each MAPK is made of a module of three kinases as follows: a MAPK kinase kinase, which phosphorylates and activates the second MAPK kinase (MAPKKK), which in turn phosphorylates and activates a third MAPK (MAPKKK). Among the three MAP kinases, extracellular signal-regulated kinase (ERK) 1/2 is involved in cell proliferation, while p38MAPK and c-Jun N-terminal kinase (JNK) are activated in response to environmental stress. One of the best characterized MAPK pathways is the Ras-dependent activation of extracellular signal-regulated protein kinases (ERKs) in response to growth factors, wherein tyrosine-phosphorylated transmembrane receptors associate with the SH2 domain of the adapter protein Grb2 [59,60] and target nucleotide exchange factor (SOS) to the membrane-bound small G-protein Ras [61]. Activated Ras recruits Raf-1 (a MAPK kinase kinase) to the membrane, thus activating Raf-1 [62]. On activation, Raf-1 phosphorylates and activates a dual specificity kinase MEK (a MAPK kinase), which in turn activates ERK. Apart from tyrosine kinase receptors, certain G-protein-coupled receptors and protein kinase C can also activate ERK cascade [63]. Another important MAPK is JNK, modulated by a parallel signalling module, consisting of MEKK1/MKK4 (or SEK1, JNKK)/JNK [64]. Unlike the ERK pathway, JNK cascade is partially activated by growth factors and phorbol esters and strongly activated by various stress signals [65,66]. Hence, JNK is also termed a stress-activated protein kinase. Both ERK and JNK activation lead to phosphorylation of downstream cytosolic and nuclear factors, such as transcription factors, c-Myc, p62TCF/Elk-1, c-Jun, and ATF2, consequently eliciting changes in gene expression [67]. Since MAPKs are activated by a wide variety of factors, the MAPK signalling cascade may mediate as a common integrative mechanism for other signalling pathways as well to control cellular activities in response to various extracellular stimuli.

6.3.3 Modulation of Cellular Signaling and Inflammation by Resveratrol

6.3.3.1 Anti-Inflammatory Role of Resveratrol

The anti-inflammatory role of polyphenols has been studied both *in vitro* and *in vivo*. Recently in studies involving rat lungs challenged with lipopolysaccharide (LPS), it has been shown that resveratrol could inhibit inflammatory cytokine expression *in vivo* [68]. Resveratrol was also shown to inhibit both NF-κB and activator protein 1 (AP-1) activation in monocytic U937 and alveolar epithelial A549 cells [69,70]. Although resveratrol had no effect on NF-κB–DNA binding, it did block the TNF-induced translocation of the p65 subunit of NF-κB and

reporter gene transcription. Similarly, the activation of JNK and its upstream kinase mitogen-activated protein (MEK) was inhibited by resveratrol, which may explain the probable mechanism by which resveratrol suppresses AP-1 activation. Previously, phorbol 12-myristate 13-acetate (PMA)-induced COX-2 was shown to be blocked by resveratrol [71]. This gene is known to be regulated by NF-κB activation, which in turn also regulates the expression of iNOS gene. Thus, it is possible that resveratrol suppresses both COX-2 and iNOS expression by a common mechanism involving modulation of NF-κB activation. Besides COX-2 and iNOS, various other genes integral to inflammation, such as matrix metalloproteinase-9 (MMP-9), cell surface adhesion molecules (e.g., intercellular adhesion molecule-1 [ICAM-1], endothelial leukocyte adhesion molecule 1 [ELAM-1], and vascular cell adhesion molecule-1 [VCAM-1]), are also regulated by NF-κB. Therefore, it may not be an exaggeration to consider that the anticarcinogenic properties assigned to resveratrol may be via suppression of NF-κB-mediated expression of such genes and their corresponding translational equivalents [72]. Since NF-κB activation also has an antiapoptotic role, the suppression of apoptosis by resveratrol may appear to be a paradox. It is to be noted, however, that NF-κB activation does not block apoptosis induced by all the agents.

Further evidence of anti-inflammatory functions of resveratrol has been obtained from studies wherein this polyphenol was found to reduce lung tissue neutrophilia by way of decreasing proinflammatory cytokines and prostanoid levels [68]. In yet another study, resveratrol was found to block TNF-α-induced activation of NF-κB in a dose- and time-dependent manner. Such an inhibition was found to be due to the suppression of TNF-α-induced phosphorylation and nuclear translocation of the p65 subunit of NF-κB, and NF-κB-dependent reporter gene transcription by resveratrol [69]. Furthermore, resveratrol was also reported to inhibit TNF-α-induced activation of mitogen-activated protein kinase kinase (MAPKK) and JNK, and diminished TNF-α-induced cytotoxicity, caspase activation, and free radical mediated injuries [69]. In a novel study using a *cis* isomer of resveratrol (c-resveratrol) in peritoneal macrophages stimulated with LPS and gamma interferon (IFN-γ), it was shown that c-resveratrol significantly attenuated the expression of the NF-κB family of genes, adhesion molecules and acute-phase proteins [73], inhibited transcription of Scya2, a chemokine:monocyte chemotactic peptide-1 (MCP-1) and RANTES (regulated on activation, normal T cell expressed and secreted), proinflammatory cytokines that attract monocyte-granulocyte cells, such as colony-stimulating factor 1 (M-CSF), colony-stimulating factor 2 (GM-CSF), and colony-stimulating factor 3 (G-CSF), the cytokine tumor growth factor beta (TGF-beta), and the extracellular ligand interleukin 1 (IL-1α). In contrast, c-resveratrol stimulated transcription of the proinflammatory cytokines interleukin 6 (IL-6) and TNF-α, the extracellular ligand IL-1β, and the IFN regulatory factor (IRF)-1. Therefore, it appears that c-resveratrol can significantly modulate a wide variety of proinflammatory pathways via inhibiting the activation of NF-κB alone [73]. A structural homologue of resveratrol, piceatannol, having anti-inflammatory, immunomodulatory, and anti-proliferative properties, has been

shown to modulate the cytokine signalling pathway and inhibit the TNF-induced-DNA binding activity of NF-κB [74]. In contrast, rhaponticin (another analog of piceatannol) had no effect, suggesting the critical role of hydroxyl groups.

6.3.3.2 Resveratrol and Cellular Signaling

Resveratrol has been known to modulate MAP kinase-dependent cellular signalling processes. The dose-dependent effects of resveratrol have been amply demonstrated by the observation that whereas a small amount of resveratrol could induce phosphorylation of ERK1/2 in undifferentiated cells [75], the same amount of resveratrol was found to induce an increase in ERK1/2 phosphorylation in retinoic acid treated cells. In contrast, the papillary and follicular thyroid carcinoma cell lines required higher doses of resveratrol to activate nuclear translocation of ERK1/2 [76], and at still higher concentrations (50–100 μM) resveratrol inhibited phosphorylation of MAPK. Resveratrol was found to increase phosphorylation of ERK1/2, JNKs, and p38MAPK in mouse epidermal cells, with concomitant increase in p53-serine-15 phosphorylation [77]. Resveratrol also caused a decrease in MAPK activity and reduced phosphorylation at the active sites of ERK1/2, JNK1, and p38 MAPK at still higher doses (37 mM) [78]. A related study showed that resveratrol activated JNKs at the same dose that inhibited tumor promoter-induced cell transformation [79]. A recent study employing 10 μM of resveratrol showed an enhanced phosphorylation of p38MAPK and ERK1/2 [59,80]. Therefore, resveratrol appears to activate MAPK in some cells, while it inhibits it in others, and such an effect appears to be dose dependent. In general, resveratrol is stimulatory at lower concentrations and inhibitory at higher concentrations. Mitogen- and stress-activated protein kinase-1 (MSK-1) is structurally related to the ribosomal p70 S6 subfamily and belongs to the AGC family of kinases. It is situated downstream to ERK1/2 and p38MAPK and is known to be activated by ERK1/2 and p38MAPK, and by p42/44 ERK and p38 MAPK in cultured cells [81]. During stress stimulation, MSK-1 is recruited for cAMP response element binding protein (CREB) and the closely related activating transcription factor (ATF1). Since resveratrol was found to induce CREB phosphorylation via adenosine A1 and A3 receptors through activation of the AKT survival pathway [82], it seems that resveratrol-mediated CREB activation may be routed through the phosphorylation of MSK-1. This notion is supported by another observation that inhibition of MSK-1 in turn inhibited phosphorylation of CREB.

6.3.4 Curcumin Modulation of Cellular Signaling and Inflammation

6.3.4.1 Anti-Inflammatory Effects of Curcumin

Curcumin is steadily gaining acceptance as an antioxidant and an anti-inflammatory agent. The hydroxyl and the methoxy groups of curcumin are believed to impart such characteristics to curcumin. The polyphenol has been shown to inhibit NF-κB

activation, with concomitant suppressions of IL-8 release, COX-2 expression, and neutrophil recruitment in the lungs [83]. Curcumin inhibits cigarette smoke-induced NF-κB activation by inhibiting IκBα kinase in human lung epithelial cells [84]. This observation is corroborated by an earlier study where curcumin was shown to inhibit NF-κB activation [85]. Curcumin also downregulates the expressions of several other NF-κB-regulated inflammatory and cell proliferating genes, such as leukotrienes, PLA_2, COX-2 and 5-LOX, cyclin D1 and c-myc, anti-apoptotic factors (e.g., IAP1, IAP2, XIAP, Bcl2, Bcl-xL, Bf1-1/A1, TRAF1, cFLIP), and metastatic factors such as vascular endothelial growth factor (VEGF), matrix metallo proteinases (MMP-9), and ICAM-1. In addition, curcumin has been reported to downregulate expression of iNOS, TNF-α, chemokines, and cell surface adhesion molecules; downregulate growth factor receptors, such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2); and inhibit the activity of JNK, protein tyrosine kinases, and protein serine/threonine kinases [83].

6.3.4.2 Modulation of Cellular Signaling by Curcumin

Suppression of NF-κB transactivation by curcumin is mediated via the inhibition of nuclear translocation of p65, which then sequentially suppresses IκB kinase activity and phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and p65 acetylation. Furthermore, curcumin was also shown to inhibit TNF-α-induced NF-κB-dependent reporter gene expression and also suppressed NF-κB reporter activity induced by TNFR1, TNFR2, NIK, I-kappa kinase (IKK), and the p65 subunit of NF-κB. Cigarette smoke contains many oxidants and carcinogenic agents, such as superoxide, hydroxyl radicals, H_2O_2 and benzo(a)pyrene; activates NF-κB; blocks apoptosis; and induces proliferation and carcinogenesis. Curcumin efficiently blocks the induction of NF-κB due to cigarette smoke; inhibits NF-κB binding to the DNA; blocks IKK activation, and IκBα phosphorylation and degradation; as well as NF-κB–p65 translocation [84]. NF-κB inhibition by curcumin is certainly an interesting strategy in chronic inflammatory diseases where NF-κB is not only activated but also triggers many inflammatory and apoptotic processes [87]. Curcumin also modulates a number of other signalling pathways, such as JNK, p38, AKT, JAK, ERK, and protein kinase C (PKC), in a wide variety of cell types [88]. Interestingly, curcumin and tumor necrosis factor related apoptosis-inducing ligand (TRAIL) have been reported to promote cell death in a cooperative manner [89]. Thus, curcumin appears to have a pleiotropic effect on cellular signalling processes, which in fact complicates the process of identifying which pathway is actually essential for the anti-inflammatory effects. On a different note, it may be envisaged that the ability of curcumin to prevent cross talk between the myriad of signaling pathways may be an essential prerequisite for its anti-inflammatory properties.

6.3.5 Catechins, Cellular Signaling, and Inflammation

6.3.5.1 Anti-Inflammatory Properties of Catechins

EGCG is the major catechin in green tea and predominates as a therapeutic agent. EGCG has been shown to induce apoptosis, cell-growth arrest, and deregulation of the cyclin kinase inhibitor p21WAF in studies involving many models of cancer [90]. In human epidermoid carcinoma (A431) cells, EGCG has been shown to inhibit cell cycle progression causing a G0/G1-phase arrest and a subsequent induction of apoptosis [91], probably through a mechanism mediated by NF-κB inhibition [92]. Employing a cDNA microarray analysis, it has been found that EGCG treatment of LNCaP cells resulted in induction of growth-inhibitory genes and subsequent repression of genes that belong to the G-protein signalling network [90]. In response to H_2O_2, EGCG could induce specific temporal changes in gene expression in treated cells [93]. These observations have led to a contention that EGCG may differentially modulate oxidative environments in tumor versus normal epithelial cells. However, the roles that EGCG, H_2O_2, and intracellular catalase play in the epithelial system are largely unknown [94]. Because EGCG can suppress malignant transformation in PMA-stimulated mouse epidermal JB6 cell line, this led to the speculation that EGCG may elicit such action via modulation of NF-κB/AP-1 activity. Indeed, such speculation gained credence from the observation that EGCG could inactivate both AP-1 [95] and/or NF-κB [96]. In yet another study, expression of genes related to angiogenesis, such as VEGF and those related to metastasis (e.g., MMP-2 and MMP-9), was also found to be inhibited by green tea or purified EGCG when administered via drinking water to mice [97]. Catechins, especially EGCG, has also been shown to downregulate CD11b expression on CD8+ T cells and thereby also inhibits infiltration of these cells into the sites of inflammation [98]. Green tea polyphenols thus appear to modulate a myriad of inflammatory pathways and may be employed as novel anti-inflammatory therapeutic alternatives.

6.3.5.2 Catechins and Cellular Signaling

IL-8 is a major human neutrophil chemoattractant and inflammatory mediator. IL-8 expression is dependent on NF-κB activation by IL-1β. The role of EGCG as a cell signal modulator became evident from the observation that EGCG could markedly inhibit IL-1β-mediated IL-1β receptor-associated kinase (IRAK) degradation. In addition, EGCG could also inhibit signalling events downstream from IRAK, such as IKK activation, IκBα degradation, and NF-κB activation [99]. The functional consequence of this inhibition was reflected by inhibition of IL-8 gene expression. Such signal modulation by EGCG was found to be due to proximal mechanisms involving inhibition of IRAK-dependent signalling and phosphorylation of p65. In HepG2 cells, green tea polyphenols were also reported to stimulate MAPK pathways [100]. Further evidence of cell signal modulating ability of green tea polyphenols was obtained from the observations that green tea extract could

increase mRNA levels of immediate-early genes such as c-jun and c-fos. However, it appears that not all green tea polyphenols may have similar activities and their effects appeared to be structurally related to the 3-gallate group [101]. The degree of activation of MAPK by the five tea polyphenols was related to the structure, dose, and time. Of the five predominant polyphenols, only EGCG showed potent activation of all three MAPKs (ERK, JNK, and p38) in a dose- and time-dependent manner, and EGC activated ERK and p38 only. The oligodynamic nature of catechins was evident from the observation that lower concentrations of EGCG activated MAPK, whereas higher concentrations of EGCG sustained activation of MAPK/JNK leading to apoptosis [101].

6.4 POLYPHENOLS AND CELLULAR REDOX SYSTEM

The candidature of antioxidant polyphenolic compounds as an important therapeutic strategy against various chronic inflammatory diseases gains support from the fact that a variety of such diseases encounter increased generation of oxidants, free radicals, and cell damaging aldehydes. Alternatively, polyphenols may be used as antioxidant reservoirs to increase the endogenous antioxidant defense potential and thus modulate the redox status in a cell. Therefore, it would be interesting to consider how polyphenols may modulate the redox system and its components in a cell during normal and pathological conditions. In the following section we will discuss the effects of polyphenols on nuclear factor-erythroid 2 p45 related factor 2 (Nrf2), as this important cellular redox-dependent transcription factor not only acts as a sensor of oxidative stress in a cell but also regulates the expression of several genes involved in the modulation of inflammatory processes in response to oxidative stress.

6.4.1 Nrf2

Nrf2 is expressed in a wide range of tissues, many of which are sites of expression for phase 2 detoxification genes. Nrf2, a member of the cap 'n' collar family of transcription factors, binds to the nuclear factor-erythroid derived 2 (NF-E2) binding sites (GCTGAGTCA) and thus regulates the expression of erythroid specific genes. The NF-E2 consists of a subset of antioxidant response elements (ARE) having the sequence GCNNNGTCA. AREs are regulatory sequences embedded in the promoters of several phase 2 detoxification genes that are inducible by a host of xenobiotics and antioxidants. AREs render cytoprotection against chemically induced oxidative/electrophilic stress by mediating and coordinating expression and induction of many antioxidant enzymes, which is a critical mechanism of protection against free radicals, aldehydes and electrophiles [102].

Curcumin, caffeic acid phenethyl ester (CAPE), 4′-bromoflavone, and other polyphenols are potential chemopreventive agents and are also known to be selective activators of Nrf2-Keap1-ARE [103]. Mechanistically, interaction of Nrf2 with Keap1 enables Nrf2 to translocate to the nucleus, bind to the ARE, and initiate the transcription of detoxifying and cytoprotective genes. Such cellular responses

are also triggered by other electrophilic compounds including polyphenols and plant-derived constituents. Curcumin and CAPE have been identified to induce a redox sensitive protein heme oxygenase-1 (HO-1) that provides protection against various forms of stress [104]. Both curcumin and CAPE stimulate the expression of Nrf2 in a concentration- and time-dependent manner. Evidence is available about curcumin (and, by inference, CAPE) being able to stimulate HO-1 gene expression by promoting dissociation of the Nrf2-Keap1 complex. This leads to an increased binding of Nrf2 to the resident HO-1 AREs. Recently, the role of Nrf2 in the transcriptional regulation of rat glutamate-cysteine ligase catalytic subunit (GCLC) has also been investigated [105]. GCLC codes for the rate-limiting glutamate cysteine synthase, an enzyme involved in GSH synthesis. Furthermore, Nrf2 regulation of rat GCLC promoter was found to be under the influence of a key AP-1 family of proteins. Interestingly, earlier work from the authors' laboratory has revealed that curcumin could increase GSH synthesis in A549 cells via increasing the expression of GCL gene. Since curcumin is also known to stimulate Nrf2 expression [104,105], it appears that the antioxidant function of curcumin may be mediated via Nrf2-ARE-GCLC axis. It could therefore be surmised that polyphenols exhibit a positive relationship with Nrf2 and ARE, and may therefore increase the levels of GSH (a major cellular thiol antioxidant) by modulating the activity status of Nrf2. EGCG also induced transcriptional activation of phase II detoxifying enzymes through ARE/EpRE via activation of all three MAPK pathways (ERK, JNK, and p38) [100]. Resveratrol has also been shown to stimulate Nrf2 in PC12 cells by MAP kinase signal transduction pathways [103,106]. It is evident from this discussion that polyphenols, in addition to their antioxidant function, also can modulate a wide range of signalling processes in a host of different types of cells. Since the signaling effects of polyphenols vary with the dose administered, time, and cell type, it becomes more interesting to consider the therapeutic potential of each type of a polyphenol for a specific oxidative stress situation.

6.5 POLYPHENOLS AND GLUCOCORTICOID SIGNALING

Corticosteroids have been the most effective therapeutic strategies for the control of asthma and other chronic inflammatory and immune diseases. However, a small proportion of asthmatics still fail to respond to higher doses of oral corticosteroids. Other inflammatory and immune diseases, including rheumatoid arthritis and inflammatory bowel disease, have also been reported to be resistant to corticosteroid treatments. Such patients exhibiting corticosteroid resistance, although uncommon, present considerable problems in their management. Patients with chronic obstructive pulmonary disease (COPD) show a poor clinical response to corticosteroids and have a largely steroid-resistant pattern of inflammation [107]. Recent studies involving many chronic and inflammatory diseases have provided new insights into the mechanisms, whereby corticosteroids suppress chronic inflammation [108].

A spate of evidence is available as to the possible involvement of oxidative stress in corticosteroid resistance observed in COPD and asthma (Figure 6.3).

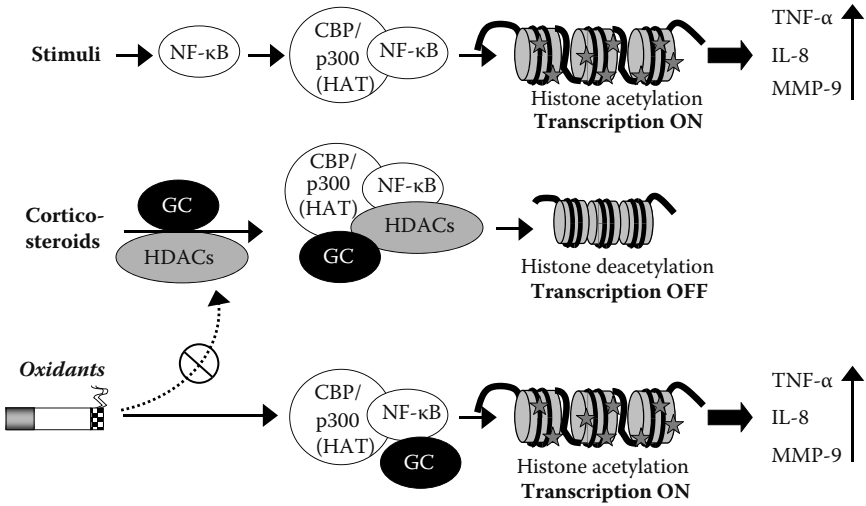

FIGURE 6.3 Impact of oxidative stress on the regulation of chromatin structure and proinflammatory gene expression. Proinflammatory cytokines activate transcription factors, such as NF-κB, recruiting transcriptional coactivator molecules CBP/p300 containing intrinsic HAT activity resulting in histone acetylation and DNA unwinding, allowing DNA polymerases access to the DNA and proinflammatory gene expression. Activated corticosteroid receptors recruit HDAC into the transcriptome complex promoting histone deacetylation, chromatin condensation, and expulsion of DNA polymerases, shutting off gene expression. Oxidative stress inhibits HDAC activity as well as activating NF-κB, facilitating histone acetylation by the transcriptome complex even in the presence of activated glucocorticoid receptor.

Recent studies in alveolar macrophages derived from smokers have revealed that histone acetylation and deacetylation play an important role in translating the beneficial effects of corticosteroids [109]. Interestingly, oxidants have been found to modulate the activity of histone deacetylase (HDAC) and inflammatory cytokine gene transcription. Furthermore, work from our laboratory has shown that both cigarette smoke/H_2O_2 and TNF-α caused an increase in histone acetylation (HAT activity) leading to IL-8 expression in monocytes and alveolar epithelial cells both *in vitro* and *in vivo* in rat lungs [110]. Suppression of inflammatory genes by glucocorticoid requires recruitment of histone deacetylase-2 (HDAC2) to the transcription activation complex, which is mediated by the glucocorticoid receptor [111]. The resultant deacetylation of histones winds back the DNA leading to diminished inflammatory gene transcription. Surprisingly, HDAC2 levels were found to be diminished in the alveolar macrophages of the smokers, which explained the resistance to glucocorticoid therapy in such subjects [110,111]. Since smokers are exposed to elevated levels of oxidative stress, it may be said that oxidative stress is associated with resistance to corticosteroid therapy in some patients. This contention was lent credence by the observations of Culpitt and

coworkers who have shown increased release of IL-8 and GM-CSF in macrophages from COPD subjects when treated with cigarette smoke solution, a pathology that was not inhibited by dexamethasone [112]. They attributed this lack of efficacy of corticosteroids in COPD to steroid insensitivity of macrophages in the respiratory tract. Thus, cigarette smoke/oxidant-mediated decrements in HDAC2 levels recorded in alveolar epithelial cells and macrophages will not only augment expression of the inflammatory gene but will also cause a decrease in glucocorticoid efficiency in patients with COPD. HDAC activity has also been measured in bronchial biopsies and alveolar macrophages from COPD patients and smoking controls, demonstrating a significant decrease in HDAC activity, the magnitude of which increased with severity of the disease [113]. Moreover, HDAC2 protein expression levels were also decreased in COPD patients. Hence, one of the potential strategies to treat COPD subjects would be to increase HDAC2 expression and activity so that steroids could regain their anti-inflammatory activity. In line with this thought, theophylline, a green tea component, has been shown to increase HDAC2 expression in macrophages and therefore resensitize the cells to the action of the steroids [114]. Methods and mechanisms to upregulate HDAC2 activity would be of great clinical interest for potential combination therapies of the future.

Previously, dietary polyphenols, such as curcumin and resveratrol, bronchodilator theophylline, and glucocorticoids, had been shown to control inflammation by modulating either NF-κB activation or chromatin remodeling. Such anti-inflammatory effects of polyphenols have now been attributed to their ability to enhance HDAC activity in lung epithelial cells. Recent reports from the authors' laboratory have shown that restoration of glucocorticoid functions against oxidative stress introduced by cigarette smoke could be achieved via polyphenol upregulation of HDAC activity in monocyte/macrophage (U937) and MonoMac6 cell lines [115,116]. These outcomes were associated with restoration of HDAC1, HDAC2, and HDAC3 levels. The authors further suggested that the outcome was due to a possible formation of HDAC-p65 complex with glucocorticoid receptor, which renders NF-κB ineffective. The authors also made a similar observation for curcumin, wherein the flavonoid was shown to inhibit inflammation and restore glucocorticoid efficacy in response to oxidative stress, through upregulation/restoration of HDAC-2 activity and expression in U937 and MonoMac6 cells. These observations have been corroborated by other independent reports that the anti-inflammatory actions of curcumin are mediated via inhibition of HAT activity, thus preventing NF-κB-mediated chromatin remodeling [117]. The stimulatory impact of curcumin on HDAC activity and steroid function in pre-ROS stressed cells was not believed to be due to the antioxidant properties of curcumin, as these occurred at concentrations >10 μM, which were at least 1000-fold higher than the observed effects on HDAC activity. It is possible that curcumin renders protection to posttranslationally modified HDAC2 via induction of tyrosine denitrase, carbonyl reductase, or aldo-keto reductase activity, enzymes that can uphold the normal functions of HDAC. This speculation needs investigation for further validation. Overall, it appears that in addition to their antioxidant and anti-inflammatory

properties, dietary polyphenols and flavonols may possibly act on chromatin remodeling, which in turn helps in reducing resistance to glucocorticosteroid in COPD.

The beneficial anti-inflammatory effects of polyphenols have been studied in humans in large-scale clinical trials. In one such Finnish study involving over 10,000 participants, a significant inverse correlation was observed between polyphenol intake and the incidence of asthma [118]. Similar beneficial outcomes were recorded in COPD subjects in a study encompassing over 13,000 adults [119]. In this study Tabak et al. reported that increased polyphenol intake correlated with improved symptoms, as assessed by cough, phlegm production, and breathlessness, and improved lung function as measured by forced expiratory volume in one second (FEV_1) [119]. Two more studies supported these findings. The first of them showed a beneficial protective effect against COPD symptoms after an increased intake of fruits high in polyphenol and vitamin E contents [120]. In the second more recent study, a standardized polyphenol extract administered orally was reported to be effective in reducing oxidant stress and increasing PaO_2, as well as improve FEV_1 between enrolment and the end of the study [121]. It is noteworthy that while a single component intake such as catechin was independently associated with FEV_1 and all three COPD symptoms, flavonol and flavone intake were independently associated with chronic cough only. The study thus probably throws a caution as to the choice of a polyphenol for a particular condition. Walda and colleagues further substantiated the results of the trials by demonstrating the protective effect of fruit containing polyphenols and vitamin E intake against COPD symptoms in a 20-year COPD mortality study from three European countries consisting of Finnish, Italian, and Dutch cohorts [120]. These important clinical studies involving human subjects certainly encourage further multinational clinical studies to further substantiate the beneficial effects of a high intake of polyphenols/bioflavanoids against COPD symptoms and other inflammatory diseases.

6.6 ADVERSE EFFECTS OF POLYPHENOLS

Adverse effects of polyphenols have still not been well documented and this may be due to the relatively low bioavailability, rapid metabolism, and elimination of most flavonoids and polyphenols. Also since no long-term studies with polyphenols and flavonoids have been undertaken in humans, it would be premature to consider polyphenols and flavonoids devoid of any adverse effects. Despite several beneficial effects attributed to polyphenols, sporadic reports of ill effects of polyphenols and flavonoids are available. For example, headache, nausea, and sensations in the extremities have been reported in people consuming up to 1000 mg/day of quercetin a month [122]. The same was observed in cancer patients in a phase I clinical trial [123]. In another trial on cancer patients, caffeinated green tea extracts (6 g/day in 3–6 divided doses) caused mild to moderate gastrointestinal discomfort, including nausea, vomiting, abdominal pain, and diarrhea [124,125]. However, the side effects were associated with the caffeine in the green

tea extract [126], which was much alleviated in a trial using decaffeinated green tea extracts (800 mg/day of EGCG) [127]. As evident by inhibition of cytochrome P450 CYP3A4, an important intestinal drug detoxifying system by grapefruit juice, polyphenols and flavonoids have been reported to interfere with the drug metabolism ability in humans [128]. Furanocoumarins, particularly dihydroxybergamottin, active components of grape juice and certain flavonoids naringenin and quercetin have been found to inhibit CYP3A4 *in vitro*. Such an inhibition of CYP3A4 can in turn increase the bioavailability and toxicity of a number of drugs, such as HIV protease inhibitors, immunosuppressants, HMG-CoA reductase inhibitors, calcium channel antagonists, antiarrhythmic agents, antihistamines, anticonvulsants, and anxiolytics [129]. Therefore, subjects using any of these therapies are advised to practice caution when consuming polyphenolic diets during the course of treatment [128].

An important mechanism by which toxic effects of drugs are reduced is by their efflux and decreased absorption mediated by P-glycoproteins in the intestinal tract. Quercetin, naringenin, and the green tea flavanol (EGCG) have been reported to inhibit the efflux activity of P-glycoprotein in cultured cells [130]. Several drugs, such as digoxin, antihypertensive, antiarrhythmic, chemotherapeutic, antifungal agents, HIV protease inhibitors, immunosuppressive agents, H2 receptor antagonists, and some antibiotics, are known to be substrates of P-glycoproteins [131]. Hence, high intake of such flavonoids/polyphenols may adversely increase the bioavailability and the risk of toxicity of such drugs. Certain flavanols present in purple grapes and dark chocolates are reported to inhibit platelet aggregation *in vitro* [132], thereby increasing the risk of bleeding when taken with anticoagulant, antiplatelet, and nonsteroidal anti-inflamatory drugs (NSAIDs). Flavonoids can also bind nonheme iron and inhibit intestinal absorption of iron from food [133], along with inhibiting transport of vitamin C into cells [134]. In view of these undesired effects of polyphenols, more in-depth and systematic investigations are required to determine the significance of the findings in humans.

6.7 SYNTHETIC ANALOGUES OF POLYPHENOLS

Clinical trial of a novel putative therapeutic agent needs to be based on robust preclinical information. Such information should relate to the mechanism of action, pharmacodynamics, and pharmacokinetics of the agent under study. Considering the complexities of polyphenol absorption and metabolic transformations and still less understood aspect of polyphenol bioavailability, it becomes more important to address the following questions: (1) What doses of polyphenols should be taken, since, for example, biological effects for resveratrol have mostly been reported at >10 mM? (2) What postabsorption metabolic modifications would render the polyphenols bioactive? (3) Since most reports on the beneficial effects of polyphenols are based on *in vitro* studies, will identical doses yield similar effects in whole animal? It is perhaps surprising that, to our knowledge, there has been hardly any attempt to probe the link between target organ levels of

the polyphenols, efficacy *in vivo,* and activity observed in cells *in vitro*. Moreover, poor absorption characteristics, robust metabolic transformations, less bioavailability, and possible adverse effects of polyphenols have recently led workers to design new varieties of polyphenolic compounds, which can overcome these problems and exhibit better clinical efficacy at lower doses.

Most preclinical and clinical studies on polyphenols were conducted with polyphenols extracted or purified from green tea leaves. Synthesis of EGCG was long awaited so that mechanism of EGCG and its activities could be elucidated by structural manipulations. Only recently have synthetic forms of green tea polyphenols including (−)-EGCG and its synthetic analogs thereof (including (+)-EGCG) have been created by one of the group of workers [135]. These synthetic analogues of EGCG were designed to exhibit proteasome-inhibitory activity *in vitro* and *in vivo*. In addition, the synthetic analogues were also reported to inhibit cell growth, induce apoptosis, and hinder transformation of tumor cells. Enantiomeric analogs of natural green tea polyphenols (+)-EGCG and (+)-GCG (gallocatechin-3-gallate) potently and specifically inhibited the chymotrypsin-like activity of purified 20S proteasome and the 26S proteasome in tumor cell lysates, while benzyl protected forms of the analogue; Bn-(+)-EGCG did not [136]. Treatment of leukemic Jurkat T or prostate cancer LNCaP cells with either (+)-EGCG or (+)-GCG accumulated p27 and IκBα proteins, associated with an increased G1 population. (+)-EGCG treatment also accumulated the proapoptotic Bax protein and induced apoptosis in LNCaP cells expressing high basal levels of Bax, but not prostate cancer DU-145 cells with low Bax expression. These synthetic analogues also significantly inhibited colony formation by LNCaP cancer cells.

Curcumin and rosmarinic acid as efficient *in vitro* amyloid inhibitor polyphenols were suggested primarily by Yamada and coworkers [137]. In this study, the similarity of curcumin to Congo red structure is mentioned as a possible factor for its inhibition characteristics [138]. Furthermore, the structural similarity of curcumin and chrysamine G, a brain permeable compound, is mentioned as a possible factor for blood–brain barrier permeability. It is also shown that curcumin has structural similarity to a β-sheet breaker, N,N′-bis(3-hydroxyphenyl)pyridazine-3,6-diamine (named RS-0406), which was found using high throughput screening of approximately 113,000 compounds. Compound RS-0406 was described to significantly inhibit 25 lM b-amyloid1 fibrillogenesis [139].

Studies with resveratrol and its analogues have recently reported their potent chemopreventive effect in multiple carcinogenesis models both *in vivo* and *in vitro* [140]. Resveratrol, and to a lesser extent its analogue piceatannol, potently upregulated spermindine/spermine N1-acetyltransferase (SSAT) activity in a study on polyamine metabolism during colorectal cancer, indicating that these hydroxystilbenes induce polyamine degradation. After 24 h of treatment with resveratrol, an increased intracellular putrescine and N1-acetylspermidine concentrations was observed, whereas the levels of spermine and spermidine did not change significantly [141]. The resveratrol analogue *cis*-3,5,4-trimethoxystilbene decreased ornithine decarboxylase (ODC) and *S*-adenosyl-L-methionine decarboxylase (SAMDC) activities at a concentration of 0.3 mM with a concomitant reduction

of putrescine concentrations after 24 h [142]. As is evident from the preceding section, more research into the synthesis of novel polyphenol analogues would circumvent the problem of postabsorption transformation and decreased bioavailability of these compounds. The great structural flexibility of phenols also allows them to be derivatized into a structure that may specifically interact with a given target molecule and at the same time be required in lower doses.

6.8 CHALLENGES FOR RESEARCH ON POLYPHENOLS

A wide range of polyphenols with antioxidant activity are potential contributors to the antioxidant mechanisms in humans and animals. Although these compounds have been shown to impart beneficial health effects, there is still not enough information on food composition data, bioavailability, interaction with other food components, and their biological effects. Especially, evidence is available as to the transformation of polyphenols by the intestinal microflora and that the transformed metabolites are also absorbed. The known metal chelating capacity of polyphenols raises further questions as to their participation in aspects related to metal metabolism and pathology [143].

Cross-reactivity of polyphenols with other biological antioxidants is another area that needs to be addressed on a priority basis. For example, the demonstration of cross-reaction between ascorbate and catechin [144] has led to the hypothesis that polyphenol antioxidants are part of the antioxidant network of the organism. Although attempts have been made to estimate the relative contribution of polyphenols to the total antioxidant capacity in plasma, insufficient knowledge on the nature and concentration of circulating polyphenol species render such results only speculative. Another rapidly developing aspect of free radical metabolism is whether polyphenols mediate and regulate cellular functions independent of their antioxidant properties or work in tandem with naturally occurring antioxidants of the cells. Satisfactory answers to these questions would not only provide better understanding of how polyphenols exert their biological effects but would also enable the design of more effective and specific therapeutic strategies for the control of various inflammatory diseases.

6.9 CONCLUSION

Polyphenols and flavonoids are important metabolic modulators and influence several cellular processes such as signaling, proliferation, apoptosis, redox balance, and differentiation (Figure 6.4). It is important to note that most beneficial effects have been obtained from *in vitro* studies. Although abundant in most dietary sources, such as fruits, vegetables, tea, and wine, true absorption and bioavailability of polyphenols is yet to be properly determined. The fact that polyphenols undergo a considerable degree of chemical modifications during digestion and absorption and that the modified forms may have altered biological properties and potencies, caution should be practiced before any pharmacological applications can be claimed. Polyphenols have also been shown to have adverse effects.

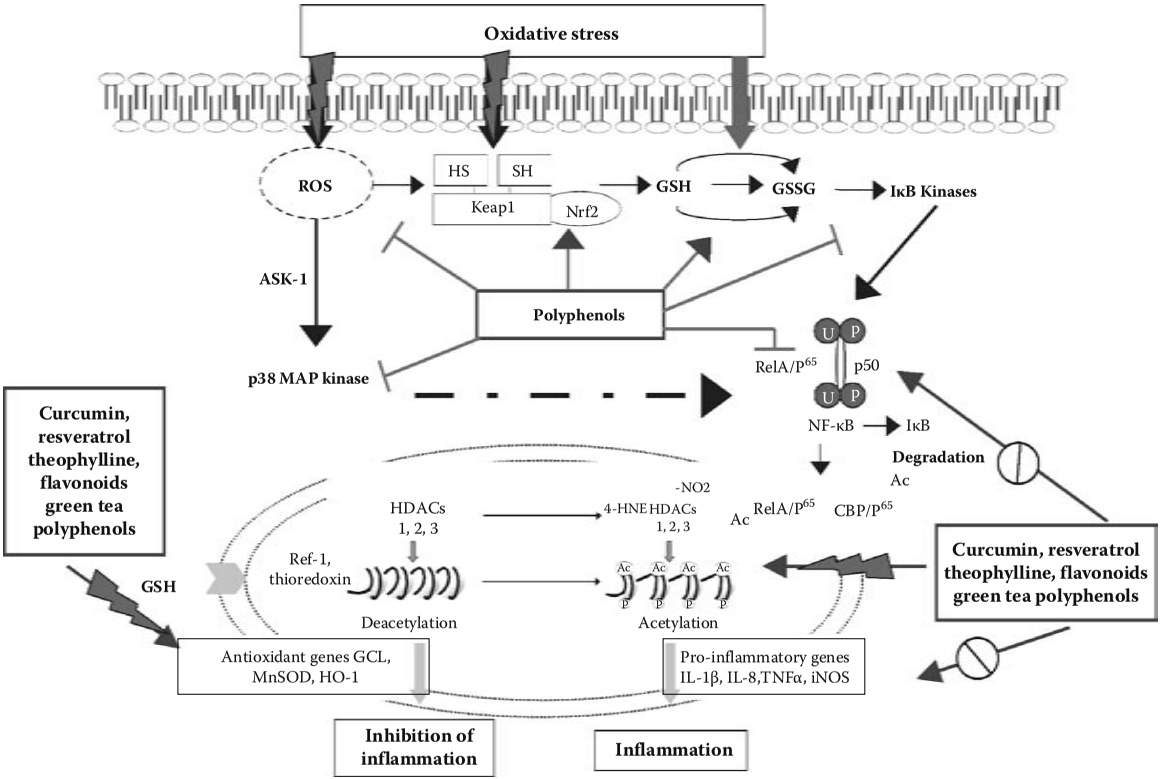

FIGURE 6.4 A schematic model for polyphenols and flavonoids mediated modulation of cell signaling. Oxidative stress induced inflammation is mediated by NF-κB activation and MAP kinases, and affect a wide variety of cellular signaling processes leading to generation of inflammatory mediators and chromatin remodeling. The latter allows expression of proinflammatory genes such as IL-1β, IL-8, TNF-α, and iNOS. On the other hand, to counter the effects of oxidative stress, the cells also concomitantly express protective antioxidant genes such as GCL, MnSOD, and HO-1. Polyphenols and flavonoids inhibit the transactivation of proinflammatory genes via inhibition of IκB, thus inhibiting NF-κB transactivation and activating histone deacetylases on one hand and on the other induce the expression of antioxidant genes such as GCL, MnSOD, and HO-1 via modulation of the MAPK-Nrf2-ARE pathway.

The anti-inflammatory, antioxidant abilities and the capacity to modulate important inflammatory and anti-inflammatory signaling pathways and influence glucocorticoid efficacy hold great promise for the potential of polyphenols and flavonoids as therapeutic candidates for controlling lung inflammation and other chronic inflammatory diseases. Polyphenols and flavonoids may thus be perceived as future pharmacological alternatives and may be used as enforcements to combat oxidative challenges. Indeed, elucidating the mechanism of action for some of the naturally occurring antioxidants, such as the potent enzyme mimetics and polyphenols, may lead to new therapeutic targets that can be modulated through more conventional pharmacological approaches.

ABBREVIATIONS

AP-1	activator protein-1
ARE	antioxidant response elements
ATF1	activating transcription factor
CAPE	caffeic acid phenethyl ester
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CRE	cyclic AMP response element
CREB	CRE-binding protein
EC	(2)-epicatechin
EGC	epicatechin gallate
EGCG	epigallocatechin gallate
EGFR	epidermal growth factor receptor
ELAM-1	endothelial leukocyte adhesion molecule-1
EpRE	electrophilic response element
ERK	extracellular signal-regulated kinase
FEV_1	forced expiratory volume in one second
GCLC	glutamate-cysteinke ligase catalytic subunit
G-CSF	colony-stimulating factor 3
GM-CSF	colony-stimulating factor 2
GSH	reduced glutathione
H_2O_2	hydrogen peroxide
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDAC2	histone deacetylase-2
HER2	human epidermal growth factor receptor 2
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
HO-1	heme oxygenase-1
ICAM-1	intercellular adhesion molecule-1
IFN-γ	gamma interferon
IKK	I-kappa kinase
IL-1α	interleukin 1α
IL-1β	interleukin 1β

IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IRAK	IL-1β receptor-associated kinase
IRF-1	IFN regulatory factor
IκBα	inhibitory kappa B-alpha
JNK	c-Jun N-terminal kinases
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MCP-1	monocyte chemotactic peptide-1
M-CSF	colony-stimulating factor 1
MEK	mitogen-activated kinase
MHC	major histocompatibility complex
MMP-9	matrix metallo proteinases
MSK-1	mitogen- and stress-activated protein kinase-1
NADH	nicotinamide adenine dinucleotide reduce form
NF-E2/Nrf2	nuclear factor erythroid derived 2
NF-κB	nuclear factor-kappa B
NIK	NF-κB inducing kinase
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drugs
O_2^-	superoxide anion
ODC	ornithine decarboxylase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PUFA	polyunsaturated fatty acids
RANTES	regulated on activation, normal T cell expressed and secreted
ROS	reactive oxygen species
SAMDC	*S*-adenosyl-L-methionine decarboxylase
SOD	superoxide dismutase
SOS	nucleotide exchange factor
SSAT	spermindine/spermine N1-acetyltransferase
TGF-beta	tumor growth factor beta
TNF	tumor necrotic factor
TRAIL	tumor necrosis factor related apoptosis-inducing ligand
VCAM-1	vascular cell adhesion molecule-1

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7 Dietary Factors in Food: Induction of Nrf2-Mediated Defense Genes in Normal Cells versus Inhibition of Cell Growth Genes in Tumor Cells

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CONTENTS

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7.1 INTRODUCTION

Evolutionarily, animals have been ingesting plants. This "animal–plant" warfare has resulted in an elaborated system of detoxification and defense mechanisms evolved by animals, including humans. Animal cells respond to these dietary phytochemicals by "sensing" this chemical stress typified by "thiol modulated" cellular signaling events leading to gene expression of pharmacologically beneficial effects but sometimes also unwanted cytotoxicity. Our laboratory has been studying two groups of dietary cancer chemopreventive compounds, isothiocyanates and polyphenols, which are effective against chemical-induced, as well as genetically modified, animal carcinogenesis models [1,2]. These compounds typically generate "cellular stress" and modulate gene expression, including phase II detoxifying/antioxidant enzymes. Indeed, reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by electrophiles or xenobiotics including these dietary phytochemicals have been proposed as second messengers in the activation of several signaling pathways leading to gene expression responses that are necessary for cell survival and cell death.

The basic principle of cancer chemoprevention strategy is to inhibit, reverse, or retard the process of multistage carcinogenesis by using defined nontoxic chemical substances [3,4]. However, current chemopreventive regimens have been questionable for their undesirable side effects after long-term administration. Therefore, cancer chemoprevention studies in human populations using natural chemopreventive agents from food, which are fairly safe, have become an area of great interest. Based on the three different stages of cancer development—initiation, promotion, and progression—chemopreventive agents can be categorized broadly either as cancer-blocking agents (which impede the initiation stage) or cancer-suppressing agents (which arrest or reverse the promotion and progression of cancer) [5]. Interestingly, some potential chemopreventive agents can target or act on all stages of cancer development. Using numerous cancer cell lines and animal carcinogenesis models, several important cellular mechanisms/events contributing to the overall cancer preventive effects of these dietary phytochemicals have been discovered, such as increasing expression of phase II detoxifying/antioxidant enzymes, disrupting the cell cycle progression/cell proliferation, inducing cell differentiation/apoptosis, inhibiting expression and functional activation of oncogenes, increasing expression of tumor-suppressor genes, and inhibiting angiogenesis/metastasis [3,5].

It has been more widely accepted that the cellular signaling network often goes awry in various disease processes, including cancer. Several intracellular-signaling cascades are disrupted or deregulated during carcinogenesis, particularly malignant transformation of cells and cancer metastasis [3], therefore identification of dietary chemopreventive agents that upregulate expression of gene-mediated cellular protective effects against oxidative stress or carcinogenic damages of normal cells, or simultaneously modulate differentially overexpressed growth signaling molecules/pathways culminating the apoptotic or autophagic cell death of tumor cells has become extremely important for achieving chemoprevention. In addition, research directed toward elucidating the differential signaling/gene expression between "normal" versus "abnormal" cells elicited by these dietary compounds may yield important insights into the varied biological responses and pharmacological effects, and the toxicodynamic effects of these natural chemopreventive agents.

In this review, we will focus on the molecular basis of two potential groups of dietary cancer chemopreventive compounds: isothiocyanates and polyphenols. We will briefly review three important redox-sensitive transcription factors—activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB), and nuclear factor-E2-related factor 2 (Nrf2)—and their redox regulators that are responsible for the cancer chemopreventive effects of these dietary compounds. We will also discuss the Nrf2 paradigm in differential gene expression elicited by these dietary compounds, with special emphasis on the gene categories involving cellular protective mechanisms and cell death mechanisms.

7.2 ISOTHIOCYANATES AND POLYPHENOLS: PROMISING DIETARY CANCER CHEMOPREVENTIVE COMPOUNDS

Two major groups of dietary chemopreventive compounds that have been well recognized as detoxifying enzyme inducers are isothiocyanates and polyphenols. Dietary isothiocyanates, the naturally occurring sulfur-containing compounds, are derived *in vivo* from the hydrolysis of glucosinolates present in cruciferous vegetables. Sulforaphane (SFN) from broccoli; phenethyl isothiocyanate (PEITC) from turnips and watercress; allyl isothiocyanate from brusssels sprouts; and benzyl isothiocyanate (BITC) from garden cress, papaya, and Brassica vegetables are the most extensively investigated isothiocyanates. Polyphenols, the complex substances with two or more phenolic rings joined together, can be divided into more than 10 subtypes based on their chemical structure [6]. Examples of powerful chemopreventive polyphenols, which are abundantly found in food, include caffeic acid phenethyl ester (CAPE) from honeybee propolis, curcumin from turmeric, epigallocatechin-3-gallate (EGCG) from green tea, genistein from soy, resveratrol from grapes, and quercetin from citrus fruits. Our laboratory has worked extensively toward understanding the molecular mechanisms by using *in vitro* cell lines and determining the chemopreventive efficacy by using *in vivo* animal carcinogenesis models of polyphenols (e.g., curcumin and EGCG) and isothiocyanates (e.g., PEITC and SFN). Interestingly, certain combination regimens

of these phytochemicals can elicit better chemopreventive effects, that is, maximum efficacy with minimum toxicity [7].

7.2.1 Isothiocyanates

Several studies conducted over the last two decades have provided convincing data suggesting that isothiocyanates can lower the incidence of many cancers, including lung, pancreas, bladder, prostate, ovarian, skin, stomach, and colon [8–11]. Isothiocyanates have been shown to exert cancer chemopreventive effects by inducing antioxidant response element (ARE)-driven phase II detoxifying/antioxidant enzyme expression through activation of transcription factor Nrf2 leading to enhanced cellular detoxification of carcinogens as well as blocking carcinogen activation. Therefore, isothiocyantes, as cancer-blocking agents, can impede the initiation stage of cancer development. We have shown that inhibition of p38 mitogen-activated protein kinase (MAPK) by SFN can cause induction of ARE-mediated heme oxygenase-1 (HO-1) gene expression in human hepatoma HepG2 cells [12]. Besides, induction of ARE-mediated HO-1 gene expression in human prostate cancer PC-3 cells by PEITC requires activation of extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways [13]. This observation suggests that the prerequisite of MAPK modulation by isothiocyanates is in part involved in the expression of ARE-mediated phase II detoxifying/antioxidant enzymes through the Nrf2 pathway. Other representative examples of phase II detoxifying/antioxidant enzymes induced by isothiocyanates include glutathione *S*-transferase (GST), NAD(P)H-quinone oxido reductase-1 (NQO-1), and UDP-glucuronosyltransferase (UGT). Recently, "anti-inflammatory" effects of isothiocyanates have also been addressed. SFN and PEITC can inhibit lipopolysaccharide (LPS)-induced NF-κB activation in human colon cancer HT-29 cells stably transfected with an NF-κB luciferase reporter construct [14].

It is well accepted that ROS or oxidative stress can stimulate cell proliferation and induce genetic instability, and their increase in cancer cells is often viewed as an adverse event. Trachootham et al. [15] showed that oncogenic transformation of ovarian epithelial cells with H-Ras(V12) or expression of Bcr-Abl in hematopoietic cells elevated ROS generation and rendered the malignant cells highly sensitive to PEITC. Excessive ROS causes oxidative mitochondrial damage, inactivation of redox-sensitive molecules, and massive cell death. *In vivo*, PEITC exhibits therapeutic activity by prolonging animal survival. Isothiocyanates also can act as cancer-suppressing agents because they have other preferable pharmacological effects such as modulation of cell cycle arrest [16] and induction of apoptotic cell death either via the caspase pathway [17] or via the p53-dependent pathway [18]. We have reported that PEITC can induce apoptosis in HT-29 cells via the mitochondrial caspase cascade and JNK activation appears to be a prerequisite for the initiation of the apoptotic processes [19]. *In vivo* cancer chemopreventive activities of isothiocyanates have been shown in several chemical-induced as well as genetically modified animal carcinogenesis models. SFN inhibited adenoma formation in the gastrointestinal tract of genetically mutant ApcMin/+ mice [2,20].

In addition, PEITC also exhibited significant cancer-preventive activities in NCr immunodeficient (nu/nu) mice bearing xenografts of PC-3 cells [7].

7.2.2 Polyphenols (Curcumin and EGCG)

Polyphenols have been well known to possess antioxidant, anti-inflammatory, and chemopreventive properties by modulating various events in cellular signaling. The key molecular mechanisms underlying anti-inflammatory activity of polyphenols are inhibition of cytokine release and downregulation of intracellular signal transduction molecules and transcription factors that regulate expression of proinflammatory genes. We have found that curcumin and EGCG can inhibit LPS-induced NF-κB activation in HT-29 cells stably transfected with NF-κB luciferace reporter gene [14]. The antitumor promoting effects of curcumin and EGCG are also attributable to their inhibition of cellular signaling mediated by NF-κB and the upstream kinases. We have shown that EGCG causes mitochondrial damage and induces apoptotic cell death through activation of JNK [21]. A combination of curcumin and PEITC was found to have an additive effect in the induction of apoptosis in PC-3 cells [21]. *In vivo*, curcumin alone or in combination with PEITC showed significant cancer-preventive activities in NCr immunodeficient (nu/nu) mice bearing subcutaneous xenografts of PC-3 cells [7].

7.3 REGULATION OF REDOX-SENSITIVE TRANSCRIPTION FACTORS BY ISOTHIOCYANATES AND POLYPHENOLS

Since low levels of oxidants are constantly generated for essential biologic functions, excess generation or an intracellular imbalance between oxidants and antioxidants can produce a common pathophysiologic condition known as oxidative stress [22]. Persistent oxidative stress can cause abnormal or improper activation or silencing of cellular signaling/transcription factor controls leading to uncontrolled cell proliferation, differentiation, and malignant transformation. It is important to note that many dietary chemopreventive agents, which increase cellular protective effects against oxidative stress or carcinogenic damages through induction of phase II detoxifying/antioxidant enzyme inducers, also have pro-oxidant properties, meaning that they could generate oxidative stress/electrophilic stress by themselves in cells. Interestingly, cellular stress generated by dietary chemopreventive agents appears to be dose dependent; at low concentrations, stress is mildly generated, however, sufficient enough to activate the cellular defense mechanisms. At very high or toxic concentrations, a large level of cellular stress can cause unwanted adverse effects, such as DNA damage, mutagenicity, or cytotoxicity. Therefore, cellular stress levels or cellular redox status can directly influence the intracellular signaling pathway and transcriptional regulation of many genes essential for cellular homeostasis.

More and more evidence supports the idea that the oxidation or covalent modifications of thiol groups present in key representative redox-sensitive transcription

factors (e.g., AP-1, NF-κB, and Nrf2) and their regulating molecules are important events of the cellular signaling network. Because of their highly conserved cysteine residues (Cys-SH), these redox-sensing proteins can respond to subtle alterations in the cellular redox status caused either by external stimuli or during normal physiological processes. To serve as "redox sensors," these cysteine residues are normally kept reduced; however, under oxidative stress, they can often undergo redox reactions leading to disulfide formation. There are three major thiol/disulfide couples, namely, glutathione (GSH)/glutathione disulfide (GSSG), reduced thioredoxin [Trx-$(SH)_2$]/oxidized thioredoxin (Trx-SS), and cysteine (Cys)/cystine (CySS), that function as control nodes to return the oxidized cysteine derivatives to their original reduced form. Due to a relatively high intracellular concentration of small molecular-weight thiol GSH, the GSH/GSSG ratio is the most important factor determining the cellular redox-status [23,24].

The conserved cysteine residues in transcription factors AP-1, NF-κB, and Nrf2 are sensitive to changes in either the cellular redox status or the levels of redox-related effector molecules [25]. Such sensitivity involves at least two redox-sensitive steps, one in activation of the signaling cascade and another in DNA binding capabilities, and possibly additional redox-sensitive nuclear processes such as nuclear import and export [25].

7.3.1 Activator Protein-1 (AP-1)

AP-1 is a dimeric transcription factor comprised of basic leucine-zipper family members including the Jun, Fos, ATF, and Maf protein families [26,27]. Its activity is regulated by transcriptional regulation of individual subunits, dimer composition, posttranslational modification of the subunits, and the interactions between the dimer and other proteins. A large variety of stimuli, including proinflammatory cytokines, growth factors, oxidative stress, and tumor promoters, can regulate AP-1 activity [28]. AP-1-mediated gene transcription is involved in a wide array of cellular functions, including tumor promotion and/or progression stages [29]. AP-1 activity is also regulated by redox-dependent mechanisms. The reduced state of critical cysteine residues present in the DNA-binding domain of AP-1 proteins is essential for DNA binding at the 12-O-tetradecanoylphorbol-13-acetate (TPA)-response element (TRE). Those critical conserved cysteine residues are Cys269 and Cys320, which are found in homodimeric c-Jun DNA binding domain, and Cys154, found in heterodimeric Fos-Jun. With regard to the signaling pathways that regulate AP-1 activity in response to proinflammatory cytokines or exogenous stress, the major MAPK signaling pathways ERK, JNK, and p38 MAPK cascades appear to be critical [30].

Several isothiocyanates have been studied for their regulatory effects on AP-1. For instance, BITC can increase AP-1 binding activity and induction of AP-1 activity associated with earlier activation of JNK kinase [31]. Using the same cell line HT-29 cells, we have shown that AITC, PEITC, and SFN induce AP-1-luciferase activity [32]. Interestingly, the expression of endogenous cyclin D1, a gene target of AP-1 transcriptional activation, positively correlated with AP-1 luciferase

activity induced by these isothiocyanates. A recent study on N-acetylcysteine (NAC) conjugates of BITC (BITC-NAC) and PEITC (PEITC-NAC) compounds in benzo(a)pyrene-induced lung tumorigenesis in A/J mice has provided the first *in vivo* evidence that activation of MAPK and AP-1 transcription factors may be involved in the chemopreventive activity of these compounds. Curcumin has also been shown to inhibit the activation of TPA-induced AP-1 in HL-60 cells and Raji DR-LUC cells [33,34]. Besides, curcumin suppresses constitutive AP-1 activity in the prostate cancer cell lines. Inhibition of AP-1 transcriptional activity by curcumin administration also correlates with inhibition of Lewis lung carcinoma (LLC) invasion in the orthotopic implantation model [35]. However, it appears that the effects of curcumin on AP-1 may be dependent on the cell types, external stimuli, and the compound concentrations.

7.3.2 Nuclear Factor kappa B (NF-κB)

The heterodimeric protein NF-κB is a ubiquitous redox-regulated transcription factor that can exert pleiotropic effects via numerous intracellular signal transduction pathways. The basic characteristics and regulation mechanisms of NF-κB have been extensively reviewed elsewhere [36]. The NF-κB/Rel family includes six members: NF-κB1, NF-κB2, RelA, RelB, c-Rel, and v-Rel. All NF-κB members can form homodimers as well as heterodimers with one another; however, the most prevalent activated form found in eukaryotes is the p50/p65 (NF-κB1/RelA) heterodimer. Similarly, NF-κB contains a redox-sensitive critical cysteine residue (Cys62) in the p50 subunit that is involved in DNA binding [37]. In resting or unstimulated cells, NF-κB is normally sequestered in the cytoplasm by the inhibitory protein IκB, but under oxidative conditions, this IκB is rapidly phosphorylated by IκB kinase (IKK), and subsequently degraded via the ubiquitin-proteasome pathway [38–40]. The resulting free NF-κB dimmers translocate to the nucleus and bind a specific consensus sequence of DNA. It is noticeable that NF-κB binds DNA preferentially when its critical cysteine residue is in reduced form, that redox-related modification of cysteine thiols can disrupt DNA-binding ability of NF-κB. Therefore, low ROS level appears to be necessary to initiate the events leading to the dissociation of the NF-κB/IκB complex [25], but excessive ROS level results in the oxidation of Cys62, which directly interferes with DNA binding and decreases gene expression [41].

Although the role of NF-κB signaling pathways in regulating immunoregulatory functions has been well accepted, accumulated evidence has also indicated the involvement of NF-κB signaling pathways in tumorigenesis/carcinogenesis [36,40]. In many malignant tumors, abnormally elevated NF-κB activation is observed, which is causally linked to resistance to apoptosis, invasion, and metastasis. Therefore, the NF-κB pathway has become an important target of cancer chemoprevention as well as therapy. Of the natural chemopreventive agents, our laboratory gives major attention to isothiocyanates and phenolic compounds. We found that SFN dose dependently inhibited the LPS-induced NF-κB-luciferase reporter activity in HT-29 cells, and those inhibitory effects were associated with

the blocking of LPS-induced IκBα phosphorylation [14]. Whereas another group suggested that SFN reduced DNA binding of NF-κB in macrophages without interfering with LPS-induced degradation of the IκBα or with nuclear translocation of NF-κB [42]. Curcumin treatment decreases DNA binding of NF-κB, IκBα kinase activation, IκBα phosphorylation, and degradation induced by cigarette smoke in human lung carcinoma H1299 cells [43,44]. *In vivo*, curcumin attenuated NF-κB activation in TPA-treated ICR mouse skin by blocking the phosphorylation and degradation of IκBα [42]. Additional intensive studies on the regulation of NF-κB and other signaling pathways by these compounds are required.

7.3.3 Nuclear Factor-E2-Related Factor 2 (Nrf2)

Nrf2, a member of the cap 'n' collar family of basic region-leucine zipper (bZIP) transcription factors, can act as a master regulator of ARE-driven transactivation of phase II detoxifying/antioxidant genes [45,46]. Under homeostatic conditions, Nrf2 is mainly sequestered in the cytoplasm as an inactive complex with a cytoskeleton-binding protein called Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1) [47–49]. Keap1 is a cysteine-rich protein that interacts with the ETGE motif within the N-terminal Neh2 domain of Nrf2 [50]. Dissociation of Nrf2 from the inhibitory protein Keap1 is a prerequisite for nuclear translocation and subsequent DNA binding of Nrf2 [48]. A widely accepted model for nuclear accumulation of Nrf2 or its activation, upon exposure to the threshold or higher levels of chemopreventive agents or oxidative stress, involves modification of Keap1 cysteine residues, which facilitates the dissociation of the Nrf2-Keap1 complex. Zhang and Hannink have identified that two cysteine residues, Cys273 and Cys288, located in the liker domain of Keap1, are essential for Keap1-induced ubiquitination and repression of Nrf2 [51]. In addition, they also identified a third cysteine residue located in the BTB domain of Keap1, Cys151, which is uniquely required for inhibition of Keap1-dependent degradation of Nrf2.

Recently, Li et al. [47] proposed a new model for Nrf2 redox signaling. According to this model, the importance of nuclear import and export in controlling the subcellular localization of Nrf2 has been suggested. Under unstimulated conditions, Nrf2 exhibits a predominantly whole-cell distribution due to balancing between nuclear exporting forces and nuclear importing forces. The nuclear exporting forces are a combination of canonical redox-insensitive NES (NES_{zip}, ^{537}LKKQLSTLYL^{546}) located in the leucine zipper (ZIP) domain of the Nrf2 protein and a redox-sensitive NES (NES_{TA}, ^{173}LLSIPELQCLNI^{186}) in the transactivation (TA) domain [47]. On the other hand, nuclear importing force is from a bipartile nuclear localization signal (bNLS, ^{494}RRRGKQKVAANQCRKRK^{511}) located in the C-terminus of Nrf2 protein. Upon exposure to oxidative stress, a redox-sensitive NES_{TA}, which possesses a reactive cysteine residue (Cys183), is disabled leading to an imbalance of exporting and importing forces and triggering Nrf2 nuclear translocation. These discoveries suggest that while Keap1 provides the major regulation of Nrf2 activation, both in basal and inducible conditions,

Nrf2 itself may be able to transduce redox signals in a Keap1-independent manner.

Similar to other redox-sensitive transcription factors, thiol modulations of several upstream-signaling kinases MAPK, protein kinase C (PKC), and phosphatidyl inositol 3-kinase (PI3K) are prerequisite for the activation of Nrf2-mediated gene expression. However, different signaling kinases have different effects on Nrf2 transcriptional activity [52]. We have also found that different segments of the Nrf2 transactivation domain have different transactivation potential. SFN showed a concentration-dependent disruption effect on the interaction between Keap1 and the Neh2 domain of Nrf2 in a native gel electrophoresis assay [53]. In human hepatoma HepG2 cells, inhibition of p38 MAPK by SFN can cause induction of ARE-mediated HO-1 gene expression. Besides, in human prostate cancer PC-3 cells, induction of ARE-mediated HO-1 gene expression by PEITC requires activation of ERK and JNK pathways [13]. *In vivo*, SFN blocked benzo[a]pyrene-induced forestomach tumors in ICR mice, and this protection resulted in part from the induction of phase II detoxifying/antioxidant enzymes since the blocking effect was abrogated in the Nrf2 knockout mice.

7.4 *IN VIVO* GLOBAL GENE EXPRESSION PROFILES IN RESPONSE TO ISOTHIOCYANATES AND POLYPHENOLS

It is important to note that many phytochemicals particularly those cancer-blocking agents, exert their chemopreventive effects, by inducting the expression of Nrf2/ARE-regulated genes. In several models of chemical carcinogenesis [54] Nrf2-deficient (*Nrf2*$^{-/-}$) mice are prone to have higher susceptibility toward cancer development and they are also refractory to the protective actions of some chemopreventive agents. These observations have highlighted the importance of the Keap1-Nrf2-ARE signaling pathway as a molecular target for prevention.

Recently, our laboratory performed many analyses of EGCG, curcumin, SFN, and PEITC in the small intestine and liver of Nrf2 knockout and wild-type C57BL/6J mice to determine the role of Nrf2 in the global gene expression profiles elicited by these chemopreventive agents. It has been observed that, *in vivo*, the regulation of Nrf2-mediated gene expression is both tissue/compartment-dependent and time-dependent levels. For example, the dietary chemopreventive agent curcumin did not modulate the transcription of glutathione S-transferase alpha 2 (GSTα2) in liver at 3 h; however, GSTα2 was induced by curcumin at 12 h [55]. The relatively delayed induction of the other phase II and antioxidant genes was also observed, which may reflect either the possibility of differential kinetics of curcumin-regulated gene response (time-dependent specificity) or effect of the *in vivo* pharmacokinetics of disposition after curcumin oral administration. However, GSTα2 was modulated differentially at 3 h in response to curcumin between small intestine and liver [55] indicating certain tissue/compartment-dependent control of gene expression. These differences in

the gene expression patterns between small intestine and liver tissues could be attributed to either a complex of physiological factors, such as partitioning across the gastrointestinal tract, intestinal transit time, and uptake into the hepatobiliary circulation, or the possibility of differential tissue/organ-dependent expression of endogenous Nrf2 in conjunction with other tissue-specific/general nuclear coregulators and corepressors. Similar phenomena were also observed in microarray studies with EGCG [56], SFN [57], and PEITC [31].

There is a renewed interest in dissecting the interacting partners of Nrf2, such as coactivators and corepressors, which are coregulated with Nrf2 to better understand the biochemistry of Nrf2. According to data from our microarray studies, several nuclear coregulators and corepressors are either upregulated as well as downregulated after treatment with curcumin [55], EGCG [56], SFN [57], and PEITC[31] in an Nrf2-dependent manner. For example, we found that the nuclear transcriptional coactivator CREB-binding protein (CBP) was upregulated in mice after treatment with EGCG. In addition, we have demonstrated that CBP, which can bind to the Nrf2 transactivation domain and can be activated by the ERK cascade, shows synergistic stimulation with Raf on the transactivation activities of Nrf2. Although microarray expression profiling cannot provide evidence of binding between partners, it could potentially suggest which corepressors or coactivators may serve as putative nuclear interacting partners of Nrf2 in eliciting the cancer chemopreventive effects of dietary phytochemicals [58].

7.4.1 Phase I, II, and III Xenobiotic Metabolizing Enzymes/Transporter Genes and Antioxidant Genes Coordinately Regulated via Nrf2

We found that these dietary chemopreventives could coordinately regulate the phase I, II, and III xenobiotic metabolizing enzyme genes as well as antioxidant genes through Nrf2-dependent pathways *in vivo* [31,55–57]. The example of major phase I and II genes as well as antioxidant genes modulated via Nrf2 by EGCG, curcumin, SFN, and PEITC are cytochrome P450, GST, UDP-glucuronosyltransferases, NAD(P)H:quinone reductase, epoxide hydrolase, thioredoxin reductase, gamma-glutamylcysteine synthetase, glutathione peroxidase, HO-1, leukotriene B4 dehydrogenase, aflatoxin B1 dehydrogenase, ferritin, and so on. Overall, these compounds increase the expression of a broad range of genes, in an Nrf2-dependent manner, that act to directly detoxify toxins as well as generate essential cofactors such as glutathione. Interestingly, phase III transporter genes, especially ATP-binding cassette (ABC) genes and solute carrier family genes, are reported to be induced via Nrf2. Those transporters function as efflux pumps to export the carcinogens and their metabolites out of cells. Theoretically, the coordinated regulation of these genes could have significant effects on prevention of tumor initiation by enhancing the cellular defense system, preventing the activation of procarcinogens/reactive intermediates, and increasing the excretion/efflux of reactive carcinogens or metabolites.

7.4.2 Major Clusters of Genes Coordinately Regulated via Nrf2

Using *Nrf2*$^{-/-}$ mice coupled with microarray analyses, we have identified many new target genes that are regulated through Nrf2 in response to chemopreventive agents EGCG, curcumin, SFN, and PEITC. Interestingly, these compounds can upregulate more Nrf2-dependent genes than downregulate, meaning that exposure to stress can cause diverse cellular responses. Based on their biological functions, the products of those genes can be categorized into ubiquitination/proteolysis, apoptosis/cell cycle, electron transport, detoxification, cell growth/differentiation, transcription factors/interacting partners, kinases and phosphatases, cell adhesion, biosynthesis/metabolism, RNA/protein processing and nuclear assembly, and DNA replication genes.

Several genes related to apoptosis/cell cycle and cell growth/differentiation control that are critical in the etiopathogenesis of many cancers have been shown to be regulated through Nrf2 [31,55–57]. For example, cadherins, cyclins, cyclin-dependent kinases, cytochrome *c* oxidase subunits, transforming growth factors, and fibroblast growth factors are induced by these compounds. Dysregulation of the cadherin family is strongly associated with cancer metastasis and progression, thus induction of cadherin genes through the Nrf2/ARE pathway could be another potential mechanism of exerting cancer chemoprevention and therapy with these chemopreventive agents. In addition, our studies also clearly indicated that EGCG, curcumin, SFN, and PEITC can regulate the gene expression of many kinases and phosphatases through Nrf2. PI3K, PKC, and MAPK that are induced by SFN *in vivo* have been previously suggested to be upstream-signaling kinase cascades for ARE-driven HO-1 gene expression *in vitro*. Although microarray expression profiling cannot provide evidence of functional proteins, it could potentially suggest which target proteins may serve as downstream effectors of Nrf2 in eliciting the cancer chemopreventive effects of these compounds [58].

7.5 CONCLUSION

In summary, the concept of cancer intervention by phytochemicals is more widely accepted as it offers great potential in the fight against cancer; however, the elucidation of molecular and cellular targets of dietary chemopreventive agents still remains a major challenge. These phytochemicals typically generate "cellular stress" and exert their chemopreventive activities through redox-mediated signaling mechanisms. EGCG, curcumin, SFN, and PEITC increase the overall cellular protective effects of the normal cells against oxidative stress or carcinogenic damages via Nrf2/ARE-mediated pathway. They also modulate two other important redox-sensitive transcription factors NF-κB and AP-1, which regulate a wide array of genes involved in many cellular events, such as cell cycle control, differentiation, transformation, apoptosis, and tumorigenesis. Although experimental data from cell culture models provide valuable information about the molecular and cellular mechanisms involved in the modulation of Nrf2, NF-κB, and AP-1 signaling pathways, the differential signaling/gene expression between "normal" versus

"abnormal tumor" cells as well as transduction cascades between phytochemicals and transcription factors are still not fully understood. Using *Nrf2*$^{-/-}$ mice coupled with Affymetrix microarray analyses, many categories of important genes modulated by these compounds are identified, which can be basic knowledge for the future of pharmacodynamic and toxicodynamic studies of dietary chemopreventive agents. While chemoprevention studies using animal models certainly provide promising results, future confirmatory human clinical trials coupled with epidemiological data would be needed to support their eventual chemopreventive potentials.

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8 The Isothiocyanate Sulforaphane Induces the Phase 2 Response by Signaling of the Keap1–Nrf2–ARE Pathway: Implications for Dietary Protection against Cancer

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CONTENTS

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8.1 ROLE OF THE PHASE 2 RESPONSE IN PROTECTION AGAINST ELECTROPHILE AND OXIDANT TOXICITIES

In the course of evolution, all eukaryotes have developed sophisticated defense systems that allow their survival and coevolution with other competing organisms. These include the biosynthesis of a wide array of small molecules (secondary metabolites) with extraordinarily sophisticated chemistry in plants, as well as elaborate enzymatic systems capable of coping with the toxicities of electrophiles and oxidants (phase 2 enzymes) in both plants and animals. Phase 2 enzymes catalyze enormously versatile chemical reactions that collectively lead to detoxification of various electrophiles and oxidants. Together with housekeeping antioxidant enzymes (e.g., catalase, superoxide dismutase) and small molecular mass direct antioxidants (e.g, ascorbic acid, tocopherol, glutathione), phase 2 enzymes constitute an integral part of the cellular defense. Furthermore, the discoveries that (1) phase 2 enzymes can be induced selectively (without concomitant induction of phase 1 enzymes) by a wide variety of stimuli that we now simply call "inducers" and (2) this "induced state" that we now refer to as "the phase 2 response" could explain how so many diverse chemical agents could block carcinogenesis in various animal models led to the birth of the hypothesis that induction of phase 2 enzymes could be a powerful strategy for protection against cancer and other chronic diseases [1–3].

In contrast to phase 1 enzymes (mostly cytochromes P450), some of which can convert innocuous procarcinogens, such as polycyclic aromatic hydrocarbons, to highly reactive electrophilic "ultimate" carcinogens (e.g., diolepoxides) that can *damage* susceptible centers of macromolecules and initiate carcinogenesis, phase 2 enzymes *protect* DNA, proteins, and lipids against electrophiles and oxidants by a wide variety of mechanisms (Figure 8.1) [4–8]. Examples include (1) direct inactivation of toxic electrophiles by conjugation with endogenous ligands such as GSH (by glutathione *S*-transferases, GSTs) and glucuronic acid (by UDP-glucuronosyltransferases); (2) inactivation of reactive centers of toxic molecules such as hydrolysis of epoxides, and reduction of electrophilic quinones to hydroquinones by NAD(P)H:quinone oxidoreductase 1 (NQO1); (3) reduction of toxic alkyl hydroperoxides and DNA base hydroperoxides by the peroxidase activities of GSTs; (4) reduction by GST of hydroxyalkenals, which are free radical-initiated lipid peroxidation products; (5) salvage by GST of adenine and thymine propenals, which are produced by radiation and hydroxyl radicals; (6) direct inactivation of oxidants such as peroxides or superoxides by the glutathione peroxidase functions of GST, Se-dependent glutathione peroxidase, and superoxide dismutase; (7) elevation of cellular glutathione by upregulation of γ-glutamatecysteine ligase, the enzyme catalyzing the rate-limiting step in the biosynthesis of glutathione. Glutathione regeneration in the reduced form is additionally stimulated through the enzymatic activities of NADPH-glutathione reductase, glucose 6-phosphate dehydrogenase, and the selenocysteine-containing flavoprotein thioredoxin reductase; (8) generation of powerful small antioxidants such as bilirubin and carbon monoxide by the sequential actions of heme oxygenase-1

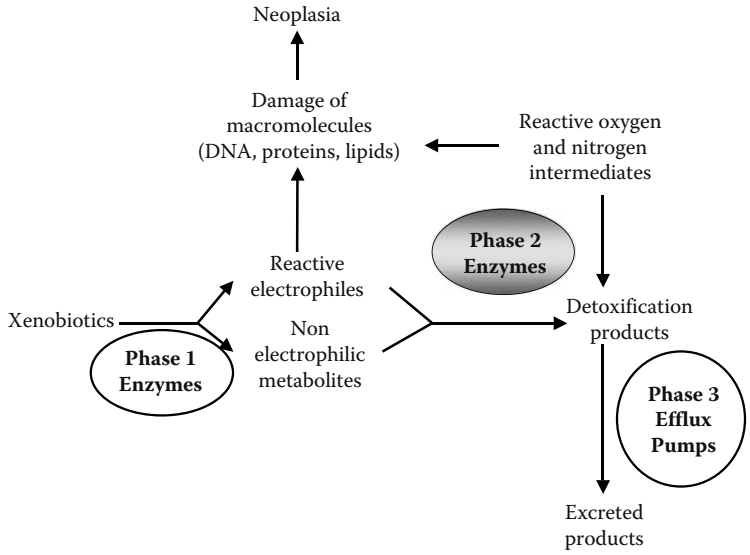

FIGURE 8.1 The protective role of phase 2 enzymes in the metabolism of xenobiotics. Upon entry in cells xenobiotics become substrates for phase 1 enzymes (mainly cytochromes P450) that catalyze their conversion to either benign nonelectrophilic or damaging electrophilic products. Reactive oxygen and nitrogen intermediates are also sources of potentially damaging agents. DNA, lipids, and proteins are protected against the damaging effects of electrophiles and oxidants by phase 2 enzymes that detoxify electrophiles and oxidants and facilitate their excretion. The ultimate detoxification products are finally exported through the action of phase 3 efflux pumps.

and biliverdin reductase; (9) enhancement of export of toxic drugs by increasing the levels of multidrug transporters; (10) elevation of ferritin levels to counteract oxidative stress caused by iron overload; (11) inhibition of cytokine-induced inflammatory processes; and (12) recognition, repair, and clearance of damaged proteins. As can be seen, several of these systems are related to glutathione, many have multiple and overlapping enzymatic activities, and nearly all ultimately function to afford protection against the toxicities of electrophiles and oxidants. Only rarely do phase 2 enzymes generate toxic species, for example, the *S*-(halomethyl)glutathione or glutathione episulfonium ions resulting from GST-catalyzed reaction of glutathione with haloalkanes and haloalkenes [9].

8.2 ISOLATION OF SULFORAPHANE AS A POTENT INDUCER OF THE PHASE 2 RESPONSE

The correlation between phase 2 inducer activity and protection against carcinogenesis of a series of phenolic antioxidants and azo dyes[10] suggests the importance of

identifying new inducers as chemoprotectors. A microtiter plate bioassay was developed that offered several advantages over using animal systems: (1) it is highly quantitative; (2) it is rapid and has a high throughput; (3) toxicity can be simultaneously determined; and (4) it can distinguish whether a particular inducer is monofunctional (i.e., it selectively induces phase 2 enzymes only) or bifunctional (i.e., it induces both phase 1 and phase 2 enzymes). The microtiter plate bioassay involves evaluation of the activity of a prototypic phase 2 enzyme, NAD(P)H: quinone oxidoreductase 1 (NQO1), after Hepa1c1c7 murine hepatoma cells have been plated in 96-well microtiter plates and exposed to a series of concentrations of a potential inducer [11,12]. Today, we refer to this assay as the "Prochaska test" [13]. The response of NQO1 to inducers in this assay mimics the response of rodent tissues *in vivo* and thus provides a quick and highly quantitative system for screening pure compounds, as well as complex mixtures, such as plant extracts, for their inducer activity. The concentration that doubles the activity of NQO1 (CD value) is a characteristic quantitative parameter of this bioassay and is extremely useful in comparing inducer potencies.

In addition to their nutritional value, edible plants have long been recognized as a primary and very rich source of biologically active natural products. The development of chemoprotective inducers based on the plethora of phytochemicals present in edible plants offers numerous advantages over the development of synthetic agents, including high probability of low toxicity that is essential for long-term use; relatively low economic cost; and possible rapid direct translation of laboratory findings to humans without requirement for extensive toxicity testing. Because of these advantages, one of the first applications of the Prochaska test was a quantitative screening test for identification of inducers from plant extracts. Thus, extracts of various organically grown vegetables were prepared and evaluated for their phase 2 inducer potencies [12]. The selected plants cover almost the entire spectrum of vegetables commonly consumed in Europe and the United States and belong to many different plant families, that is, Chenopodiaceae (beets, spinach), Compositae (red leaf lettuce), Cruciferae (cauliflower, bok choi, broccoli, cabbage, kale, radish), Cucurbitaceae (zuccini), Leguminosae (green beans, peas), Liliaceae (asparagus, green onions, leeks), Rosaceae (apples), Solanaceae (green peppers, potatoes, tomatoes), Umbelliferae (carrots, celery), and Zingiberaceae (ginger). This extensive screening revealed that crucifers (and especially those of the *Brassica* genus) had the highest inducer activity. Subsequently, the application of the bioassay as "activity-guided fractionation" led to the isolation of the isothiocyanate sulforaphane as the principal phase 2 inducer from extracts of broccoli (*Brassica oleracea* var. *italica*) [14] (Figure 8.2) and to the finding that 3-day-old broccoli sprouts are an especially rich source with an average of 50-fold higher inducer potency than the mature plant [15].

The microtiter plate assay made possible the screening of numerous compounds for their potential phase 2 inducer activity. During that process it became clear that inducers belong to at least 10 different chemical classes: (1) Michael acceptors; (2) oxidizable diphenols and diamines; (3) conjugated polyenes; (4) hydroperoxides; (5) trivalent arsenicals; (6) heavy metals; (7) isothiocyanates;

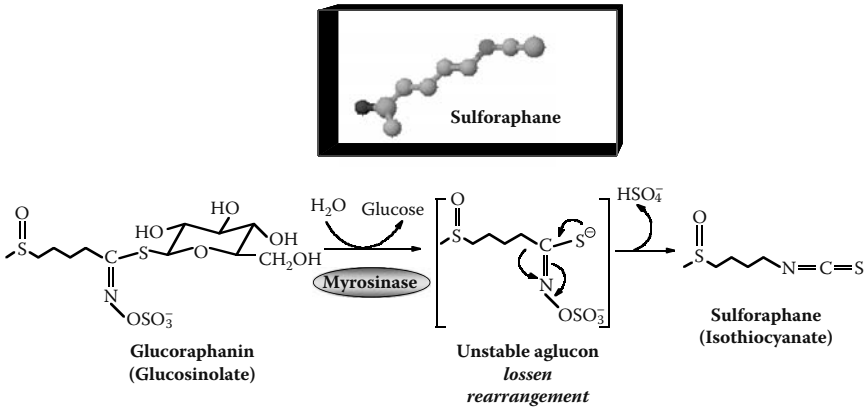

FIGURE 8.2 The myrosinase-catalyzed conversion of glucoraphanin (glucosinolate) to sulforaphane (isothiocyanate). In plant tissues, glucoraphanin coexists with but is physically segregated from a hydrolytic β-thioglucosidase (myrosinase) enzyme. Enzyme and substrate come in contact upon tissue damage, resulting in hydrolysis of glucoraphanin to liberate glucose and sulfate, and form an unstable aglucone that, at neutral pH, spontaneously undergoes Lossen rearrangement to form the isothiocyanate sulforaphane.

(8) dithiolethiones; (9) dithiocarbamates; and (10) vicinal dimercaptans [16,17]. Prominent among inducers are several classes of natural products, for example, isothiocyanates, ferulic acid derivatives, chalcones, flavonoids, coumarins, curcuminoids, carotenoids, chlorophylls, withanolides, brassinins, and organosulfur compounds [17–19].

8.3 GLUCOSINOLATE TO ISOTHIOCYANATE CONVERSION: A FASCINATING BIOLOGICAL PHENOMENON

Some of the most potent naturally occurring inducers known to date are isothiocyanates. Although it was isolated from broccoli, the isothiocyanate sulforaphane is not present in the intact plant, but is actually derived from its precursor glucosinolate glucoraphanin (Figure 8.2). The Cruciferae family represents a rich source of glucosinolates (approaching several percent by weight, especially in selected seeds). Glucosinolates are *S*-β-thioglucoside *N*-hydroxysulfates with more than 120 unique side chains derived from common amino acids: Phe or Tyr give rise to aromatic glucosinolates; Ala, Leu, Ile, Met, or Val, to aliphatic glucosinolates; and Trp to indole glucosinolates [20,21]. The last named do not give rise to stable isothiocyanates. The first documented introduction of a glucosinolate to the scientific community dates to 1831 when Robiquet and Boutron reported the isolation of sinalbin from the seeds of white mustard (*Sinapis alba*) [22]. In plants, glucosinolates coexist with, but are physically segregated from β-thioglucosidase

enzymes known as myrosinases (EC 3.2.3.1). Thus, in the flower stalk of *Arabidopsis thaliana* extraordinary high concentrations of glucosinolates (>100 mM) have been detected in specialized S-(sulfur-rich) cells that are positioned between the phloem and the endodermis, whereas myrosinase resides in the adjacent phloem parenchyma cells [23]. Enzyme and substrate come in contact upon damage to plant tissues such as during injury or chewing, resulting in hydrolysis of the glucosinolates to liberate glucose and sulfate and form unstable aglucones that spontaneously rearrange to form isothiocyanates, thiocyanates, nitriles, epithionitriles, or oxazolidinethiones (Figure 8.3). The nature of the hydrolysis products depends on the structure of the glucosinolate side chain, the plant species, and the reaction conditions [24–27]. At neutral pH and upon inactivation of a protein factor, for example, the epithiospecifier protein, the products are principally isothiocyanates [26,28].

The glucosinolate–myrosinase–specifier protein(s) relationship represents a fascinating biological phenomenon and is often referred to as the "mustard oil bomb" [29]. Curiously, certain insects that feed on crucifers have developed various strategic mechanisms to protect themselves against its potential "explosion." Thus, the larvae of the cabbage white butterfly *Pieris rapae* avoid the potential toxic effects of isothiocyanates by diverting the glucosinolate conversion in the

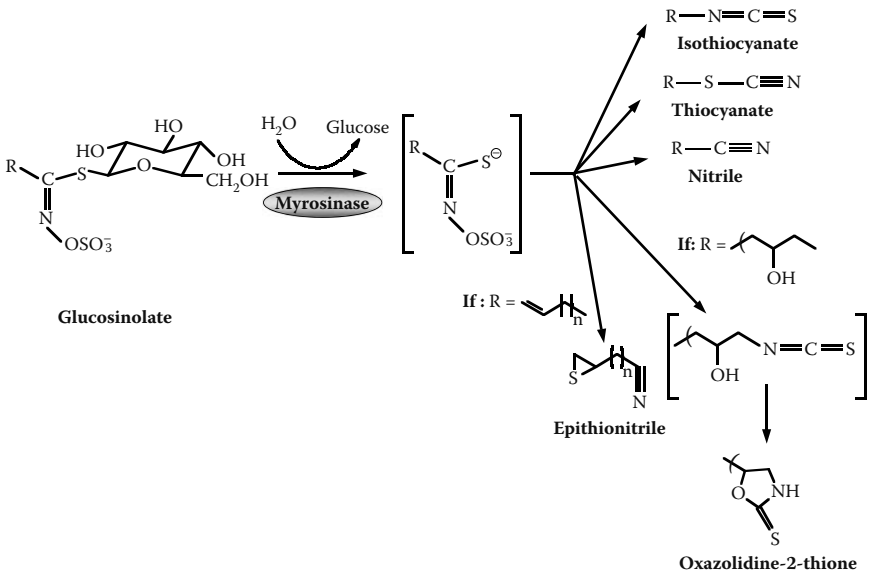

FIGURE 8.3 Possible products of myrosinase-catalyzed hydrolysis of glucosinolates. Glucosinolates are hydrolyzed by myrosinase to glucose and unstable aglucones, which, depending on the reaction conditions, the presence of protein factors (e.g., epithiospecifier protein, nitrile-specifier protein), and the structure of the glucosinolate side chain (R), can give rise to isothiocyanates, thiocyanates, nitriles, epithionitriles, or oxazolidinethiones.

direction of production of nitriles because of the unique presence of nitrile-specifier protein in their midgut [30,31]. The diamondback moth *Plutella xylostella* utilizes a preventive strategy: these insects have sulfatase activity with a very broad substrate specificity (which enables them to feed on various cruciferous plants) and convert glucosinolates to desulfo-glucosinolates that cannot serve as myrosinase substrates and, in addition, the released sulfate is a competitive inhibitor of the plant myrosinase [29]. Interestingly, the aphids *Brevicoryne brassicae* and *Lipaphis erysimi* can sequester glucosinolates from their host plants and subsequently employ them in their own defense against predators. These insects have their distinct myrosinases that are compartmentalized into crystalline microbodies and, much like the case with their host plants, the enzyme comes into contact with its substrate only upon damage of the insect tissues [32]. Yet another mechanism operates in the larvae of the sawfly *Athalia rosae*: they do not seem to have any myrosinase or sulfatase activities, but instead rapidly sequester intact glucosinolates from the host plant into the larval hemolymph and subsequently excrete (within 14 h after ingestion) glucosinolate metabolite(s) whose exact nature remains unknown [33].

The existence of myrosinase was first reported by Bussy [34], who found a substance with "albumin character" that was involved in the hydrolysis of the glucosinolate sinigrin. Myrosinases have been purified and characterized from several plant sources, including white mustard (*S. alba*) [35], rapeseed (*Brassica napus*) [36,37], cress (*Lepidium sativum*) [38], yellow mustard (*Brassica juncea*) [39], horseradish (*Armoracia rusticana*) [40], and daikon (*Raphanus sativus*) [41]. Purified plant myrosinases are dimeric 120–150 kDa-glycoproteins. Interestingly, the activity of myrosinase increases by >100-fold in the presence of ascorbic acid, but not its oxidation product dehydroascorbic acid, in an unusual "uncompetitive manner," that is, with increases in both V_{max} and K_m. The structure of *S. alba* myrosinase has been solved at 1.6 Å resolution: the protein folds into a characteristic $(\beta/a)_8$ barrel structure and contains one zinc atom at the interface of the two subunits [42]. The elegant work of Burmeister et al. [43]. has subsequently established the mechanism of ascorbate-activated catalytic activation of myrosinase. Ascorbic acid acts as a cofactor that is recruited to the active site of the covalent glycosyl enzyme intermediate where it then functions as a catalytic base, a highly unusual case of its utilization in contrast to the much more common use as a reducing agent.

Notably, nearly all of the biological activities of glucosinolates (except indole), in both plants and animals, are attributable to their cognate isothiocyanate hydrolytic products. Although mammalian tissues do not contain myrosinases, glucosinolate hydrolysis in mammals is mediated by the microflora of their gastrointestinal tract [44]. People who undergo antibiotic treatments may not be able to benefit fully from the protective effects of cruciferous vegetables during the time of treatment, especially when these vegetables are not raw and the plant myrosinase is inactivated during food processing. Antibiotic and mechanical bowel preparation almost completely eliminates the conversion of glucosinolates to isothiocyanates in humans [44].

8.4 SULFORAPHANE, A POTENT INDUCER OF THE PHASE 2 RESPONSE

Since its isolation as an inducer of the prototypic phase 2 enzyme NQO1, sulforaphane still remains one of the most potent naturally occurring inducers known to date. In addition to NQO1, sulforaphane has been shown to induce other members of the phase 2 response in various cell lines and animal models. The first report involved induction of NQO1 in Hepa1c1c7 cells in the Prochaska test [14]. In the same cell line, in addition to NQO1, sulforaphane treatment also induces GST activity [25,45]. GSTA1 and UDP-glucuronosyltransferase were elevated in Caco-2 colon cancer cells [46]. Using a reporter assay in the human hepatoma cell line HepG2 stably transfected with the chloramphenicol acetyltransferase reporter gene under the transcriptional control of the rat GSTYa promoter, Fei et al. [47] showed that exposure to sulforaphane induces the reporter gene in a dose-dependent manner. In the same cell line, sulforaphane treatment also resulted in increased transcription of thioredoxin reductase 1 in a time- and dose-dependent manner [48], and in combination with selenium the treatment resulted in significant protection against paraquat (an oxidative cycler)-induced cell death. In primary human and rat hepatocytes, sulforaphane also induced the transcription of GSTA1/2 mRNA [49,50].

With the use of ^{14}C-labeled 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP), it was shown that treatment with sulforaphane inhibited the formation of PhiP-DNA adducts in HepG2 cells and primary human hepatocytes [51,52]. This protective effect correlated with transcriptional upregulation of UDP-glucuronosyltransferase and GSTA1, whereas no changes in DNA repair systems were observed. Importantly, protection was also demonstrated in primary cultures of human hepatocytes prepared from liver biopsies, even though there were large interindividual variations [52].

Sulforaphane induced NQO1 enzymatic activity in several human prostate cancer cell lines [45,53,54]. Transcriptional induction was transient; it was detected as early as 4 h after exposure, reached a peak at 8 h, and returned to basal levels by 12 h. However, enzyme activity remained elevated for up to 5 days after treatment. In addition, the levels of γ-glutamatecysteine synthetase light chain mRNA, glutathione, and GSTs were also upregulated.

Induction of phase 2 enzymes by sulforaphane has also been demonstrated to occur *in vivo*. Daily doses of 15 μmol of sulforaphane *per os* for 5 days resulted in induction of NQO1 and GST activities in several mouse tissues, including the liver, forestomach, glandular stomach, small intestine, and lung [14]. Similar observations were made in liver, colon, and pancreas of rats given either 200–1000 [25] or 40 μmol/kg of sulforaphane *per os* for 5 days [55]. Especially striking was the magnitude of induction in the bladder [55]. Supplementing the diet with sulforaphane at a dose of 3 μmol/g diet for 14 days induced the activities of NQO1 and GST in the small intestine, whereas an identical treatment was without effect in mice that lack Nrf2, the major transcription factor that controls phase 2 gene expression (see Section 8.5) [56].

Global gene expression profiling has confirmed that sulforaphane upregulates phase 2 genes both in cell cultures and *in vivo* [57–59]. Thus, exposure of Caco-2 human colon cancer cells to 50 μM sulforaphane (which reduced cell viability by 26%) elevated the expression of 106 genes and inhibited the expression of 63 genes [59]. The most prominent changes were in genes encoding for proteins that are concerned with cellular defense, inhibition of cell proliferation, and induction of differentiation, for example, there was upregulation of NQO1, thioredoxin reductase 1, γ-glutamatecysteine ligase, heme oxygenase-1, aldo-ketoreductase 1, p21, Nrf2, activating transcription factor 3, Krüppel-like factor (a transcription factor associated with induction of differentiation and reduction in cellular proliferation), and downregulation of members of the minichromosome maintenance family (MCM4 and MCM7) that are associated with DNA synthesis. In contrast, when Caco-2 cells were exposed to a 10-fold lower concentration of sulforaphane (5 μM), fewer genes were affected, primarily those of the aldo-ketoreductase family.

Thimmulappa et al. [57] treated 10-week-old female ICR mice with 9 μmol of sulforaphane daily for 1 week *per os*, and 24 h after the last dose, used microarray analysis to generate the transcriptional profile of the small intestine. This approach revealed the coordinate transcriptional upregulation of NQO1, GST, γ-glutamatecysteine ligase, UDP-glucuronosyltransferases, epoxide hydrolase, biosynthetic enzymes of the glutathione and glucuronidation conjugation pathways, various xenobiotic metabolizing enzymes, antioxidant proteins (glutathione peroxidase, glutathione reductase, ferritin, and haptaglobin), as well as genes encoding for cellular NADPH regenerating enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme).

In a pharmacokinetic study, Hu et al. [58] administered a single oral dose of 50 μmol of sulforaphane to F344 rats and observed a peak plasma concentration of 20 μM 4 h postdosing with a half-life of ~2.2 h. Microarray analysis of liver samples confirmed induction of phase 2 genes, but the kinetics of induction were rather complicated. Thus, the expression of metallothionein genes MT-1/2 and MT-1a increased by >10-fold between 2 and 4 h after dosing, while the gene expression of GSTA3, UDP-glucuronosyltransferase, aflatoxin B1 aldehyde reductase, and aldehyde oxidase was highest at 12 h. Recently, the same group performed a similar study in C57BL/6J mice treated with a single dose of sulforaphane of 90 mg/kg *per os* and observed, in addition to induction of liver phase 2 genes, transcriptional upregulation in genes encoding for heat shock proteins, ubiquitin/26 S proteasome subunits, and lipid metabolism [60]. Overall, the data from global gene expression profiling have confirmed that sulforaphane administration results in the upregulation of phase 2 genes with consistent increases in the expression of NQO1, GSTs, UDP-glucuronosyl transferase, γ-glutamatecysteine ligase, heme oxygenase 1, epoxide hydrolase, thioredoxin reductase 1, and multidrug resistant protein. In addition, many other genes can be up- or downregulated, depending on the experimental conditions, for example, dose, time of sampling after dosing, or cell or tissue type examined.

8.5 MOLECULAR MECHANISM OF INDUCTION OF THE PHASE 2 RESPONSE

The overall scheme of induction of the phase 2 response by sulforaphane involves three essential cellular components [61–65] (Figure 8.4).

1. The antioxidant response elements (AREs) are upstream regulatory sequences that are present on all phase 2 genes either in single or multiple copies and contain the consensus core sequence TGACNNNGC.
2. These sequences are recognized by nuclear factor-erythroid 2-related factor 2 (Nrf2), a 66-kDa transcription factor that binds to the ARE as a heterodimer with members of the small Maf family of transcription factors, subsequently recruiting the general transcriptional machinery to initiate both basal as well as inducible expression of phase 2 genes.
3. The third essential player is Kelch-like ECH-associated protein 1 (Keap1), the cellular sensor for phase 2 inducers, a cytosolic repressor protein that binds to the Neh2 domain of transcription factor Nrf2 and

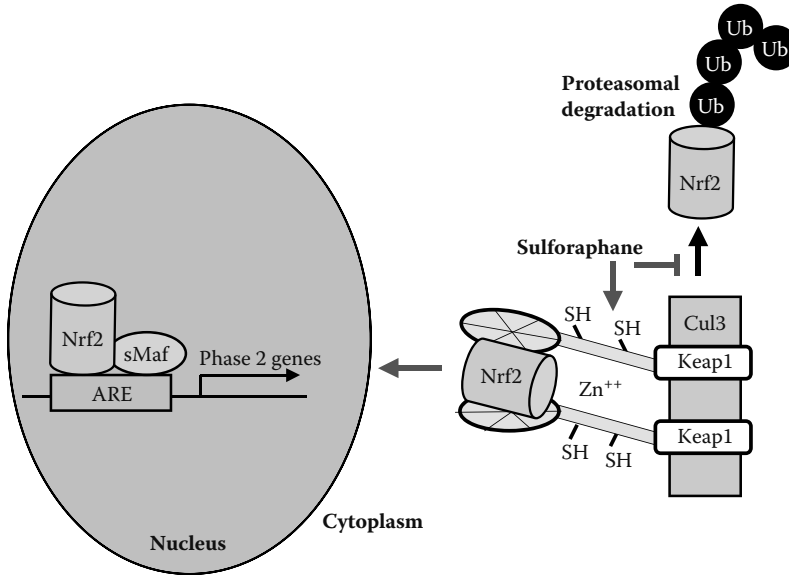

FIGURE 8.4 General scheme for the mechanism of phase 2 enzyme induction. In the absence of inducing stimuli, the sensor protein Keap1 binds and targets transcription factor Nrf2 for ubiquitination and proteasomal degradation via association with the Cullin 3 (Cul3)-based E3 ubiquitin ligase complex. Inducers (e.g., sulforaphane) react and chemically modify specific highly reactive cysteine residues of Keap1. Consequently, Keap1 loses its ability to repress transcription factor Nrf2, allowing its translocation to the nucleus, binding to the ARE (in heterodimeric combinations with members of the small Maf family of transcription factors), and ultimately the transcriptional activation of phase 2 genes.

targets it for proteasomal degradation via association with Cullin 3 to form an E3 ubiquitin ligase complex. Inducers react with Keap1 leading to loss of its repressor activity and subsequently allowing Nrf2 to undergo nuclear translocation and activate transcription of phase 2 genes.

On the basis of its sequence, Keap1 has five distinct domains: (1) NTR, N-terminal region, amino acids 1–60; (2) BTB (broad complex, tramtrack, bric-à-brac) dimerization domain, amino acids 61–179; (3) IVR, intervening region, amino acids 180–314; (4) DGR, double glycine repeat, also known as Kelch domain, amino acids 315–598; and (5) CTR, C-terminal region, amino acids 599–624. Keap1 forms a dimer through its BTB domain and "holds" one molecule of Nrf2 through its Kelch domain. The crystal structure of the Kelch domain of Keap1 has been solved [66,67]. It forms a six-bladed propeller with multiple potential protein–protein interaction sites. Interestingly, each Nrf2 molecule presents two distinct motifs (the ETGE and the DLG) that recognize overlapping surfaces on the Keap1 dimer, and the seven lysine residues of Nrf2 that act as ubiquitin acceptors are positioned between them [68,69].

Keap1 is a cysteine-rich zinc metalloprotein with 25 and 27 cysteine residues among the 624 amino acids of the murine and human homologues, respectively [63,70]. Because all phase 2 inducers react with thiol groups, by oxidation, reduction, or alkylation, Keap1 emerged as "the perfect candidate" for the cellular sensor for inducers. Using dexamethasone 21-mesylate as a probe, my colleagues and I identified C257, C273, C288, and C297 as the most reactive cysteine residues of Keap1, all located within its IVR domain [71], and subsequently demonstrated that C273 and C288 are absolutely essential for the repressor activity of Keap1 [72].

Sulforaphane has been shown to react directly with cysteine sulfhydryls of Keap1 *in vitro* [71,73]. Exposure to sulforaphane leads to nuclear translocation of Nrf2 in cultured cells and animal tissues [73–75]. In addition to C273 and C288 which are essential for the repressor activity of Keap1, Zhang and Hannink [75] identified C151 as a critical cysteine required for the inhibition of Keap1-dependent degradation of Nrf2 by sulforaphane. Perhaps the most convincing evidence that the chemoprotective effects of sulforaphane are mediated by induction of the phase 2 response through signaling of the Keap1/Nrf2/ARE pathway was provided by the demonstrations that whereas sulforaphane administration to mice resulted in substantial protection against bezo[*a*]pyrene-induced stomach carcinogenesis [76] and DMBA/TPA-induced skin carcinogenesis [77], the corresponding identical treatments in both models were without effect in *nrf2* knockout mice.

8.6 SULFORAPHANE INHIBITS TUMOR DEVELOPMENT IN ANIMAL MODELS

Since its discovery as a potent phase 2 inducer, sulforaphane has been shown to inhibit tumor development in at least nine different animal models (Table 8.1).

TABLE 8.1
Inhibition of Tumor Development by Sulforaphane in Animal Models

Animal Model	Tumor Model (Carcinogen)	Sulforaphane Dose and Route of Administration	Reference
Sprague-Dawley rats	Mammary tumors (DMBA)	75, 100, or 150 μmol/day for 4 days before to 1 day after the last dose of carcinogen, by gavage	[78]
Fischer rats	Colonic aberrant crypt foci (azoxymethane)	20 μmol/day for 3 days before the carcinogen or 5 μmol three times/week for 8 weeks after the carcinogen, by gavage	[79]
Syrian hamsters	Pancreatic tumors (*N*-nitroso-bis(2-oxopropyl) amine)	4.5 mmol/kg diet for 3 weeks, including 1 week before and 1 week after the carcinogen, by feeding	[82]
ICR mice	Gastric tumors (benzo[*a*]pyrene)	7.5 μmol/day for 7 days before to 2 days after the last dose of carcinogen, by feeding	[76]
A/J mice	Lung adenoma to carcinoma progression (benzo[*a*]pyrene and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone)	1.5 or 3 mmol/kg diet for 21 weeks after the carcinogen, by feeding	[80]
SKH-1 hairless mice	Skin tumors (DMBA/TPA)	1, 5, or 10 μmol/day, topically, twice a week, from 1 week after the carcinogen until the end of the study (15 weeks) or from 7 days before the carcinogen until the end of the study	[81]
C57BL/6 mice	Skin tumors (DMBA/TPA)	100 nmol/day topically, for 14 days before DMBA	[77]
SKH-1 hairless mice	Skin tumors (UV light)	1 μmol/day, topically, 5 days a week for 11 weeks starting after completion of 20-week UV irradiation schedule	[86]
Apc^{min} mice	Intestinal tumors	6 μmol/day for 10 weeks, by feeding, or 300 or 600 ppm in diet for 3 weeks	[83,84]

1. First, Zhang and colleagues [78] demonstrated that oral administration of sulforaphane at 75, 100, or 150 μmol/day for 4 days before to 1 day after the last dose of carcinogen (7,12-dimethylbenz[*a*]anthracene, DMBA) reduces the incidence, multiplicity, and burden of mammary tumors in female Sprague-Dawley rats.
2. Fahey et al. [76] showed that stomach carcinogenesis in ICR mice was markedly inhibited by feeding sulforaphane in the diet at 7.5 μmol/day for 7 days before to 2 days after the last dose of the carcinogen benzo[*a*] pyrene. Importantly, *nrf2* knockout mice were much more susceptible to the carcinogenic effect of benzo[*a*]pyrene, and sulforaphane had no effect on tumor development in these animals, providing evidence that, at least in this model, the protective effect of sulforaphane is primarily due to induction of the phase 2 response which is ultimately dependent on functional *nrf2* gene.
3. Sulforaphane inhibited the formation of azoxymethane-induced colonic aberrant crypt foci in male Fischer rats when given by gavage at 20 μmol/day for 3 days before the carcinogen or 5 μmol three times/week for 8 weeks after the carcinogen [79].
4. Conaway et al. [80] showed that dietary sulforaphane also inhibited the malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice when given at 1.5 or 3 mmol/kg diet for 21 weeks after administration of the carcinogen.
5. In the two-stage chemical skin carcinogenesis model that involves a single dose of DMBA as an initiator followed by multiple doses of TPA as a promoter, sulforaphane protected SKH-1 hairless mice against the development of skin tumors when administered topically twice a week at levels of 1, 5, or 10 μmol/mouse during the promotion stage [81].
6. In a similar model of skin carcinogenesis, pretreatment with 100 nmol of sulforaphane topically for 14 days before DMBA application decreased tumor incidence from 60% to 20% and multiplicity from 1.35 to 0.35 tumors per mouse in C57BL/6 mice [77]. Similar to the benzo[*a*]pyrene-induced gastric carcinogenesis model [76], *nrf2* knockout mice developed many more skin tumors much more rapidly and sulforaphane had no effect on tumor outcome measures.
7. Sulforaphane at a dose of 4.5 mmol/kg diet was effective in inhibiting the development of pancreatic tumors when administered before or during the initiation stage in male Syrian hamsters treated with *N*-nitroso-bis (2-oxopropyl)amine [82].
8. Of note, the protective effects of sulforaphane are not only restricted to chemical carcinogenesis, but are also observed in genetic models. Myzak et al. [83] and Hu et al. [84] demonstrated inhibition of the development of intestinal adenomas in mice in which the *apc* tumor suppressor gene is truncated, a condition that makes them genetically highly predisposed to multiple intestinal neoplasia, by feeding sulforaphane in the diet at doses of

6 μmol/mouse daily for 10 weeks [83], or 300 ppm (~4.25 μmol/mouse) or 600 ppm (~8.5 μmol/mouse) for 3 weeks [84].

9. In a mouse model of UV light-induced skin carcinogenesis [85], we showed that topical application of broccoli sprout extracts, 5 days a week for 11 weeks, containing the equivalent of 1 μmol of sulforaphane reduced by 50% tumor incidence, multiplicity, and total tumor burden in SKH-1 hairless mice that had been rendered "high-risk" for skin cancer development by prior chronic exposure (20 weeks) to low doses (30 mJ/cm^2) of UVB light [86].

Notably, in addition to its potent induction of the phase 2 response through the Keap1/Nrf2/ARE pathway, sulforaphane is a pleiotropic agent for which multiple other biological activities have been described that could potentially contribute, independently or in combination, to the inhibition of tumor development. Examples include induction of cell cycle arrest and apoptosis [87]; inhibition of angiogenesis [88]; inhibition of phase 1 enzymes [89]; suppression of proinflammatory responses [90]; and inhibition of histone deacetylase [83,91].

8.7 SULFORAPHANE METABOLISM

The central carbon atom of the isothiocyanate group of sulforaphane is highly electrophilic and can undergo facile addition reactions with nucleophiles, such as sulfhydryl groups. Although sulforaphane reacts with glutathione nonenzymatically, it is also a substrate for human GSTs [92,93]. Examination of the kinetic properties of four distinct human GSTs revealed that GSTP1-1 was the most efficient catalyst of the four isoenzymes, whereas GSTM1-1 and A1-1 are less active by one and GSTM4-4 by two orders of magnitude. Curiously, sulforaphane was the poorest substrate among 14 structurally related isothiocyanates even though its nonenzymatic reaction rate with GSH was among the highest. Nevertheless, the large enhancement of the enzymatic reaction rate and the abundance of the enzymes suggested that the GSTs play a major role in the metabolism of sulforaphane. The finding that the enzymatic conjugation of sulforaphane is relatively slow is particularly important since the active species that reacts with Keap1 is most likely to be the isothiocyanate and not its conjugated metabolites.

The suggestion that the GSTs participate in the metabolism of sulforaphane was further strengthened by epidemiological data from studies conducted in the United States that have concluded that individuals with *GSTM1*-positive genotype who consume cruciferous vegetables are more effectively protected against the development of prostate [94], colon [95], and lung [96,97] neoplasia than individuals with *GSTM1*-null genotype. It is now generally accepted that the glutathione *S*-transferases catalyze the first step in the metabolism of sulforaphane that subsequently proceeds via the mercapturic acid pathway. The initial conjugation with glutathione (GSH) is followed by successive cleavage of the γ-glutamyl residue (by γ-glutamyltransferase), removal of the glycine residue (by cysteinylglycinase), and *N*-acetylation (by *N*-acetyltransferases) to give the *N*-acetylcysteine conjugates

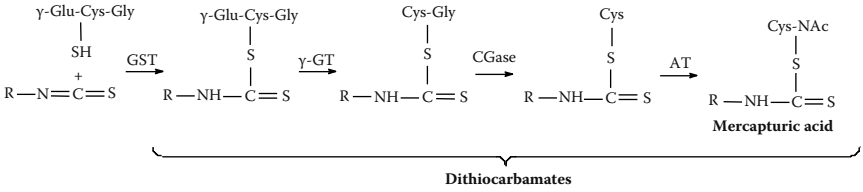

FIGURE 8.5 Sulforaphane metabolism. Sulforaphane is metabolized by the mercapturic acid pathway that involves initial conjugation with glutathione (catalyzed by GSTs) and the sequential catalytic activities of γ-glutamyltransferase (γ-GT), cysteinylglycinase (CGase), and *N*-acetyltransferase (AT) to ultimately form *N*-acetylcysteine conjugate (mercapturic acid). Sulforaphane and all of the glutathione-derived conjugates, collectively known as dithiocarbamates, are detected by the cyclocondensation reaction with 1,2-benzenedithiol.

(mercapturic acids; Figure 8.5). Sulforaphane and its conjugates, which are collectively known as dithiocarbamates (DTC), can all be quantified by cyclocondensation with the vicinal dithiol, 1,2-benzenedithiol, to give rise to 1,3-benzodithiole-2-thione, which has highly favorable properties for spectroscopic determination [98,99]. The cyclocondensation reaction is widely used to standardize the sulforaphane and glucoraphanin content of broccoli sprout extracts and to quantify the levels of sulforaphane and its metabolites in cells, tissues, plasma, and urine of animals and humans [44,100–105]. In addition, two methods that involve gradient liquid chromatography coupled with tandem electrospray ionization mass spectrometry were recently developed to permit the separation and individual quantification of sulforaphane and its metabolites [106–108].

Because broccoli is already present in the human diet, it can be used as a delivery vehicle for the administration of glucoraphanin and sulforaphane to humans. The glucosinolate content in mature broccoli varies enormously, however. Thus, when the inducer potencies of extracts of 22 fresh broccoli plants randomly collected from local supermarkets in the Baltimore area were examined, they were found to vary by at least eightfold [15]. This finding prompted the systematic examination of the inducer activity of broccoli at different stages of the development of the plant, which revealed that (1) the highest concentration is found in seeds; (2) 3-day-old broccoli sprouts contain as much inducer activity as 10–100 times larger quantities of mature plants; and (3) the inducer activity declines as the plant develops reaching the activity of mature broccoli heads at about 15 days of age [15]. It appears that the biosynthesis of glucosinolates in broccoli takes place almost exclusively in the seed and, at least under laboratory conditions, there is very little, if any, additional biosynthesis as the plant grows and develops. My colleagues and I have chosen to use 3-day-old broccoli sprouts and extracts from them as delivery vehicles for glucosinolates and isothiocyanates in our human studies because of their high and uniform glucosinolate content.

By the use of the cyclocondensation assay, the disposition of glucosinolates and isothiocyanates was examined in healthy human subjects who received single

doses of either nonhydrolyzed or hydrolyzed broccoli sprout extracts containing the equivalent of 111 μmol of glucosinolates or isothiocyanates, respectively [100]. Cumulative urinary dithiocarbamate excretion was 88.9 ± 5.5 and 13.1 ± 1.9 μmol for subjects receiving isothiocyanate and glucosinolate preparations, respectively. The efficiency of microbial conversions that takes place in the gastrointestinal tract varied markedly among individuals and could be almost completely eliminated by reduction of the bowel microflora by mechanical cleansing and antibiotic treatment [44]. In contrast, the conversion of directly consumed isothiocyanates to dithiocarbamates was much higher, with very small interindividual variation. In a dose escalation study, dithiocarbamate excretion was linear over a 25 to 200 μmol dose range of isothiocyanates and occurred with first-order kinetics [100].

Efforts to enrich the glucosinolate content of the mature plant have led to the production of "super" broccoli, which exhibited up to 80-fold higher inducer activity in the NQO1 bioassay than conventional broccoli [109]. Gasper et al. [107] conducted a randomized crossover study on 16 healthy human subjects to whom they administered by mouth a soup of either conventional or "super" broccoli that contained the equivalent of ~16 and 52 μmol sulforaphane, respectively. The plasma concentration of sulforaphane and its metabolites increased rapidly after dosing, reaching a maximum concentration at 1.5 and 2 h for the conventional and "super" broccoli soup, respectively. After reaching C_{max}, the plasma concentration decreased rapidly and was approaching baseline levels at 24 h postdosing. The values for both the area under the concentration curve (AUC) and the maximum concentration (C_{max}) in plasma were dose-dependent, that is, they were approximately threefold higher after consumption of "super" broccoli in comparison with conventional broccoli. Of note, the plasma C_{max} reached 2.2 ± 0.8 and 7.3 ± 2.9 μM after consumption of conventional and "super" broccoli, respectively. The same study also revealed that although there was no difference in C_{max}, in comparison to *GSTM1*-positive individuals, *GSTM1*-null subjects had slightly larger statistically significant AUC of total dithiocarbamates in plasma, a greater rate of urinary excretion during the first 6 h after oral dosing, and a higher percentage of total urinary excretion at 24 h. Because this result was somewhat surprising, the authors proposed that a portion of sulforaphane could be retained within the tissues and subsequently eliminated via the mercapturic acid pathway as well as alternative and hitherto unidentified pathways.

We conducted a randomized, placebo-controlled, double-blind clinical Phase 1 study of safety, tolerance, and pharmacokinetics of broccoli sprout extracts containing either glucosinolates (principally glucoraphanin) or isothiocyanates (principally sulforaphane) [105]. There were three cohorts, each comprising three treated individuals and one placebo recipient. All were inpatients in the Clinical Research Unit of the Johns Hopkins School of Medicine throughout the duration of the study and were fed a control diet devoid of crucifers and other sources of inducers of phase 2 enzymes. After a 5-day acclimatization period, the volunteers received the extracts by mouth at 8-h intervals for 7 days (a total of 21 doses). The individual doses for each of the three cohorts were (1) 25 μmol of glucosinolates, or a total of 75 μmol per day per subject; (2) 100 μmol of glucosinolates, or a total

of 300 μmol per day per subject; and (3) 25 μmol of isothiocyanates, or a total of 75 μmol per day per subject. The subjects were monitored throughout the intervention period and for 3 days after the last treatment. The mean cumulative excretion of dithiocarbamates as a fraction of dose was 17.8 ± 8.6% (±S.D.) and 19.6 ± 11.7% (±S.D.) for the low- and the high-glucosinolate dose-receiving cohorts, respectively, with a rather high interindividual variation. For the isothiocyanate dose-receiving cohort, the mean cumulative excretion of dithiocarbamates as a fraction of dose was 70.6 ± 2.0%, a value much more consistent among individuals. Examination of 32 types of hematology or chemistry tests that were done before, during, and after the treatment period, including indicators of liver (transaminases) and thyroid (TSH, T3, and T4) functions, revealed no evidence of systematic, clinically significant, adverse events that could be attributed to ingestion of the sprout extracts.

A randomized, placebo-controlled, double-blind chemoprevention trial in Qidong Province in the People's Republic of China examined the effect of 400 μmol glucoraphanin (in the form of aqueous broccoli sprout extract) that was given once a day for 2 weeks, on the metabolic disposition of aflatoxin and phenanthrene in 200 healthy human subjects [104]. When the urinary levels of dithiocarbamates were measured, striking interindividual differences were found. Thus, in this study population, the cumulative urinary excretion varied from 1% to 45% of the administered dose. This interindividual variability in the conversion is probably the reason why no overall difference between intervention arms (glucoraphanin vs. placebo) was observed. Indeed, there was an inverse association for excretion of dithiocarbamates and aflatoxin-DNA adducts ($P = 0.002$; $R = 0.31$) in individuals receiving broccoli sprout glucosinolates. Similarly, excretion of dithiocarbamates correlated inversely with the urinary levels of trans, anti-phenanthrene tetraol, a metabolite of the combustion product phenanthrene ($P = 0.0001$; $R = 0.39$). Taken together, these findings highlight the importance of fully understanding and controlling the factors that determine conversion and bioavailability of glucosinolates and isothiocyanates in the design of future chemoprevention studies.

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ABBREVIATIONS

ARE antioxidant response element
DMBA 7,12-dimethylbenz[*a*]anthracene

DTC	dithiocarbamate
GSH	glutathione
GST	glutathione *S*-transferase
Keap1	Kelch-like ECH-associated protein 1
NQO1	NAD(P)H:quinone oxidoreductase 1 (EC 1.6.99.2)
Nrf2	nuclear factor-erythroid 2-related factor 2
TPA	12-*O*-tetradecanoylphorbol 13-acetate

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9 Nutritional Redox Homeostasis and Cellular Stress Response: Differential Role of Homocysteine and Acetylcarnitine

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CONTENTS

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9.1 INTRODUCTION

Oxidative stress is correlated with a plethora of cellular alterations including the accumulation of oxidized-damaged molecules and increased levels of dysfunctional macromolecules, which impact cellular homeostasis. Oxidative stress occurs when there are insufficient levels of antioxidants to prevent reactive oxygen species (ROS) from promoting deleterious levels of oxidative damage. Examples of ROS include superoxide anion (O_2^-), hydroxyl radical (OH), singlet oxygen ($1O_2$), and hydrogen peroxide (H_2O_2) [1]. Each of these ROS is highly reactive and unstable due to the fact that they contain an unpaired electron in their outer electron shell. This conformation promotes their ability to rapidly interact with cellular macromolecules such as proteins, lipids, and nucleic acids [1]. Thus, when cells are unable to sufficiently regulate the levels of ROS, or are unable to adequately remove or replace oxidized macromolecules, cellular dysfunction can occur via oxidative stress. Interestingly, the propensity or sensitivity of cells to undergo oxidative stress appears to be cell-type specific, with cells exhibiting dramatic differences with regard to their sensitivity to accumulate oxidized molecules and undergo toxicity during periods of high ROS exposure [2–4]. The basis for this cell-type specificity is poorly understood but is clearly an important topic for aging, hepatic, cardiovascular, cancer, and neuroscience research [5–10]. As mentioned previously, there are several mechanisms by which ROS may be generated including aerobic respiration, nitric oxide synthesis, and NADPH oxidase pathways during inflammation. In aerobic respiration, the mitochondrial respiratory chain produces ROS as it transfers electrons during the reduction of molecular oxygen to water. During this process, some electrons escape the electron transport chain and interact with oxygen to generate superoxide, hydrogen peroxide, or hydroxyl radical [11]. Activated neutrophils can also release the enzyme myeloperoxidase, which produces the highly active oxidant hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions. In addition to these biological mechanisms of ROS generation, there are also exogenous sources of free radicals including drugs, manmade pollutants, environmental toxins, cigarette smoke, and radiation. The nitric oxide synthase (NOS) enzymes produce nitric oxide (NO) via the deamination of L-arginine to L-citrulline. NO is not highly reactive *per se* but can interact with other intermediates like oxygen, superoxide, and transition metal generating products that affect the functionality of macromolecules [12–15]. Recently, the term *nitrosative stress* has been used to indicate the cellular damage elicited by nitric oxide and its congeners peroxynitrite, N_2O_3, nitroxyl anion, and nitrosonium (all can be indicated as reactive nitrogen species or RNS) [16–18]. From a molecular point of view, the cell is able to fight against oxidant stress using many resources, including vitamins (A, C, and E), bioactive molecules (glutathione, thioredoxin, flavonoids), enzymes (heat shock protein-32, superoxide dismutase, catalase, glutathione peroxidases, thioredoxin reductase, etc.), and redox-sensitive protein transcriptional factors (AP-1, NFκB, Nrf-2, HSF, etc.). The heat shock proteins (Hsps) are one of the more studied defenses systems active against cellular damage.

In this chapter, we describe the more recent discoveries about the biochemical changes occurring in the central nervous system (CNS), when brain cells are

exposed to chronic oxidative insult as well as the key role played by the heat shock response, particularly the heme oxygenase (Hsp32) and Hsp70 pathways. Increasing evidence underscores the high potential of the Hsp system as a target for new neuroprotective strategies, especially those aimed at minimizing deleterious consequences associated with oxidative stress, such as in neurodegenerative disorders and brain aging. We review here the evidence for the emerging role of homocysteine in the pathogenesis of neurodegenerative damage as well as the role of acetylcarnitine in modulating redox-dependent mechanisms leading to upregulation of vitagenes in brain, and hence potentiate brain stress tolerance.

9.2 HEME OXYGENASE-1

Heme oxygenase-1 (HO-1), also referred to as heat shock protein-32, is the redox-sensitive inducible isoform of the HO family. Heme oxygenase is a microsomal enzyme and catalyzes the degradation of heme in a multistep, energy-requiring system. The reaction catalyzed by HO is the α-specific oxidative cleavage of heme moieties to form equimolar amounts of ferrous iron, carbon monoxide (CO), and biliverdin. This latter is then reduced by the cytosolic enzyme biliverdin reductase in bilirubin (BR), which is then conjugated with glucuronic acid and excreted [19].

Increasing evidence suggested that the HO-1 gene is redox regulated (Figure 9.1) and contains in its promoter region the antioxidant responsive element (ARE), similar to other antioxidant enzymes [20]. In fact, HO-1 can be induced by several

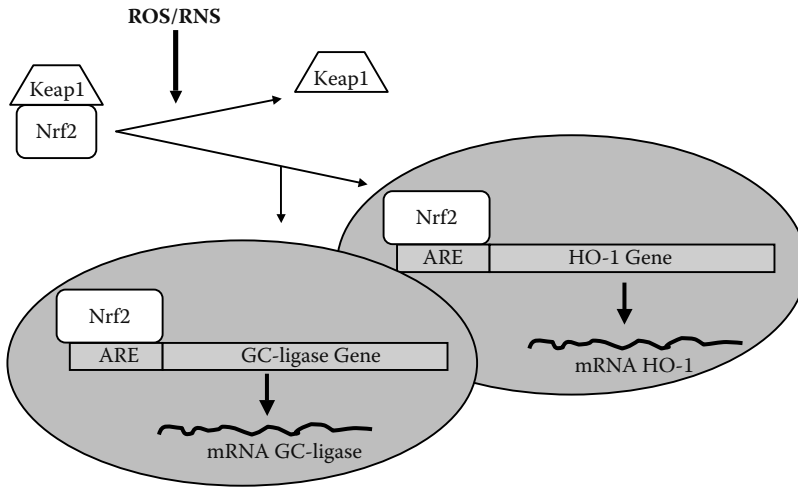

FIGURE 9.1 Regulation of Nrf-2-related genes. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor responsible for the induction of several genes related to the cellular stress response, including HO-1 and GC-ligase. Under normal conditions, Nrf2 is sequestered in the cytoplasm by an actin-binding protein, Kelch-like ECH associating protein 1 (Keap1), but upon exposure of cells to oxidative stress or nitrosative stress Nrf2 dissociates from Keap1, translocates to the nucleus, binds to antioxidant responsive elements (AREs), and activates HO-1 and GC-ligase genes.

stimuli including oxidative and nitrosative stress, ischemia, heat shock, LPS, hemin, and the neuroprotective agent Neotrofin [21–23]. HO-1 induction is one of the earlier cellular responses to tissue damage and is responsible for the rapid transformation of the pro-oxidant heme into CO and BR, two molecules with anti-inflammatory and antioxidant activity [24–27].

HO-1 gene is induced by a variety of factors (Figure 9.2), including metallophorphyrins and hemin, as well as ultraviolet A (UVA) irradiation, hydrogen peroxide, pro-oxidant states, and inflammation [28,29]. This characteristic inducibility of HO-1 gene strictly relies on its configuration: the 6.8-kilobase gene is organized into four introns and five exons. A promoter sequence is located approximately 28 base pairs upstream from the transcriptional site of initiation. In addition, different transcriptional enhancer elements, such as heat shock element and metal regulatory element, reside in the flanking 5′ region. Also, inducer-responsive sequences have been identified in the proximal enhancer located upstream the promoter and, more distally, in two enhancers located 4 and 10 kb upstream the initiation site [30]. The molecular mechanism that confers inducible expression of *ho-1* in response to numerous and diverse conditions has remained elusive. One important clue has recently emerged from a detailed analysis of the transcriptional regulatory mechanisms controlling the mouse and human *ho-1* genes. The induction of *ho-1* is regulated principally by two upstream enhancers, E1 and E2 [31]. Both enhancer regions contain multiple stress (or antioxidant) responsive elements (StRE, also called ARE) that also conform to the sequence of the Maf recognition element (MARE) [32] with a consensus sequence (GCnnnGTA) similar to that of other antioxidant enzymes [33]. There is now evidence to suggest that heterodimers of NF-E2-related factors 2 (Nrf2) and one or another of the small Maf proteins (i.e., MafK, mafF, and MafG) are directly involved in induction of *ho-1* through these MAREs [32]. A possible model, centered on Nrf2 activity, suggests that the *ho-1* locus is situated in a chromatin environment that is permissive for activation. Since the MARE can be bound by various heterodimeric basic leucine zipper (bZip) factors including NF-E2, as well as several other NF-E2-related factors (Nrf1, Nrf2, and Nrf3), Bach, Maf, and AP-1 families [31] random interaction of activators with the *ho-1* enhancers would be expected to cause spurious expression. This raises a paradox as to how cells reduce transcriptional noise from the *ho-1* locus in the absence of metabolic or environmental stimulation. This problem could be reconciled by the activity of repressors that prevent nonspecific activation. One possible candidate is the heme protein Bach1, a transcriptional repressor endowed with DNA binding activity, which is negatively regulated upon binding with heme. Bach1–heme interaction is mediated by evolutionarily conserved heme regulatory motifs (HRM), including the cysteine-proline dipeptide sequence in Bach1. Hence, a plausible model accounting for the regulation of *ho-1* expression by Bach1 and heme is that the expression of the *ho-1* gene is regulated through antagonism between transcription activators and the repressor Bach1 (Figures 9.1 and 9.2). While under normal physiological conditions, expression of *ho-1* is repressed by Bach1/Maf complex, increased levels of heme displace Bach1 from the enhancers and allow activators, such as heterodimer of Maf or Keap with NF-E2 related

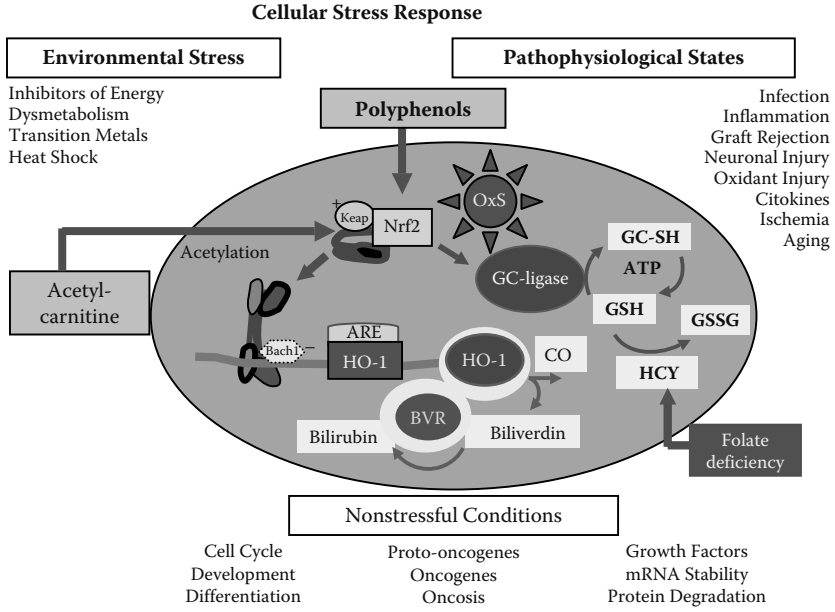

FIGURE 9.2 Heme oxygenase and the pathway of cellular stress response. Environmental stress factors, such as heavy metals, cytokines, heat shock, dysmetabolic conditions or energy inhibitors, or pathophysiological conditions of oxidant antioxidant balance perturbation (e.g., inflammation, graft rejections, neuronal damage, ischemia, and brain aging) are all situations associated with induction of cellular stress response. Hsp response is also involved in cellular homeostasis during various physiological conditions, such as during brain development and differentiation, cell cycle, apoptosis and oncosis, oncogene, and growth factors action, as well as mRNA and protein half-life. The heme oxygenase system represents a therapeutic funnel for cellular stress tolerance and can be activated by non-noxious stimuli, such as nutritional antioxidants or acetylcarnitine. Acetylcarnitine, through activation (via acetylation) of the redox-sensitive transcription factor Nrf2, and its consequent binding to the ARE (antioxidant responsive element) in the HO gene, upregulates HO-1 and glutathione synthesis, thus counteracting nitrosative stress and NO-mediated neurotoxicity. Also indicated are the respective roles of protein factors Bach-2 (positive) and Keap1 (negative) in the Nrf2 activation, the redox cycling between Bilirubin and biliverdin through the enzyme biliverdin reductase (BVR), as well as the role of folate deficiency in inducing neurotoxicity via increasing levels of the neurotoxin homocysteine (Hcy), and the consequent glutathione depletion associated with mitochondrial and DNA damage. OxS, oxidative stress; HO-1, heme oxygenase.

activators (Nrf2), to the transcriptional promotion of the *ho-1* gene [31] (Figure 9.2). To our knowledge, the Bach1–*ho-1* system is the first example in higher eukaryotes that involves a direct regulation of a transcription factor for an enzyme gene by its substrate. Thus, regulation of *ho-1* involves a direct sensing of heme levels by Bach1 (by analogy to *lac* repressor sensitivity to lactose), generating a simple feedback loop whereby the substrate affects repressor-activator antagonism.

The promoter region also contains two metal responsive elements, similar to those found in metallothionein-1 gene, which respond to heavy metals (cadmium and zinc) only after recruitment of another fragment located upstream, between −3.5 and 12 kbp (CdRE). In addition, a 163-bp fragment containing two binding sites for HSF-1, which mediates the HO-1 transcription, is located 9.5 kb upstream of the initiation site [34]. The distal enhancer regions are important in regulating HO-1 in inflammation, since, as has been demonstrated, are responsive to endotoxin. In the promoter region also resides a fragment 56 bp that responds to the STAT-3 acute-phase response factor, involved in the downregulation of HO-1 gene induced by glucocorticoid [35,36].

9.2.1 HO-1, Oxidative Stress, and Neurodegenerative Disorders

The mechanisms responsible for neuronal death are not completely elucidated, even if many studies suggest that ROS are primarily involved in the genesis of neurodegenerative disorders [11,12,37–39]. Due to its strong antioxidant properties and wide distribution within the CNS, HO-1 has been proposed as a key enzyme in the prevention of brain damage [21,22,40]. Recently, Panahian et al., using transgenic mice overexpressing HO-1 in neurons, demonstrated the neuroprotective effect of this enzyme in an experimental model of ischemic brain damage [41]. The neuroprotective effects of overexpressed HO-1 can be attributed to (1) an increase in cGMP and bcl-2 levels in neurons; (2) inactivation of p53, a protein involved in promoting cell death; (3) an increase in antioxidant sources; and (4) an increase in the iron sequestering protein ferritin [41]. Particularly interesting is the role played by HO-1 in Alzheimer's disease (AD), a neurodegenerative disorder that involves a chronic inflammatory response associated with both oxidative brain injury and β-amyloid associated pathology. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles and also HO-1 mRNA was found at increased levels in AD neocortex and cerebral vessels [42,43]. HO-1 increase was not only in association with neurofibrillary tangles, but also colocalized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains [44]. It is plausible that the dramatic increase in HO-1 in AD may be a direct response to an increase in free heme concentrations, associated with neurodegeneration, and can be considered as an attempt by brain cells to convert the highly toxic heme into the antioxidants CO and BR. The protective role played by HO-1 and its products in AD raised new possibilities regarding the use of natural substances, which are able to increase HO-1 levels, as potential drugs for the treatment of AD. In this light very promising are the polyphenolic compounds contained in some herbs and spices, for example, curcumin [45–47]. Curcumin is the active antioxidant principle in *Curcuma longa*, a coloring agent and food additive commonly used in Indian culinary preparations. This polyphenolic substance has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize ROS and RNS [48]. In addition, curcumin has been shown to significantly increase HO-1 in astrocytes and vascular endothelial cells [46,49]. This latter effect on HO-1 can explain, at

least in part, the antioxidant properties of curcumin, in particular keeping in mind that HO-1-derived BR has the ability to scavenge both ROS and RNS [24,27,50,51]. Epidemiological studies suggested that curcumin, as one of the most prevalent nutritional and medicinal compounds used by the Indian population, is responsible for the significantly reduced (4.4-fold) prevalence of AD in India compared to United States [52]. Based on these findings, Lim and colleagues have provided convincing evidence that dietary curcumin given to an Alzheimer's transgenic APPSw mouse model (Tg2576) for 6 months resulted in a suppression of indices of inflammation and oxidative damage in the brain of these mice [53]. Furthermore, in a human neuroblastoma cell line it has recently been shown that curcumin inhibits NFκB activation, efficiently preventing neuronal cell death [48].

Although it is generally agreed that HO-1 overexpression is a common feature during oxidative stress, recent papers demonstrated that HO-1 can be repressed following oxidant conditions. In particular, human cells exposed to hypoxia, thermal stress, and interferon-γ treatment showed a marked HO-1 repression and this effect seems to be peculiar for humans because rodent cells overexpressed HO-1 when exposed to the same stimuli [54–57]. The importance of HO-1 repression has been corroborated by the discovery of Bach-1/Bach-2 as heme-regulated transcription factors for the HO-1 gene [58]. In fact, Bach-1 is broadly expressed in mice and human tissues and, in human cells, it is induced by the same stimuli that are able to repress HO-1 gene [54,59–61]. The reason why the cell should react to an oxidant stress by repressing the HO-1 gene is strictly related to the maintenance of a good metabolic balance during stressful conditions. The current hypothesis suggests that HO-1 repression is useful for the cell because it (1) decreases the energy costs necessary for heme degradation; (2) reduces the accumulation of CO and BR, which can become toxic if produced in excess; and (3) increases the intracellular content of heme necessary for the preservation of vital functions, such as respiration and defense [60].

9.2.2 Carbon Monoxide and Stress Response

Carbon monoxide (CO) is the gaseous product of HO and it has been found to play a role in several biological phenomena, including hippocampal long-term potentiation, nonadrenergic noncholinergic gastrointestinal relaxation, and vasodilatation, and is currently regarded as a neuromodulator in the peripheral and central nervous system (for an extensive review of CO and its functions in the nervous system, see Ref. [40]). Evidence from *in vitro* and *in vivo* studies suggests that the HO–CO pathway is involved in the modulation of the neuroendocrine mechanism of stress. Thus, increased CO generation is clearly associated with the inhibition of K^+ stimulated arginine vasopressin (AVP) and oxytocin release from rat hypothalamic explants, whereas the inhibition of HO activity significantly potentiates the LPS-induced increase in AVP circulating levels while reducing the hypothalamic content of this neuropeptide [62–64]. The effects of CO on the release of the corticotropin-releasing hormone (CRH) are contradictory because increases in CO generation induced by two HO substrates, hematin and hemin, were associated

with reduced or enhanced CRH release, respectively, in two different *in vitro* models [65,66]. As far as the intracellular mechanism(s) by which CO exerts its biological functions, it is generally agreed that this gas activates the cytosolic form of guanylyl cyclase (sGC), which in turn increases intracellular cGMP levels [22]. However, during the last 10 years many studies arose in literature demonstrating that CO signals through the activation of alternative intracellular signal transduction pathways. Studies from our laboratory suggested that the activation of another hemoprotein, cyclooxygenase (COX), plays a significant role in CO signaling in the rat hypothalamus. In these studies, we demonstrated that hemin, the precursor of CO via HO, dose-dependently increases prostaglandin E_2 (PGE_2) production from rat hypothalamus *in vitro* and this effect is specifically due to CO because it is counteracted by the HO inhibitor Sn-mesoporphyrin-IX and oxyhemoglobin, the latter being a well-known scavenger for CO [67]. The direct evidence about the stimulatory role of CO on prostaglandin (PG) production was obtained incubating hypothalami directly in CO-saturated solutions and measuring significantly increased PGE_2 levels with respect to control tissue [25]. Recently, Jaggar and colleagues, in a very elegant paper, demonstrated that exogenously or endogenously produced CO dilates cerebral arterioles by directly activating large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels primarily by increasing the coupling ratio and amplitude relationship between Ca^{2+} sparks and K_{Ca} channels [68]. Although CO is a potent and effective activator of K_{Ca} channels, the gas does not dilate arterioles in the absence of Ca^{2+} sparks. Therefore, CO appears to act by priming K_{Ca} channels for activation by Ca^{2+} sparks, and this ultimately leads to arteriole dilation via membrane hyperpolarization [68]. Finally, Otterbein and colleagues have shown that in organs and tissues other than the brain, CO exerts anti-inflammatory and antiapoptotic effects dependent on the modulation of the p38 MAPK-signaling pathway [26]. By virtue of these effects, CO confers protection in oxidative lung injury models and likely plays a role in HO-1–mediated tissue protection [69].

9.2.3 Heat Shock Protein-70

The 70 kDa family of stress proteins is one of the most extensively studied. Included in this family are Hsc70 (heat shock cognate, the constitutive form), Hsp70 (the inducible form, also referred to as Hsp72), and GRP-75 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum).

Only recently, the availability of transgenic animals and gene transfer allowed us to overexpress the gene encoding for Hsp70, thus demonstrating that overproduction of this protein leads to protection in several different models of nervous system injury [70,71]. Following focal cerebral ischemia, Hsp70 mRNA is synthesized in most ischemic cells except in areas of very low blood flow, due to scarce ATP levels. Hsp70 proteins are produced mainly in endothelial cells, in the core of infarcts in the cells that are most resistant to ischemia, in glial cells at the edges of infarcts, and in neurons outside the areas of infarction [72]. It has been suggested that this neuronal expression of Hsp70 outside an infarct can be used to define the ischemic penumbras, which means the zone of protein denaturation in the ischemic areas [72].

As mentioned earlier, Hsps are induced in many neurodegenerative disorders mainly in the view of its cytoprotective function. Hsp72 was overexpressed in postmortem cortical tissue of AD patients and an increase in Hsp70 mRNA was found in the cerebellum hippocampus and cortex of AD patients during the agonal phase of the disease [73–75]. Recently, Kakimura et al. demonstrated that Hsp70 induces IL-6 and TNF-α in microglial cells, and this event is associated with an increased phagocytosis and clearance of Aβ peptides [76]. The same authors hypothesize that Hsps could activate microglial cells through NFκB and p-38 MAPK-dependent pathways [76].

A large body of evidence now suggests a correlation between mechanisms of nitrosative stress and Hsp induction. We have demonstrated in astroglial cell cultures that cytokine-induced nitrosative stress is associated with an increased synthesis of Hsp70 stress proteins. The molecular mechanisms regulating the NO-induced activation of heat shock signal seem to involve cellular oxidant/antioxidant balance, mainly represented by the glutathione status and the antioxidant enzymes [77,78].

9.3 ACETYLCARNITINE

Mitochondria are cellular organelles involved in many metabolic processes—such as pyruvate oxidation, the tricarboxylic acid cycle, and the fatty acid β-oxidation—and are the common final pathway of oxidative phosphorylation, which generates most of the cellular energetic source, ATP. It has been proposed that accumulation of mitochondrial DNA (mtDNA) mutations during life is a major cause of age-related disease and this is because of its high mutagenic propensity. The lack of introns and protective histones; limited nucleotide excision and recombination DNA repair mechanisms; and location in proximity to the inner mitochondrial membrane, which exposes mt DNA to an enriched free radical milieu, are all factors contributing to a 10-fold higher mutation rate occurring in the mtDNA than in the nuclear DNA. Relevant to mitochondrial bioenergetics, in fact, is the finding of a significant decrease in the state 3: state 4 ratio, which has been observed to occur in brain as function of age [79]. Since this ratio relates to the coupling efficiency between electron flux through the electron transport chain and ATP production, an increase in state 4 would result in a more reductive state of mitochondrial complexes and, consequently, to an increase in free radical species production. A decrease in state 3: state 4 respiration during aging has been found to be associated with a significant decrease in cardiolipin content in brain mitochondria [80]. This loss could play a critically important role in the age-related decrements in mitochondrial function, and appears to be associated with both quantitative and qualitative region-specific protein changes, which are parallel to structural changes (e.g., a decrease of the inner membrane surface, smaller as well as sparser cristae, decreased fluidity, and increased fragility). Modifications in cardiolipin composition are recognized to accompany functional changes in brain mitochondria, which include all proteins of the inner mitochondrial membrane that generally require interaction with cardiolipin for optimal catalytic activity [81].

Acetylcarnitine (LAC; Figure 9.3) is an ester of the trimethylated amino acid, L-carnitine, and is synthesized in the brain, liver, and kidney by the enzyme

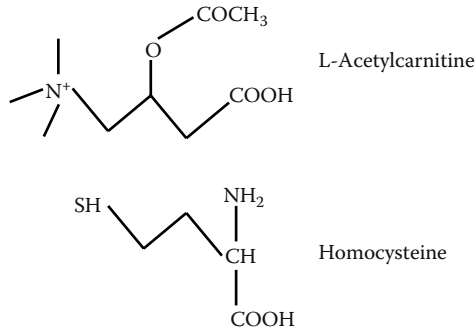

FIGURE 9.3 Chemical structures of L-acetylcarnitine and homocysteine.

LAC-transferase. LAC facilitates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation, enhances acetylcholine production, and stimulates protein and membrane phospholipid synthesis [82]. Studies have shown that LAC is a compound of great interest for its wide clinical application in various neurological disorders. It may be of benefit in treating Alzheimer's dementia, chronic fatigue syndrome, depression in the elderly, HIV infection, diabetic neuropathies, ischemia and reperfusion of the brain, and cognitive impairment of alcoholism and aging [83–85]. The neuroprotective benefits of this compound have been observed in the hippocampus, prefrontal cortex, substantia nigra, and muscarinic receptor portions of the brain [86]. These include antioxidant activity, improved mitochondrial energetics, stabilization of intracellular membranes, and cholinergic neurotransmission [87]. Promising therapeutic applications of LAC are derived from observations that this compound crosses the blood–brain barrier through a saturable process in a sodium-dependent manner and improves neuronal energetic and repair mechanisms, while modifying acetylcholine production in the CNS [88]. LAC treatment restores the altered neurochemical abnormalities, cerebral energy metabolites in ischemia and aging, and, in particular, ammonia-induced cerebral energy depletion [87]. In addition, it increases the responsiveness of aged neurons to neurotrophic factors in the CNS and it has preventive and corrective effects on diabetic neuropathology. Its beneficial effects have been also observed on EEG, evoked potentials, and long-term synaptic potentiation in aged animals [89]. Moreover, LAC is commonly used also for the treatment of painful neuropathies; it exerts a potent analgesic effect by upregulating metabotropic glutamate receptors [90]. There are experimental data that LAC improves memory function in Alzheimer's patients and it influences attention, learning, and memory in the rat [91]. Chronic treatment enhances spatial acquisition in a novel environment of rats with behavioral impairments and has a slight effect on retention of the spatial discrimination in a familiar environment [92]. More recently, it has been observed that LAC produces sustained changes of nonassociative learning of sensitization and

dishabituation type in the invertebrate *Hirudo medicinalis*, and it has been suggested that LAC might exert its effects by means of new protein synthesis, through qualitative and quantitative changes of gene expression. Furthermore, recent evidence has reported that it influences expression of glyoxylase 1, a gene involved in the detoxification of metabolic by-products, and increases p75-mRNA in Alzheimer's disease mutant transgenic mouse model Tg2576 [93]. Recently, by using suppressive subtractive hybridization (SSH) strategy, a PCR-based cDNA subtraction procedure particularly efficient for obtaining expressed transcripts often obscured by more abundant ones, it was reported that LAC modulates specific genes in the rat CNS, such as the hsp72 gene, the gene for the isoform of 14-3-3 protein and that encoding for the precursor mitochondrial P3 of ATP synthase lipid-binding protein [94].

Acetylcarnitine fed to old rats increased cardiolipin levels to that of young rats and also restored protein synthesis in the inner mitochondrial membrane, as well as the cellular oxidant/antioxidant balance, suggesting that administration of this compound may improve cellular bioenergetics in aged rats [95]. Fascinatingly, caloric restriction, a dietary regimen that extends the lifespan of rodents, maintains the levels of 18:2 acyl side chains, and inhibits the cardiolipin composition changes [96]. In addition, caloric restriction retarded the aging-associated changes in oxidative damage, mitochondrial oxidant generation, and antioxidant defenses observed during aging [97].

Interestingly, we have recently demonstrated that acetylcarnitine treatment of astrocytes induces HO-1 in a dose- and time-dependent manner and that this effect was associated with upregulation of other Hsps, as well as high expression of the redox-sensitive transcription factor Nrf2 in the nuclear fraction of treated cells. In addition, we showed that addition of LAC to astrocytes, prior to LPS and INFγ-induced nitrosative stress, prevents changes in mitochondrial respiratory chain complex activity, protein nitrozation, and antioxidant status induced by inflammatory cytokine insult [82]. Very importantly, this new envisioned role of LAC as a molecule endowed with the capability of potentating the cellular stress response pathways appears to provide an alternative therapeutic approach for those pathophysiological conditions where stimulation of the HO pathway is warranted [7]. Although clinical application of compounds potentiating the action of stress responsive genes should be fully considered, a better understanding of how HO mediates its action will guide therapeutic strategies to enhance or suppress HO effects. A role of Hsp70 as a vehicle for intracytoplasmic and intranuclear delivery of fusion proteins or DNA to modulate gene expression has been reported [98,99]. In addition, exists evidence that binding of HO protein to HO-1 DNA modifies HO expression via nonenzymatic signaling events associated with CO and P-38-dependent induction of Hsp70 exists [100]. These notions open intriguing perspectives, as it is possible to speculate that synergy between these two systems might represent a possible important target for acetylcarnitine action, with a possible impact on cell survival during times of oxidative stress. Hence these considerations may contribute to activation of cell life programs and to the extent of cellular stress tolerance.

9.4 HOMOCYSTEINE

Homocysteine (Hcy; Figure 9.3) is a nonprotein amino acid naturally occurring in the plasma, which has been implicated as a risk factor for numerous diseases. In particular, increased levels of circulating Hcy have been recognized as an independent risk factor for the development of vascular disease, and recent findings emphasize a relationship between elevated Hcy and neurodegeneration, which can be observed in some neurodegenerative diseases such as AD and Parkinson's disease (PD) [101,102].

Multiple enzymes and cofactors are involved in Hcy metabolism. Indeed, metabolic pathways consist of (1) remethylation to methionine, which requires methylenetetrahydrofolate reductase (MTHFR)/methionine synthase (MS) or betaine homocysteine methyltransferase (BHMT), and folic acid and vitamin B12 as cofactors; and (2) *trans*-sulfuration to cysteine, which requires cystathionine-beta-synthase (CBS) and pyridoxal-5′-phosphate, the vitamin B6 coenzyme [103]. Since MTHFR and BHMT are rate-limiting enzymes, vitamin deficiency may lead to increased plasma Hcy levels [104]. The other major determinants of hyperhomocysteinemia are the C677T and A1298C missense polymorphisms of the MTHFR gene. Carriers of either TT677/AA1298 or CT677/AC1298 diplotype can have dramatically elevated Hcy levels, especially if stores of folate or vitamin B12 are depleted [105].

Plasma membrane carrier-mediated uptake is present in neurons and glial cells, suggesting that Hcy can be imported from the plasma into the brain [106]. Particularly, high levels of Hcy in the CSF were found in several neuropathological conditions and paralleled those in serum; however, serum concentrations are 20–100-fold higher than concentrations in the CSF [107–109].

Therefore, it appears that mild to moderate increases in Hcy levels can be a risk factor for neuronal degeneration and thus hyperhomocysteinemia can be involved in neuronal damage. Additionally, it is noteworthy that plasma levels of Hcy increase with age and have been implicated in vascular disease as well as in brain atrophy [110–112]. Although *in vivo* and *in vitro* studies have suggested a causal role for Hcy in neurodegenerative diseases, clinical studies should be carried out to further evaluate the relationship between high levels of Hcy and cognitive decline, dementia, or AD. The overstimulation of glutamate receptors can be considered a common feature in several neurodegenerative conditions and alterations in intracellular calcium homeostasis mediate the toxicity of glutamate and NMDA for neurons. Consistently, among excitatory aminoacids, Hcy is toxic to the human and murine neuronal cell *in vitro*, [113] and its neurotoxicity is partially dependent on overstimulation of NMDA subtype glutamate receptors, which promotes increases in calcium influx. Moreover, recent attention has been focused on the cell damage produced in different cell types by Hcy exposure. It has been reported that Hcy impairs DNA repair in hippocampal neurons, and sensitizes them to oxidative stress. Hcy also potentiates the beta-amyloid-induced increase in cytosolic calcium and apoptosis in differentiated human neuroblastoma cells [114]. Additionally, in cerebellar granule cells, Hcy-mediated cell death can be

prevented by coadministration with superoxide dismutase (SOD) and catalase, or with catalase alone, suggesting that formation of hydrogen peroxide contributes to Hcy-mediated cell death. Hyperhomocysteinemia can be a consequence of folic acid deficiency that contributes to metabolic changes during nervous system development, as well as in aging process, and is involved in the pathogenesis of various diseases. In light of the data implicating increased DNA damage in neurons that degenerate in AD, it is noteworthy that folic acid deficiency and Hcy can impair DNA repair in neural and nonneuronal cells. However, folic acid deficiency and elevated Hcy levels increase the vulnerability of cultured hippocampal neurons to beta-amyloid-induced neuronal degeneration and death [115]. Both oxidative stress and DNA damage have been documented in neurons associated with beta-amyloid-containing plaques in the brains of AD patients (for review, see Ref. [116]). All this suggests that folic acid deficiency and Hcy promote the accumulation of DNA damage in neurons by impairing DNA repair mechanisms. In this context, postmitotic cells have been shown to be more vulnerable to DNA damage than mitotic cells, probably because of the lack of efficacy of DNA repair. In fact, it was shown that neurons and neuroblastoma cells become extremely UV-sensitive after terminal differentiation. Therefore, in mature neurons the mechanisms involved in the cell cycle activation can be an important component of the mechanisms associated with DNA damage leading to cell death. The exposure of cultured neurons to beta-amyloid induces caspase activation [117] and increased production of Par-4 and Bax, each of which appears to play an important role in the cell death process [118]. Apoptosis triggered by DNA damage typically involves activation of PARP and induction and activation of the tumor suppressor protein, p53 [119]. Increased PARP activity and p53 levels have been associated with degenerating neurons in AD patients and in cultured neurons exposed to beta-amyloid. Moreover, pifithrin-alpha, a p53 transactivation inhibitor, can protect neurons against beta-amyloid toxicity suggesting that, in DNA damage-responsive cell death, this pathway might play a key role in the pathogenesis of AD [120]. This evidence indicates that by impairing the DNA repair capacity in neurons, folic acid deficiency and elevated homocysteine levels lower the threshold level of DNA damage that is required to trigger neuronal death. Consistently, folic acid deficiency and elevated Hcy levels accelerated the accumulation of DNA damage that is promoted by age-related increases in oxidative stress and by accumulation of beta-amyloid [114]. Neurons are more vulnerable to DNA damage than nonneuronal cells, suggesting that the brain may be particularly sensitive to diets deficient in folic acid and other nutritional and genetic factors associated with one carbon metabolism [114].

In humans, the normal range of Hcy concentrations in plasma is 5–13 μM, and levels of Hcy in CSF and brain tissue are reported to range from 0.5 to 10 μM [121]. Plasma folic acid levels decrease and homocysteine levels increase with age, and to an even greater extent in patients with AD and PD [122]. However, in long-term patients with AD, or following acute lesions such as stroke, neurons will degenerate and axons will not be replaced. Therefore, vitamin supplementation did not improve brain function [123]. However, the involvement of Hcy as a risk

factor for stroke has been confirmed by many investigators and recent data demonstrated an improvement in stroke mortality after folate fortifications [124]. To summarize, different treatments are used to lower Hcy levels. The efficacy can be improved if the intervention is started at an early stage of the disease. Several findings suggested a causal link between hyperhomocisteinemia and neurological disorders. However, available results from treatment studies are very limited and further studies are required to demonstrate the efficacy of treatments against hyperhomocysteinemia conditions. Nevertheless, on the basis of the available results, useful efforts can be made to prevent more than reduce the effects of hyperhomocysteinemia and early intervention can play a crucial role to be beneficial. Therefore, increasing vitamin intake can be considered a neuroprotective strategy in age-related disease.

9.5 CONCLUSIONS AND PERSPECTIVES

Modulation of endogenous cellular defense mechanisms via stress response signaling represents an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Efficient functioning of maintenance and repair processes seems to be crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed *vitagenes*. Maintaining or recovering the activity of vitagenes could possibly delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy lifespan [6,7,61]. As one of the most prevalent neurodegenerative disorders, AD is a progressive disorder with cognitive and memory decline, speech loss, personality changes, and synapse loss. With an increasingly aging U.S. population, the number of AD patients is predicted to reach 14 million in the mid-twenty-first century in the absence of effective interventions [2,45]. This will pose an immense economic and personal burden on the people of this country. Similar considerations apply worldwide, except in sub-Sahara Africa, where HIV infection rates seem to be leading to decreased incidence of AD. There is now strong evidence to suggest that factors such as oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle are central to AD pathogenesis. Brain-accessible antioxidants may potentially provide the means of implementing this therapeutic strategy of delaying the onset of AD and all degenerative diseases associated with oxidative stress [47]. As one potentially successful approach, potentiation of endogenous secondary antioxidant systems can be achieved by interventions that target the HO-1/CO and/or Hsp70 systems. In this review, the importance of the stress response signaling and, in particular, the central role of HO-1 together with the redox-dependent mechanisms involved in cytoprotection were outlined. The beneficial effects of HO-1 induction result from heme degradation and cytoprotective regulatory functions of biliverdin/bilirubin redox cycling. Thus, HO-1 can amplify intracellular cytoprotective mechanisms against a variety of insults. Consequently, induction of HO-1, by increasing CO and/or biliverdin availability can be of clinical relevance.

Very importantly, HO-1 and CO can suppress the development of atherosclerotic lesions associated with chronic rejection of transplanted organs [125]. LAC, as a molecule endowed with the capability of potentiating the cellular stress response pathways, consistently appears to afford similar protective action, thereby providing an alternative therapeutic approach valuable for all those pathophysiological conditions where stimulation of the HO pathway becomes a primary target.

Aging is the most important risk factor for common neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. In the central nervous system aging has been associated with elevated mutation load in mitochondrial DNA, defects in mitochondrial respiration and increased oxidative damage. These observations support a 'vicious cycle' theory which states that there is a feedback mechanism connecting these events in aging and age-associated neurodegeneration. Despite being an extremely attractive hypothesis, the bulk of the evidence supporting the mitochondrial vicious cycle model comes from pharmacological experiments in which the modes of mitochondrial enzyme inhibition are far from those observed in real life. Furthermore, recent *in vivo* evidence does not support this model [126].

Presented here is strong evidence that a crosstalk between stress response genes is critical for cell stress tolerance, highlighting a compelling reason for a renewed effort to understand the central role of this most extraordinary defense system in biology and medicine [27–131]. All of the presented evidence supports the notion that stimulation of various maintenance and repair pathways through exogenous intervention, such as mild stress or compounds targeting the heat shock signal pathway (e.g., LAC), may have biological significance as a novel approach to delay the onset of various age-associated alterations in cells, tissues, and organisms. Hence, by maintaining or recovering the activity of vitagenes it can be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span.

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10 Dietary Factors in the Regulation of Selenoprotein Biosynthesis

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CONTENTS

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10.1 INTRODUCTION

The human genome contains 25 genes that encode about 30 selenoproteins [1]. The functions are more or less known for glutathione peroxidases, deiodinases, thioredoxin reductases, selenoprotein P, and selenophosphate synthetase-2, whereas the function of the remaining selenoproteins remains mainly unclear (Table 10.1).

The dietary factor that is almost exclusively considered in the regulation of selenoprotein expression is selenium itself. In nature, selenium occurs in many chemically diverse compounds. To be incorporated into selenoproteins, selenium

TABLE 10.1
List of Some Representative Mammalian Selenoproteins

Mammalian Selenoproteins	Common Abbreviations
Glutathione peroxidase	**GPx**
Cytosolic or classical GPx	cGPx, GPx-1
Phospholipid hydroperoxide GPx	PHGPx, GPx-4
Plasma GPx	pGPx, GPx-3
Gastrointestinal GPx	GI-GPx, GPx-2
GPx3-homolog	GPx-6
Iodothyronine deiodinases	
5′-Deiodinase, type 1	5′DI-1
5′-Deiodinase, type 2 (also called SelY)	5′DI-2
5-Deiodinase, type 3	5-DI-3
Thioredoxin reductases	
Thioredoxin reductase-1	TrxR1
Mitochondrial thioredoxin reductase	TrxR2
Thioredoxin and glutathione reductase	TGR
Selenophosphate synthetase-2	**SPS2**
15-kDa selenoprotein (T cells)	
Selenoprotein P	SelP
Selenoprotein W	SelW
Selenoprotein R (also called SelX; this protein is a methionine sulfoxide reductase)	MrsB
Selenoprotein T	SelT
Selenoprotein M	SelM

(*continued*)

TABLE 10.1 (continued)
List of Some Representative Mammalian Selenoproteins

Mammalian Selenoproteins	Common Abbreviations
Selenoprotein N (located in the endoplasmic reticulum; knockout causes muscular dystrophy with spinal rigidity and restrictive respiratory syndrome)	SelN
Selenoprotein H	SelH
Selenoprotein I	SelI
Selenoprotein K	SelK
Selenoprotein O	SelO
Selenoprotein S (human homolog of tanis)	SelS
Selenoprotein V	SelV

compounds have to be converted to selenide. By far not all of the naturally occurring selenium compounds are metabolized this way and only a few of them have been tested for their ability to function as precursors for selenoprotein biosynthesis. The diversity of selenium compounds and the unknown functions of many selenoproteins make it difficult to predict the biological effects of individual forms of selenium. Moreover, increased expression of certain selenoproteins that is mediated by factors other than selenium reveals more complex regulatory phenomena. This chapter will summarize how the expression of selenoproteins is regulated at the translational level by selenium and at the transcriptional level by other dietary compounds.

10.2 SELENOPROTEIN BIOSYNTHESIS

10.2.1 Mechanism of Translation

Selenoproteins contain selenium in form of selenocysteine (Sec), which is incorporated during translation, encoded by UGA, and presented for incorporation by a specific tRNA, $tRNA^{(Ser)Sec}$ (reviewed in Refs. [2,3]). The $tRNA^{(Ser)Sec}$ is first loaded with serine. In a mechanism that is so far not clearly understood, serine is converted into selenocysteine by means of selenophosphate catalyzed by a selenocysteine synthase. The UGA codon usually means stop and, thus, cells had to establish a complex mechanism to differentiate between UGA meaning stop and UGA meaning Sec (Figure 10.1). An important cis-acting factor is a secondary structure called Secis (for selenocysteine inserting sequence), which in mammals is located in the 3′UTR of the mRNA. When the translation process reaches the UGA, the ribosome pauses, the Secis-binding protein-2 (SBP2), binds to the Secis, and recruits the guanosine triphosphate (GTP) binding elongation factor EFsec together with the Sec-loaded $tRNA^{Sec}$ (Sec $tRNA^{Sec}$). Once associated with the ribosome, SBP2 is displaced by the ribosomal protein L30, which guides the Secis complex close to the ribosomal A site. Thereby, a conformational change of the Secis is induced that activates EFsec to hydrolyze

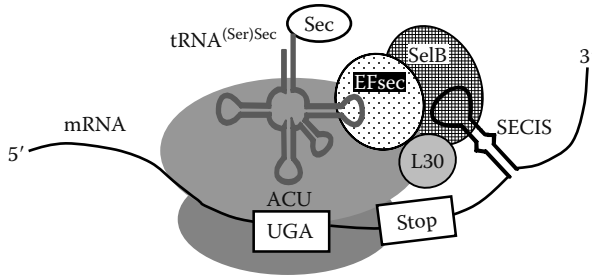

FIGURE 10.1 A simplified model for selenocysteine incorporation. The selenocysteine inserting sequence (Secis) in the 3′UTR of the selenoprotein mRNA binds the Secis-binding protein-2 (SBP2). SBP2 recruits the elongation factor EFsec and the Sec-loaded $tRNA^{Sec}$. When the complex associates with the ribosome SBP2 exchanges for the ribosome-associated protein L30. The exchange causes a conformational change in the Secis, which finally guides the tRNA to the A site of the ribosome and leads to the incorporation of selenocysteine into the growing peptide chain. See the text for further details.

GTP and to deliver the Sec $tRNA^{Sec}$ to the A site of the ribosome, where Sec can be incorporated into the growing peptide chain [4,5].

10.2.2 Hierarchy of Selenoproteins

Selenoproteins do not equally respond to selenium. Whereas protein levels of the classical glutathione peroxidase (cGPx) and selenoprotein P (SelP) rapidly decrease when selenium becomes limiting, GI-GPx, PHGPx, and 5′deiodinases remain relatively stable (reviewed in Refs. [6,7]). Reoccurrence upon refeeding of selenium is in the opposite direction. From this phenomenon, called hierarchy of selenoproteins, it has been inferred that selenoproteins ranking high in the hierarchy, that is, remaining stable under selenium restriction, might have more essential functions than those ranking low.

The selenium-dependency of the expression of a selenoprotein is determined by its mRNA stability. The Sec-encoding UGA may be recognized as premature stop codon that makes mRNAs of selenoproteins potential substrates for the nonsense mediated decay (NMD) [8]. However, not all selenoprotein mRNAs are degraded, but are resistant to NMD, particularly those of selenoproteins ranking high in the hierarchy [3,6]. Several hypotheses have been tested to explain this phenomenon:

1. The structure of Secis elements might contribute, but is not sufficient since 3′UTRs from instable mRNAs can confer instability to stable mRNAs but not vice versa [9,10].
2. SBP2 contains domains homologous to RNA-binding regions and a yeast suppressor of translation termination [11,12], which points to a function in the prevention of termination. Thus, affinity of SBP2 to individual Secis elements might play a role. Accordingly, the affinity of SBP2 to six different Secis elements has been measured by electrophoretic mobility

shifts [13], but differed maximally by a factor of 2, which cannot comply either with the positions of the selenoproteins in the hierarchy.
3. Import of SBP2 to the nucleus and binding to the Secis immediately after its transcription has been suggested to prevent the degradation of the selenoprotein mRNA [14].
4. The interaction of NSEBP-1 (nuclease sensitive element binding protein-1) with the Secis has been suggested to prevent degradation [15].

So far, however, none of these mechanisms explains why mRNA stability depends on selenium. The differential response of an individual selenoprotein to the selenium supply can only be achieved within a complex that contains (1) a selenium compound; (2) a selenium sensor, which may be or activate and (3) an mRNA stabilizing factor. The only known selenium-containing component of the Secis complex still is Sec $tRNA^{(Ser)Sec}$. In bacteria, Sec $tRNA^{Sec}$ suppresses the translation of selenocysteine synthase (SelA) and of SelB, the bacterial Secis binding protein, by interaction with a 5′UTR Secis-like element in the SelAB transcript. Competition between the Secis-like and the Secis elements in the RNAs leads to the dissociation of the SelB-GTP-Sec $tRNA^{Sec}$ from the regulatory structure and allows production of SelA and in consequence new Sec $tRNA^{Sec}$. In this way formation of the selenocysteine synthase is suppressed at high selenium supply and increased at limiting selenium supply [16]. Although this does not satisfactorily explain the situation in mammals, it shows that the level of Sec $tRNA^{Sec}$ might have a more important role than previously considered.

In practical terms, the diverse positions of selenoproteins in the hierarchy proved to be helpful in the evaluation of the alimentary selenium status and hence were used to define the dietary reference intake (DRI). Accordingly, SeMet and selenite can easily enhance glutathione peroxidase activity in plasma or platelets. The GPxs have, therefore, been taken as biomarkers for selenium status [17]. Recently, however, GPxs turned out not to be the best biomarkers. Surprisingly, maximal selenoprotein P expression needed a much higher selenium intake than maximal plasma GPx expression [18]. These findings leave open the possibility that the DRI might be not adequate, since it is mainly based on the selenium amount required for optimal plasma and blood GPx activities, and might also question the concept that dosages of selenium required for chemopreventive effects generally exceed those required for optimizing selenoprotein biosynthesis [19,20].

10.3 DIETARY SELENIUM COMPOUNDS AS SOURCES FOR SELENOPROTEINS

10.3.1 Hydrogen Selenide: The Gateway to Selenoprotein Synthesis

As previously mentioned, selenium compounds have to be metabolized to selenide to become donors for selenoprotein biosynthesis. This means that they have to deliver selenium as hydrogen selenide. Hydrogen selenide becomes activated with ATP to selenophosphate (Figure 10.2) by selenophosphate synthetase (SPS) [21].

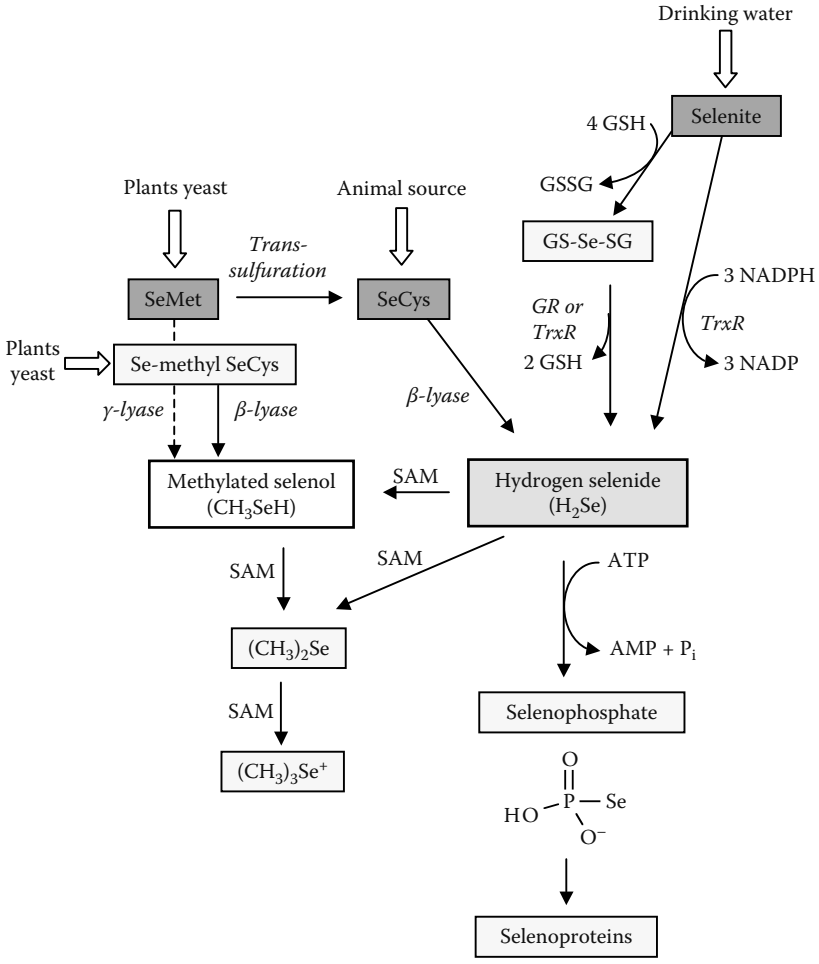

FIGURE 10.2 Pathways of selenium metabolism. Selenite reacts with glutathione to form selenodiglutathione (GS-Se-SG), a major metabolite of inorganic selenium salts in mammalian tissue [108]. GS-Se-SG can be reduced by NADPH and glutathione reductase (GR) [109] and more efficiently by thioredoxin reductase (TrxR) [110]. Alternatively, selenite can be directly reduced to selenide by TrxR and NADPH [110]. The SeCys β-lyase splits selenocysteine (SeCys) into selenide and alanine [111]. Methionine γ-lyase catalyzes α-γ-elimination of selenomethionine (SeMet) into α-ketobutyrate, ammonia, and methylselenol [112]. Se-methyl SeCys is split by a cysteine conjugate β-lyase [113]. Hydrogen selenide is the precursor for selenoprotein biosynthesis or is methylated for excretion by methyltransferases and S-adenosylmethionine (SAM) as is methylated selenol. See the text for further details.

Apart from the selenophosphate synthetases 1 and 2, which directly phosphorylate selenide [22], a phosphoseryl-tRNA$^{(Ser)Sec}$ kinase may be involved that phosphorylates serine bound to tRNA$^{(Ser)Sec}$ [23].

The major forms of selenium occurring in food are the organic, mostly protein-associated selenomethionine (SeMet, in plants and animal sources) and selenocysteine (in animal sources). Selenocysteine is cleaved by a β-lyase directly into selenide and alanine, which is further metabolized into pyruvate and ammonia [24], whereas SeMet has first to be "transsulfurated" into selenocysteine (Figure 10.2). In contrast to the tRNA^{Cys}, the tRNA^{Met} does not differentiate between sulfur and selenium in the methionine. Thus, SeMet can nonspecifically be incorporated into proteins without the production of functional selenoproteins. Inorganic salts such as selenate and selenite are predominantly provided by drinking water. At least selenite can be reduced directly to selenide by thioredoxin reductase or glutathione reductase (Figure 10.2).

10.3.2 Methylated Selenols

Selenium accumulating plants predominantly produce selenomethionine (reviewed in Refs. [25,26]). With technical progress, however, many more selenium compounds have been identified in plants. Many of them are compounds methylated at the selenium moiety from which methylselenol can be liberated. Whereas selenium enriched yeast mainly produces selenomethionine [27], Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine are the major compounds present in selenium-enriched garlic [28]. The predominant form of selenium in selenium-enriched onion is γ-glutamyl-Se-methylselenocysteine in bulb and Se-methylselenocysteine in leaves (reviewed in Ref. [29]). The anticarcinogenic effect of selenium has been attributed to these latter compounds, since selenium-enriched garlic proved to have better anticarcinogenic properties than selenium-enriched yeast [30]. Furthermore, Se-methylselenocysteine [31,32], Se-alkylated or Se-allylated selenocysteine [33], and methylseleninic acid [30] were particularly effective. Methylselenol can be formed by methylation of hydrogen selenide as part of the selenium excretion pathway; from SeMet in a γ-lyase-catalyzed reaction or an α,γ-elimination; or from γ-glutamyl-Se-methylselenocysteine by the β-lyase (reviewed in Ref. [34]). Alk(en)ylated selenocysteine can be split into alk(en)ylated selenides by the β-lyase [35]. Unfortunately, it has only been sporadically tested whether selenoproteins are upregulated by these compounds. There is, however, one report on the induction of glutathione-S-transferases by selenocysteine Se-conjugates most probably by the activation of Nrf2 [36], the same mechanism by which certain selenoproteins can be upregulated at the transcriptional level (see Section 10.4.1).

Methylated selenium compounds have been extensively tested in cell culture studies for their anticarcinogenic mechanism. The reported responses comprise inhibition of VEGF expression, inhibition of androgen receptor expression and androgen signaling, induction of caspase-mediated apoptosis, induction of cell arrest, and inhibition of MMP-2 expression (reviewed in Ref. [37]). Thus, there is

room for distinct roles of dietary selenium depending on the chemical form, that is, selenoprotein biosynthesis and effects independent thereof.

10.4 NONSELENIUM DIETARY COMPOUNDS IN THE REGULATION OF SELENOPROTEIN EXPRESSION

10.4.1 Regulation by the Nrf2/Keap1 System

Nrf2 (NF-E2-related factor 2) is a member of the NF-E2 family of basic leucine zipper transcription factors (b-ZIP). It binds to the antioxidant response element (ARE), better called the electrophile responsive element (EpRE), which is present in the promoters of genes encoding phase 2 enzymes and enzymes of the antioxidant system and activates their transcription [38]. Nrf2 interacts with Keap1, the Kelch-like ECH-associated protein-1. Keap1 contains 25 cysteine residues, which are conserved in man, rat, and mouse, some of them serving as redox sensors [39,40]. Keap1 retains Nrf2 in the cytoplasm and prevents its activation [41]. The release of Nrf2 and its subsequent nuclear translocation is achieved by a change in the conformation of Keap1 via modification of one or more of the crucial cysteine residues. Many Nrf2 activators (Table 10.2) are derived from the diet (reviewed in Ref. [42]). Their chemical structure is diverse but they all are electrophilic, can modify thiols, or chelate metal ions [43–45].

10.4.1.1 Thioredoxin Reductase-1 as Nrf2/Keap1-Regulated Selenoprotein

Thioredoxin reductases (TrxRs) are a family of NADPH-dependent selenoflavoproteins with three members so far: the cytosolic form (TrxR1), the mitochondrial

TABLE 10.2
Some Dietary Components Capable of Activating Nrf2

Dietary Activators of Nrf2	References
Isothiocyanates	[44]
Curcumin	[114]
Oxidizable hydroquinones	[44]
Carotenoids/polyenes	[115]
(−)Epigallocatechin-3-gallate	[116]
(−)Epicatechin-3-gallate	[116]
Organosulfur compounds	[117]
Xanthohumol	[118]
Chlorogenic acid	[119]
α-Lipoic acid	[120]

form (TrxR2), and the testes-specific thioredoxin and glutathione reductase (TGR; for review see Ref. [46]). TrxR is a homodimeric protein that contains two distinct redox centers in each subunit. The first one resembles typical disulfide reductases in comprising FAD with associated cysteines. The second one is situated at the C-terminus and consists of Gly-Cys-Sec-Gly-COOH [47]. Its role is to transfer the reduction equivalents from the central redox center to the substrate. TrxRs reduce Trx-S_2 to Trx-$(SH)_2$ using NADPH as reduction equivalents. Historically, Trx was identified as a key factor for DNA synthesis by directly transducing electrons to ribonucleotide reductase [48]. More generally, the Trx/TrxR couple acts as a protein disulfide reductase system that contributes to the redox regulation of transcription factor activity, for example, of NFκB or p53; to the inhibition of apoptosis by an inhibitory binding to ASK-1 (apoptosis stimulating kinase-1 [49]; for review see Ref. [50]); and to the regulation of cell growth. Actually, Trx had independently been identified as the adult T-cell leukemia factor that acts as a growth factor for lymphoid cells [51]. Finally, the Trx/TrxR system provides reduction equivalents for oxidized peroxiredoxins, a novel family of peroxidases involved in the cellular signaling and differentiation [52]. Thus, influencing the expression of Trx and/or TrxR substantially contributes to cellular redox regulation processes.

TrxR1 was the first selenoprotein shown to be directly induced by Nrf2 [53–56]. The studies were initiated by the observation that Nrf2 upregulates multiple antioxidant enzymes (e.g., NAD(P)H:quinone oxidoreductase-1, γ-glutamylcysteine synthetase, and glutathione *S*-transferase) [57], and that TrxR-activity and protein levels were induced by *t*-butylhydroquinone [58]. The TrxR1 promoter contains two putative AREs. The involvement of both AREs in the induction was demonstrated by a mutation in the respective core sequences, which resulted in a decrease in the response to sulforaphane [54]. Interestingly, also thioredoxin (Trx) is upregulated via Nrf2 [59]. The physiological role of the upregulation of the Trx/TrxR system is not clear. Although the continuous reduction of oxidized Trx by TrxR is indispensable for the survival of healthy cells, the upregulation of the Trx/TrxR system in cancer cells also lets tumor cells survive [60,61]. In fact, inhibition of TrxR1 prevented cancer cell growth *in vivo* [61], and TrxRs have been suggested as potential targets for anticancer drugs [62]. In so far, upregulation of the Trx/TrxR system might not necessarily be beneficial under all circumstances. The upregulation of the entire system by a transcription factor that is generally accepted to trigger the adaptive response, however, does not support this concern, neither does the initial consideration that the redox-active thioredoxin system prevents tumor initiation by prevention of oxidative DNA damage. At present, upregulation of Trx/TrxR is rather discussed to contribute to the inhibition of tumor initiation than to inhibit the growth of an existing tumor.

10.4.1.2 Glutathione Peroxidases and the Adaptive Response

The GI-GPx belongs to the family of glutathione peroxidases, which catalyze the reduction of hydroperoxides to the corresponding alcohol by means of glutathione

(GSH). Five of six GPxs are selenoproteins (Table 10.1). Although the catalyzed reaction appears to be the same for all known GPxs, individual GPxs differ in substrate specificity and localization (reviewed in Ref. [6]). They also differ in transcriptional regulation and function [63]. Whereas cGPx, as obvious from studies with knockout mice, acts as an antioxidant device [64], the other GPxs may have additional functions. PHGPx is indispensable for male fertility [65] and by inhibition of NFκB activity it might interfere with tumor cell growth (reviewed in Ref. [66]). All GPxs in general may dampen the Nrf2-mediated adaptive response by lowering the cellular hydroperoxide tone. One of them, however, the gastrointestinal isoenzyme, is also a target of the Nrf2/Keap1 system.

10.4.1.2.1 Gastrointestinal Glutathione Peroxidase

GI-GPx was first identified to be exclusively expressed in the gastrointestinal system and suspected to act as a barrier against hydroperoxide absorption. Yet it soon became obvious that its expression was not limited to the GI system. GI-GPx was found in several cancer cell lines and was upregulated in preneoplastic lesions in skin cancer [67] and in early stages of adenomatous polyposis in the gut [68]. Upregulation of GI-GPx might have beneficial effects. GI-GPx$^{-/-}$ mice appear to have a normal phenotype [69], but mice deficient in cGPx (GPx1) and GI-GPx showed retarded growth after weaning, exhibited severe ileocolitis [70], and developed intestinal cancer [71]. Interestingly, the development of ileocolitis and tumors was dependent on colonization with intestinal bacteria, which in wild-type mice induced GI-GPx expression [72]. Taken together, these observations point to a pivotal role of GI-GPx as anti-inflammatory enzyme that essentially contributes to the development of tolerance against the intestinal microflora [73].

Sporadically, *gpx2* appeared in lists of genes upregulated by sulforaphane or hyperbaric oxygen [74,75]. Starting from these observations, two AREs were identified in the GI-GPx promoter from which the ATG-proximal ARE proved to be indispensable for endogenous: curcumin- and sulforaphane-induced GI-GPx expression [76]. The GI-GPx promoter-driven reporter gene expression was enhanced by transfection with Nrf2 and suppressed by transfection with Keap1. The induction of endogenous mRNA and protein in Caco2 cells by sulforaphane and *t*-butylhydroquinone finally identified GI-GPx as a target for Nrf2 [76]. If it turns out that GI-GPx is indeed an anticarcinogenic enzyme, the chemopreventive action of many phytochemicals might, in part at least, be mediated by an induction of this particular selenoprotein.

A question remains: When does it makes sense to increase the mRNA of a high-ranking selenoprotein that is preferentially synthesized anyway? The most plausible answer might be under selenium-limiting conditions when the expression of high-ranking selenoproteins is decreased. The limited amounts of selenium could then be used to translate an enhanced level of mRNA. The ability of trace amounts of GI-GPx to prevent ileocolitis and, in consequence, tumor formation was convincingly demonstrated in mice with the genotypes *Gpx1*$^{-/-}$*Gpx2*$^{+/-}$ and *Gpx1*$^{+/-}$*Gpx2*$^{-/-}$ that were grown under restricted selenium supply [77]. One allele

of *gpx2* was sufficient for complete protection even under selenium restriction. Certainly, however, a combination of Nrf2 activators with selenium might be even more efficacious (see Section 10.4.2).

10.4.1.2.2 Classical Glutathione Peroxidase

The cGPx promoter contains an oxygen-responsive element [78], and the targeting binding protein (OREBP) responding to oxygen tension has been characterized [79]. Further, human cardiomyocytes [80] and endothelial cells [81] respond to hyperoxia with an upregulation of cGPx. Although increased oxygen tension might also contribute to the required oxidative modification of Keap1, a direct link to cGPx expression is not obvious, since an ARE appears to be absent in the cGPx promoter. The capacity of the cGPx/GSH system, however, is typically enhanced upon exposure to peroxides or redox cyclers by the induction of γ-glutamylcysteine synthetase, which influences signaling pathways that depend on the cellular glutathione status [82].

10.4.2 Nrf2 Activators and Selenium

Only a few Nrf2 activators have so far been tested for their capability to induce selenoproteins, and most of the studies have been performed with cultured cells. Induction of GI-GPx was observed in microarrays from sulforaphane-fed mice [74]. In another study, induction of TrxR in Fisher 344 rats was achieved by administration with both sulforaphane and broccoli [55]. Surprisingly, total GPx activity—to which mainly cGPx contributes—was decreased in the liver by the high dose of sulforaphane. The finding was explained by selenium withdrawal by the increased production of TrxR, which ranks higher in the hierarchy of selenoproteins. If GI-GPx had been simultaneously induced (which was not measured), a second selenoprotein could have deprived cGPx from selenium. Taken together, maximum upregulation of a selenoprotein by Nrf2-activators may require the activator plus selenium.

TrxR1 induction by sulforaphane was synergized by selenium supplementation [53], indicating that the increase in mRNA can be used to translate more protein if selenium is available. Vegetables serving as good sources for a high selenium and high sulforaphane supply are *cruciferae*, since they do not only accumulate glucosinolates but also selenium. Accordingly, increasing the selenium content in broccoli should raise the power of its glucosinolates. Broccoli grown on selenium-fertilized ground indeed inhibited the formation of chemically induced preneoplastic lesions in rat colon [83], of spontaneous development of intestinal tumors in mice [84], and of mammary tumors in rats [85]. But a recent *in vitro* study failed to explain the *in vivo* findings by the proposed interaction of sulforaphane and selenium in the adaptive response. Surprisingly, the sulforaphane content of broccoli grown on substrates with high, medium, and low selenium was inversely correlated with the selenium content [86]. Indeed, selenium fertilization of broccoli changed the pattern of phenolic compounds and the content of glucosinolates, especially that of glucoraphanine from which sulforaphane is derived [87]. In consequence, the extract of selenium-enriched broccoli did not induce an

adaptive response, as measured by NQO1 expression, whereas it optimized the selenium-dependent antioxidant systems, as was evident from a slight increase of TrxR1 activity and a rise of the primary antioxidant selenoenzyme cGPx [86]. The latter effect was associated with optimum protection against oxidative DNA damage in terms of DNA strand breaks. This observation might explain why selenium-enriched broccoli exerts chemopreventive efficacy *in vivo* despite a marginal or absent induction of the adaptive response.

10.4.3 Regulation by Vitamins

10.4.3.1 Vitamin A

Chu et al. [88] first identified several caudal homeobox protein-binding sequences and two retinoic acid responsive elements in *gpx2* of human breast carcinoma cells and showed that endogenous GI-GPx could be induced by retinoic acid in some (MCF-7) but not all (HT29) cells. More recently, Morbitzer and Herget [89] discovered that GI-GPx was downregulated in hepatoma cells infected with hepatitis C virus subgenomic RNA. Induction of GI-GPx by retinoic acid suppressed the HCV replicon [89], suggesting a therapeutically interesting inverse relationship of GI-GPx levels and viral replication. A second selenoprotein responding to vitamin A is human type I 5′-deiodinase [90]. The promoter of type I 5′-deiodinase was activated in HEK-293 cells that were cotransfected with expression plasmids coding for RAR and RXR upon treatment with all-*trans* retinoic acid about 2.5-fold.

10.4.3.2 Vitamin D

A differentiated display of mRNA isolated from fetal human osteoblasts treated with vitamin D ($1,25(OH)_2D3$) for 20 h revealed the induction of an mRNA that was identified as TrxR mRNA [91]. The response was transient and not reflected by an increase in TrxR activity, most probably due to a lack of selenium in usual cell culture media [92,93]. Vitamin D substantially contributes to the regulation of differentiation in osteoblasts; the identification of the TrxR gene as a vitamin D responsive gene points to a role of TrxR in the differentiation program. The response of TrxR to vitamin D is not unique for osteoblasts; it has also been found in peripheral blood monocytes and human myeloid leukemia cells where TrxR might equally contribute to the differentiation of monocytes into macrophages [94].

The need for selenium to more stably upregulate TrxR indicates that selenium is not only necessary to maintain the levels of constitutively expressed selenoproteins but also to be able to rapidly respond to an altered demand for specific selenoproteins under specific conditions, a scenario that so far has not been investigated in any detail.

10.4.3.3 Vitamin C

The effect of different phytochemicals on the promoter activity of TrxR1 has been investigated by Hintze et al. [95]. Ascorbic acid at doses above 60 μM

significantly activated the TrxR1-promoter, although the effect was much weaker compared to that elicited by sulforaphane treatment. It is not clear whether the effect of ascorbic acid is also mediated by the Nrf2/Keap1 system or by a different, still unknown mechanism.

10.4.4 Regulation by Various Dietary Factors

10.4.4.1 Phytoestrogens

Since the late 1960s it has been known that females express higher amounts of GPx than males [96]. In addition, females produce lower mitochondrial levels of reactive oxygen species and higher amounts of mitochondrial SOD and cGPx (reviewed in Ref. [97]), observations that have been attributed to the difference in lifespan between the genders. Estrogens have been made responsible for the high cGPx expression. In a microarray analysis of genistein-treated human prostate cancer cell lines LNCaP and PC-3, the cGPx gene was the most upregulated one [98]. Upregulation was confirmed by real-time polymerase chain reaction (PCR) and activity measurements. Remarkably, enhancement of cGPx activity was observed in cells not supplemented with selenium indicating that even cGPx expression can be increased under moderate selenium deficiency if the mRNA level rises. Genistein is a soy isoflavone with anticancerogenic properties but weak estrogenic activity and weak affinity for the nuclear estrogen receptor [98]. Neither MnSOD nor cGPx, which are upregulated by estrogens, contain an estrogen-responsive element in their promoters, which makes it difficult to explain the observed effects. The cell surface membrane-associated estrogen receptor might provide an explanation [99]. These receptors may activate gene expression via canonical cytosolic signaling pathways. Since the promoters of both estrogen-responsive enzymes contain NFκB-responsive elements, the following pathway has been proposed: the interaction of estrogens with a membrane estrogen receptor leads to activation of MAP kinases followed by activation of NFκB and gene expression [97].

10.4.4.2 Glucose

Selenoprotein S (SelS) turned out to be the human homolog of Tanis, a diabetes-associated protein that is downregulated in the liver and adipose tissue of the Israeli sand rat (*Psammomys obesus*), a polygenic animal model of type 2 diabetes. Tanis is a type II transmembrane protein that is predominantly localized in the endoplasmic reticulum but also found on the cell surface [100] and acts as a serum amyloid receptor [101]. Like Tanis, SelS responds to glucose-deprivation with upregulation [102]. Overexpression of SelS protected Min6 pancreatic β-cells from oxidative stress-induced cell death indicating that SelS, like many other selenoproteins, may regulate intracellular redox homeostasis [102]. Tanis/SelS is increased upon insulin stimulation in adipose tissue of subjects suffering from type 2 diabetes [101]. Emerging evidence suggests that type 2 diabetes is an

inflammatory disorder with elevated circulating concentrations of several acute phase reactants including amyloid A. SelS obviously is another selenoprotein involved in the regulation of the inflammatory response and may therefore play a role in related diseases. What its precise function is and how it responds to selenium supply await further investigation.

10.4.4.3 Metals

Two selenoproteins can be induced by metal ions: selenoprotein W and TrxR1. Selenoprotein W (SelW) is highly expressed in muscle from which it was originally purified [103]. The rat SelW promoter contains a metal responsive element (MRE) and responded to different metals. Exposure of glial cells to either copper or zinc significantly increased SelW promoter activity, whereas cadmium had no effect. A mutation of the MRE abolished the effect of zinc and copper [104].

Treatment of vascular endothelial cells with cadmium chloride resulted in increased TrxR promoter activity as well as increased TrxR mRNA levels [105]. Zinc, however, did not have an effect. The underlying mechanism does not involve a metal responsive element, as was the case for SelW, but is mediated by the ARE and, hence, by the Nrf2/Keap1 system, which responds to cadmium [106]. Cadmium is generally considered an environmental toxin without any physiological function. Induction of the adaptive response might contribute to its detoxification.

10.5 CONCLUSIONS AND PERSPECTIVES

Regulation of selenoprotein expression by the diet can be obtained at the transcriptional and translational level. The most prominent factor at the translational level is selenium itself. Selenium is provided in various chemical forms from which only those that can be metabolized to hydrogen selenide are precursors of selenoprotein synthesis. How the individual members of the selenoprotein family respond to selenium supply is only partially known; neither has the function of all selenoproteins been elucidated. Since the functional domain of selenoproteins appears to catalyse redox reactions, it does not surprise that many of them are involved in the redox regulation of cellular processes, like the regulation of the activity of transcription factors or apoptosis. Recent discoveries such as SelS as a diabetic-associated protein and SelN as key player in muscle function [107] underscore that the full scope of selenoproteins functions is just emerging.

Regulation of certain selenoproteins at the transcriptional level via the activation of Nrf2 has only recently come into focus. Induction of TrxR1 and GI-GPx by dietary compounds with chemopreventive properties together with the known upregulation of these enzymes in cancer cells opens up new perspectives for the prevention of cancer by nutrition. The point in time of an induction might be critical, since, at least for TrxR1, an enhanced activity at late stages of tumor development supports tumor cell growth. Intestinal inflammation and tumor development in GI-GPx/cGPx double knockout mice indicate an anti-inflammatory role of

these enzymes. Accordingly, protection may be expected from an optimal expression of both enzymes before or during tumor initiation, which is considered to be linked to inflammation. Also vitamins, especially those involved in regulation of cell growth and differentiation, turned out to be involved in the expression control of certain selenoproteins.

In short, an optimal expression of selenoproteins requires more than selenium alone. It can be achieved by increasing mRNA levels by dietary activators at the transcriptional level, whereas the translation has to be guaranteed by an adequate selenium supply.

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11 Regulation of Signaling Pathways by Selenium in Cancer

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CONTENTS

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11.1 INTRODUCTION

Mitogenic and survival signals initiated by polypeptide growth factors in mammalian cells are processed by receptors localized on the plasma membrane surface. These transmembrane receptors have a protein tyrosine kinase (PTK) activity domain that is localized at the cytoplasmic region of the protein molecule. The interaction of the growth factor ligands with the receptors induces their dimerization and activation through autophosphorylation. The activated PTK then activates one or multiple cytosolic signaling cascades including several protein kinase families, such as phosphatidylinositol 3-kinase-AKT (PI3K-AKT), extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and stress-activated kinase (SAPK)-1/p38 mitogen-activated protein kinase (p38 MAPK). These kinases relay the signals from the cell surface to the nucleus to activate nuclear transcriptional factors, such as activating protein-1 (AP-1), which consists of Jun/Fos heterodimers, nuclear factor-kappa B (NF-κB), and p53 tumor suppressor

protein, resulting in altered gene expression patterns and cellular responses, such as cell cycle progression and suppression of cell death. In addition to the large polypeptide growth factors that bind surface receptors, small molecule hormone ligands can enter the cell and bind tissue specific receptors, such as the androgen receptor (AR) in the prostate and the estrogen receptor in the breast, to mediate specialized signaling in their target cells and organs.

The aberrant behavior of cancer, to a large extent, reflects the accumulation of genetic and epigenetic alterations of multiple oncogenic signaling and tumor suppressing pathways at the cell membrane [e.g., bcr-abl in chronic myelogenous leukemia, Her-2/neu in familial breast cancer, epidermal growth factor receptor (EGFR) in lung cancer], cytosolic [e.g., Ki-Ras mutation in colon, pancreatic cancers; loss of the PI3K-AKT suppressor phosphatase and tension homologue deleted on chromo (PTEN) in prostate cancer] and nuclear locations (e.g., p53 mutations in most cancers), promoting cancer cell proliferation, evasion of apoptosis, stimulating angiogenesis, and enabling cancer cell motility and invasion to spread and metastasize. Since multiple signaling pathways are dysfunctional in most cancers, and cancers accumulate new oncogenic mutations as they progress, the greatest and most durable preventive benefits will likely be achieved by agents with multitargeting actions.

Selenium (Se), the subject of this review chapter, possesses multitargeting attributes. It is important to note upfront that the Se status of human or animal subjects, the chemical forms and the dosages of Se, and the organ/tissue Se metabolic capacity and specificity will determine the fates of ingested Se and the profiles of Se metabolites, and consequently, the biochemical, molecular, and cellular responses, which are also further defined by the organ site specificities of the cancer etiology and cell signaling pathways. These pieces of information must be considered to obtain a balanced understanding of the Se literature, and for the practical application of Se for cancer prevention and therapy. Here, we introduce the activities associated with and attributable to Se for cancer risk modification, and discuss how they can be related to selenoproteins and metabolite pools. We will examine key studies dealing with the effects of Se on cell signaling pathways as they relate to cancer chemoprevention and possible new applications in cancer therapy.

11.2 ANTICANCER ACTIVITIES OF SELENIUM

Converging data from epidemiological, ecological, and preclinical studies have implicated Se as a significant risk modifier for some but not all cancers [1–4]. The original report of the results of a landmark cancer prevention trial in the United States by the late Dr. Larry Clark and his coworkers, Dr. Gerald Combs and others, and subsequent updates of the extended data collection [5–10] support the plausibility that dietary Se supplement, in the form of selenized yeast (Se-yeast), is a safe and effective preventive agent against solid cancers in multiple organ sites, particularly in the prostate, colon, and lung. In the treatment arm, subjects were given once daily 200 μg Se (~4 times the recommended daily value of 55 μg per day) as selenized brewer's yeast, which consists predominantly of selenomethionine (SeMet), for a mean of 4.5 years and the subjects

were followed for a mean of 6.5 years. Subjects who received the Se-yeast supplement showed significantly lower incidences of cancers of the prostate (relative risk = 0.37, $p = 0.002$), colon ($RR = 0.42$, $p = 0.03$), and lung ($RR = 0.54$, $p = 0.04$) than the placebo group. Total cancer-related mortality was also significantly decreased by 41%. However, there was a Se-yeast–associated increase in the risk of secondary nonmelanoma skin cancers ($RR = 1.10$), which were the primary endpoints of the trial [5,9]. The protective effects of Se supplementation for prostate cancer ($RR = 0.51$, $p = 0.009$) and colon cancer ($RR = 0.46$, $p = 0.055$) persisted upon longer follow-up for a mean of 7.8 years [7]. The risk reduction for prostate cancer was greatest in the subjects entering the trial with the lowest tertile of serum Se [7,10]. The protective effects of Se-yeast supplement were also reported in a small-scale trial in China with hepatitis B patients on their liver cancer risk [3,11,12]. For further information on the analyses of these results, the reader is referred to our recent review paper [4] and a number of excellent reviews that have covered this topic [3,13,14].

The potential benefit of preventing the top two cancers that account for the cancer-related mortality in North America has stimulated great research interests that culminated into two large clinical prevention trials in the United States and Canada to validate the preventive efficacy of Se for prostate cancer and lung cancer. The Selenium and Vitamin E Cancer Prevention Trial (SELECT) [15] is a randomized, prospective, double-blind study designed to determine whether Se as SeMet, which is a principal Se component of Se-yeast [16,17], and vitamin E alone and in combination can reduce the risk of prostate cancer among healthy men. Coordinated by the Southwest Oncology Group (SWOG), SELECT is a 2×2 factorial study with an accrual goal of 32,400 men aged 55 years or older (age 50 or older for the African American men) with nonsuspicious digital rectal examination (DRE) and serum prostate specific antigen (PSA) of 4 ng/ml or lower. Enrollment began in 2001 and was accomplished in 2005, with final results anticipated in 2013.

A phase III randomized chemoprevention study of Se in participants with previously resected stage I nonsmall cell lung cancer has been initiated by the Eastern Cooperative Oncology Group (protocol ECOG-5597) [18]. Patients will be randomly assigned to one of the two groups. Patients in one group will receive Se in the form of Se-yeast (200 μg Se) by mouth once a day. Treatment will continue for up to 4 years. Patients in the other group will receive a placebo by mouth once a day. All patients will be evaluated once a year.

These trials are billed as the definitive validation tests of the preventive efficacy of Se in the forms of SeMet and Se-yeast for prostate and lung cancers, respectively. The outcomes of these two trials can be expected in about a decade. Several small-scale clinical trials and pilot studies concerning prostate cancer prevention have also been either completed or initiated in the United States [19–22] and other countries, including the Prevention of Cancer by Intervention by Selenium (PRECISE) Trial pilot studies in the United Kingdom and Denmark [14,23]. If a positive efficacy is confirmed in these major trials, the public health impact of using these forms of Se is self-evident, and this will provide an outstanding impetus for further clinical trials to identify more efficacious Se agents for even greater

preventive benefits. If negative, the quest for effective Se agents takes on more significance and urgency. A better understanding of the mechanisms of the Se actions and the targeted signaling pathways will be crucial to help predict and analyze the outcomes of these trials and for defining the choice of second generation Se agents and biomarkers in future studies.

11.3 PROPOSED MECHANISMS OF SELENIUM ACTIONS

These include antioxidant protection against lipid peroxides-driven and reactive oxygen species (ROS)-driven initiation and promotion events, enhanced carcinogen detoxification through phase I and II enzymes, enhanced immune surveillance, inhibition of cancer cell proliferation, and selective induction of apoptosis of transformed epithelial cells, to name a few [1–3]. We have proposed that inhibition of cancer angiogenesis by Se metabolites may also contribute to the chemopreventive activity [4,24–27]. Inhibition of the cyclooxygenase-2 (COX-2) pathway might play a role in the prevention of colon cancer and other cancers in which the COX-2 pathway is critically involved [28]. In terms of the Se chemical forms, the critical Se metabolite(s) hypothesis has steadily gained both *in vivo* and *in vitro* support during the past two decades [1,2,4,26,27,29]. However, much remains debatable as to what mechanisms account for the anticancer activity of Se in humans. It is likely that a combination of these mechanisms will operate depending on the host Se status as has been documented in the Clark trial [4,7,10], the forms and dosages of Se supplement, and the organ site specificities of cancer etiology and cell signaling pathways, as well as the organ site's capacity for Se metabolism.

11.3.1 Role of Selenoproteins and Antiperoxidation by Nutritional Selenium

The best studied function of Se in the nutritional range of intake is as an integral part of a cellular antioxidant enzyme family, the Se-dependent glutathione peroxidases (SeGPXs) [30,31]. Figure 11.1 shows possible metabolic pathways for Se from inorganic salts and from selenoanimo acids. For synthesis of SeCys-containing selenoproteins, hydrogen selenide is cotranslationally incorporated into the proteins by pathways that are highly conserved from bacteria to humans involving the seryl-tRNA^{UGA} codon for this so called twenty-first amino acid [30,31]. The putative connections for SeGPXs and other selenoproteins such as thioredoxin reductases (TRs) [32,33] and thyroixine deiodinases (TDIs) [34,35] to regulate ROS-driven cellular signaling events are depicted schematically in Figure 11.2.

Mutagenic oxidative stress is generally thought to be a major factor in the initiation of human carcinogenesis, as the electron-rich DNA bases are susceptible to electrophilic attack by ROS or other free radicals. These can result in genetic damages, mutant oncogenes, and tumor suppressor genes, or epigenetic changes that alter their expressions, leading to the expression of malignant phenotypes. Endogenously produced ROS include superoxide radical, hydrogen peroxide

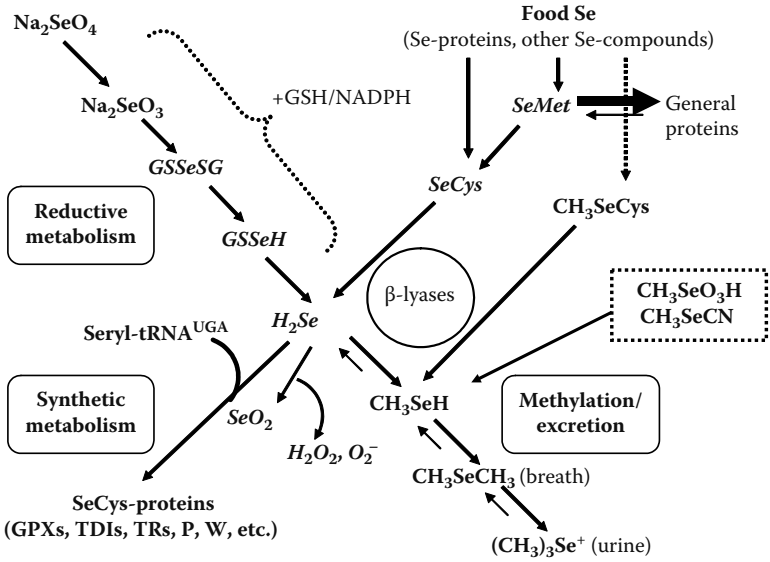

FIGURE 11.1 Possible metabolic pathways for inorganic Se and selenoanimo acids. Se salts such as selenate (+6) or selenite (+4) undergo reductive metabolism with reduced glutathione and NADPH. Tissue cysteine β-lyases release hydrogen selenide (H_2Se) and methylselenol (CH_3SeH) from selenocysteine (SeCys) and Se-methylselenocysteine (CH_3SeCys or MSeC), respectively. Hydrogen selenide is the source for incorporation into SeCys-containing selenoproteins by cotranslational incorporation into Ser-tRNA^{UGA}. It is also considered the source for triggering reactive oxygen species. Methylselenol pool may be selectively enriched by using methylselenol precursors either as purified compounds or as functional foods such as Se-garlic, bypassing the hydrogen selenide pool. The majority of selenomethionine (SeMet) is incorporated into general proteins in place of Met. Transselenation from SeMet to Ser is likely a minor pathway to yield SeCys. Methylation of hydrogen selenide constitutes an excretion pathway for excess Se. Abbreviations: GSSeSG, selenodiglutathione; GSSeH, glutathioneselenol; CH_3SeO_2H, methylseleninic acid; CH_3SeCN, methylselenocyanate; SeO_2, selenium dioxide; CH_3SeCH_3, dimethylselenide; $(CH_3)_3Se+$, trimethylslenonium.

(H_2O_2), singlet oxygen, hydroxyl radical, as well as electrophilic metabolites of xenobiotics and other reactive intermediary metabolites. Hypotheses based on antioxidant protection against ROS-driven cancer initiation and promotion are plausible for human cancer prevention, especially in individuals with marginal and deficient Se status (Figure 11.2). Especially, there has been considerable interest whether the cancer preventive activity of Se is mediated through the SeGPXs.

The second family of selenoproteins conceivably important for regulating cancer risk is thioredoxin reductase (TR) [36]. All mammalian TR isozymes are homologous to glutathione reductase (GR) and contain a conserved C-terminal elongation with a cysteine–selenocysteine sequence forming a redox-active selenenylsulfide/selenolthiol active site and are inhibited by goldthioglucose

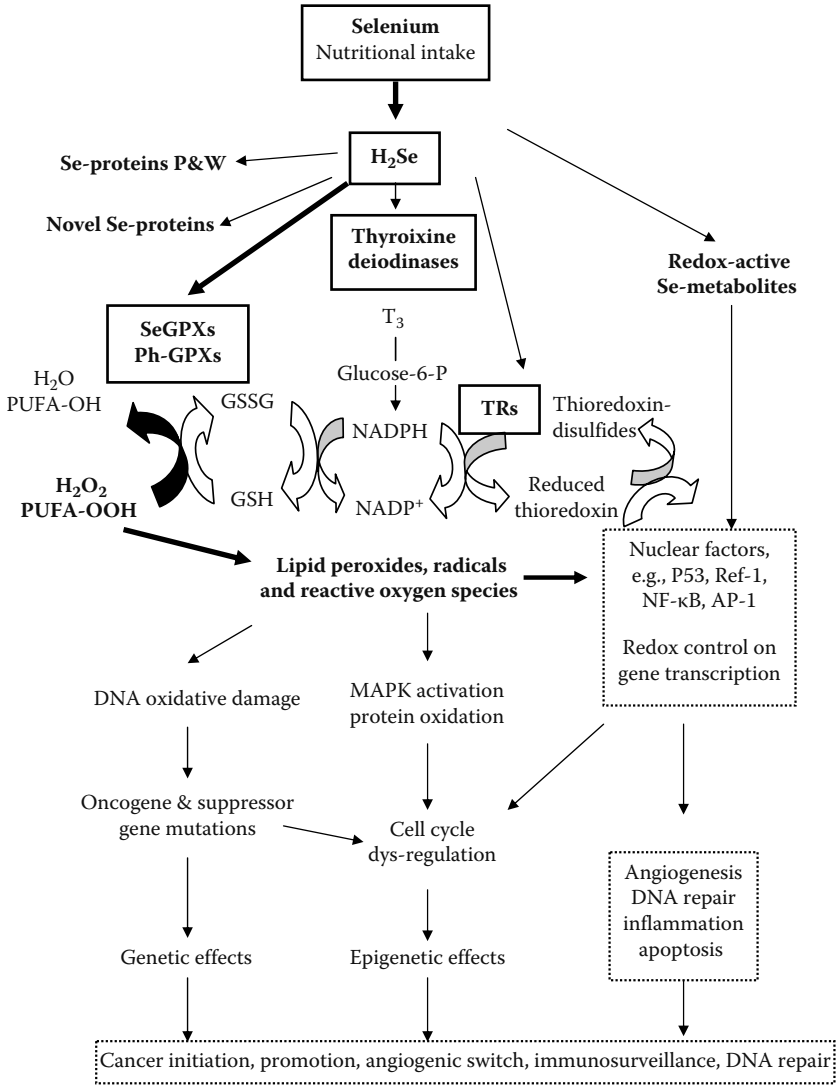

FIGURE 11.2 Proposed roles of known selenoproteins or selenoenzymes against reactive oxygen species (ROS)-driven cancer initiation, promotion, and angiogenic switch through genetic and epigenetic pathways in a nutritional dietary supplement context. Hydrogen selenide is incorporated into selenoproteins through cotranslational incorporation using the SeCys codon (Seryl-$tRNA^{UGA}$). The balance of opposing actions of two selenoprotein families may be critical for the extent of cancer risk modification. Improving SeGPX activities decreases ROS load and carcinogenic events, whereas improving TR activities increases thioredoxin activity and promotes cancer cell growth. Abbreviations: H_2Se, hydrogen selenide; SeGPx, Se-dependent glutathione peroxidases; Ph-GPx, phospholipid glutathione peroxidase; TDIs, iodothyronine 5′-deiodinases; TR's, thioredoxin reductases.

(aurothioglucose) and other clinically used drugs. TR1 is a crucial redox regulator of p53 function and other tumor suppressor activities and is also overexpressed in many malignant cells. TR1 has been proposed as a target for cancer therapy. Thioredoxins, with a dithiol/disulfide active site (CGPC), are the major cellular protein disulfide reductases. They also serve as electron donors for enzymes such as ribonucleotide reductases, thioredoxin peroxidases (peroxiredoxins), and methionine sulfoxide reductases. Thioredoxins are critical for redox regulation of protein function and signaling via thiol redox control. A growing number of transcription factors including NF-κB or the Ref-1-dependent AP-1 require thioredoxin reduction for DNA binding.

Past studies with Se-deficient rodent models of cancer have shown a modest protection at best with nutritional Se supplements on chemically induced carcinogenesis in mammary and other organ sites [1,2,4]. The interpretation of such studies, however, was complicated by how much the protective effects were exerted by improving the activities of SeGPXs, TRs, and other selenoproteins, or by the increase in nonprotein Se metabolites *per se*. The recent development of a transgenic mouse model with suppressed selenoprotein synthesis through the expression of an altered selenocysteine-tRNA deficient in isopentenyladenosine (i6A-) has made it possible to address this question with some specificity [37]. As reported in the original study, selenoprotein suppression was in a protein- and tissue-specific manner. Cytosolic SeGPX (GPX1) and mitochondrial TR3 were the most and least affected selenoproteins, while selenoprotein expression was most and least affected in the liver and testes, respectively. The defect in selenoprotein expression occurred at translation, since selenoprotein mRNA levels were largely unaffected [37].

Diwadkar-Navsariwala et al. [38] developed double transgenic mice with reduced selenoprotein levels because i6A-transgenic expression and prostate targeted expression of the SV40 large T and small t oncogenes to that organ [C3(1)/Tag]. The resulting bigenic animals (i6A-/Tag) and control WT/Tag mice were assessed for the presence, degree, and progression of prostatic epithelial hyperplasia and nuclear atypia. The selenoprotein-deficient mice exhibited accelerated development of lesions associated with prostate cancer progression with adequate dietary Se (0.1 ppm Se), implicating selenoproteins in cancer risk and development and supporting the possibility that nutritional Se prevents cancer by modulating the levels of these selenoproteins.

Irons et al. [39] examined Se homeostasis in the liver and colon of wild-type and transgenic mice fed Se-deficient diets supplemented with 0, 0.1, or 2.0 ppm Se (as selenite)/g diet. In agreement with the original study [38], they revealed that transgenic mice had reduced liver and colon SeGPX expression, but conserved TR expression compared with wild-type mice, regardless of whether the Se was at nutritional (0.1 ppm) or chemopreventive (2 ppm) supplementation. The transgenic mice had more Se in the nonprotein fraction of the liver and colon than wild-type mice, indicating a greater amount of low molecular weight Se metabolites. Compared with wild-type mice, transgenic mice had more azoxymethane-induced aberrant crypt formation (a preneoplastic lesion for colon cancer) at both 0.1 and 2 ppm levels. Supplemental Se (0.1 to 2 ppm) decreased the number of aberrant crypts and

aberrant crypt foci in both wild-type and transgenic mice. These results provide evidence that a lack of selenoprotein activity (mostly SeGPX, not TR) increases colon cancer susceptibility. Independent of the selenoprotein genotype, low molecular weight Se metabolites exert the important cancer-protective effects of Se.

To specifically assess the function of TR, Hatfield and coworkers used RNA interference technology to decrease TR1 expression in mouse Lewis lung carcinoma (LLC1) cells [40]. Stable transfection of LLC1 cells with a siRNA construct that specifically targets TR1 led to a reversal in the morphology and anchorage-independent growth properties of these cancer cells toward the normal cells. Mice fed a diet with adequate Se level inoculated with the TR1 knockdown cells showed a dramatic reduction in tumor progression and metastasis compared with those mice inoculated with the corresponding control vector. In addition, tumors that arose from the injected TR1 knockdown cells lost the targeting construct, suggesting that TR1 is essential for LLC tumor growth in mice. Since these data show that lowering the TR1 level in lung cancer cells is antitumorigenic in the presence of adequate dietary Se, the reverse implication is that improving TR1 activity by Se nutritional supplement may promote this lung cancer model.

These *in vivo* studies together suggest that the reduction of SeGPXs [39] or TR1 [40] has opposite consequences for the cancer risk. The overall balance of the activities between TR and SeGPX could therefore be a crucial factor for determining the cancer risk in the nutritional range of Se supplementation.

The Se status of individuals residing in the United States has been considered "nutritionally" adequate judging by their Se intake data and the serum Se content. According to the Third National Health and Nutrition Examination Survey (NHANES III), the mean intake of all ages was 103 μg [41], nearly twice the National Research Council's recommended daily allowance of 55 μg [42]. From the 18,597 persons for whom serum Se values were available in NHANES III [43,44], the mean concentration was 1.58 μM and the median concentration was 1.56 μM. This is much higher than the 1 μM or 80 ng/ml that was found to be the upper limit for SeGPX responses to supplemental Se in healthy adults [45]. This was also supported by the Clark study in which the placebo group had a baseline plasma Se level of 114 ng Se/ml (1.4 μM) [5,7]. In that trial, the average plasma Se level in the supplemented group was increased by some 67% to 190 ng/ml or 2.4 μM. Only two subjects (1.5%) had Se levels below 80 ng/ml [7]. In spite of an increase in plasma total Se level, the plasma SeGPX activity of selected subjects before and after Se supplementation was not increased [46].

Because Se deficiency is not a serious health concern in the United States, most animal models and *in vitro* cell culture studies since the mid-1980s have dealt with chemopreventive levels of Se and have focused on the cancerous epithelial cells as the targets of its anticancer effects. Most animal models have shown cancer chemopreventive activity of Se intake that is 20 to 50 times greater than the nutritional requirement [2]. Based on a large body of data from these studies, it has been articulated that cancer chemoprevention by Se in the nutritionally adequate subject is independent of the antioxidant activity of plasma or tissue SeGPX [1,2]. This paradigm was based on the observation that the dietary level of Se (2 ppm or greater

as selenite) needed to achieve a significant cancer preventive activity in animal models far exceeded that required (i.e., 0.1 ppm) to support maximal SeGPX in the blood (GPX3) or the target tissues from which experimental cancers arise. This view has been extended to the other selenoproteins identified subsequently in the last decade, including phospholipid glutathione peroxidase (Ph-SeGPX, aka GPX4), selenoprotein P (Sel-P), selenoprotein W (Sel-W), thyroixine de-iodinases (TDI), and TRs. The studies with transgenic suppression of selenoproteins (increased prostate and colon cancer risk with decreased SeGPXs in the presence of adequate dietary Se) and the TR1 knockdown transfectant cells (decreased lung cancer growth with knocked down TR1) cited earlier indicate likely contradicting roles of these proteins as regulators of cancer risk in the nutritional range of Se intake. The availability of these genetic models provides useful experimental tools to further address the role of selenoproteins in cancer risk modification by Se.

11.3.2 Nonselenoprotein Selenium Metabolites

Figure 11.1 shows possible metabolic pathways for Se from inorganic salts and from selenoanimo acids derived from the digestion of food proteins or from dietary supplements. Excess Se beyond the need for selenoprotein synthesis (hydrogen selenide is cotranslationally incorporated into the SeCys containing selenoproteins) is methylated into methylselenol, which is further methylated and excreted as dimethylselenide (volatile through breath) and trimethylselenonium (urine). It should be pointed out that SeMet is likely to be either incorporated into general proteins in place of Met (nonspecific substitution) or metabolized to SeCys through a transselenation pathway similar to the transulfuration pathway for Met → cysteine conversion. The efficiency of the latter pathway will be dependent on the metabolic capacity of the cell types and organs. Liver and hepatocytes are expected to be well equipped with the metabolic enzymes, whereas nonhepatic tumor cells in culture, in general, would be expected to be limited in this ability.

Milner has shown that selenodiglutathione (GSSeSG), an intermediate of reductive metabolism of selenite, was significantly more efficacious than selenite against leukemia growth *in vivo* [47]. However, the transient nature of GSSeSG under physiological pH *in vivo* plus the fact that food-derived selenoanimo acids that do not produce GSSeSG through *in vivo* metabolism have been shown to exert anticancer activity [1–3] suggest more downstream Se metabolites as the likely critical anticancer Se species.

Ip and Ganther led the efforts to identify the putative *in vivo* Se metabolite pool using a mammary chemical carcinogenesis model. They proposed that the active anticancer Se metabolites were likely monomethylated Se species (presumably methylselenol) and the chemopreventive efficacy of a given Se compound might depend on the rate of its metabolic conversion to the active Se form(s) [2,48]. Strong supporting evidence was obtained by comparing the cancer chemopreventive efficacy of forms of Se that fed into different Se metabolite pools, with precursors of methylselenol displaying greater preventive efficacy than those for hydrogen selenide or dimethylselenide in the chemically induced rodent mammary

carcinogenesis model [49,50]. In addition, arsenic was used as an inhibitor of the Se methylation steps and the data showed that blocking the conversion of hydrogen selenide to methylselenol decreased the anticancer activity, whereas inhibiting the further methylation of methylselenol increased the efficacy. Extending on the methylselenol structure–activity theme, subsequent work had shown that the alkyl-selenol and allyl-selenol precursor compounds were more active against mammary carcinogenesis than methylselenol precursors on an equal molar basis of dietary Se intake [51,52]. However, these structure–activity studies have yet to be extended beyond the mammary carcinogenesis model for assessing the general applicability of the methylselenol hypothesis in other organ sites.

Consistent with the concept of nonselenoprotein Se metabolites for chemoprevention, el-Bayoumy and coworkers undertook to develop new organoselenium cancer chemopreventive agents with less toxicity than some of the classic compounds, such as sodium selenite [53–55]. They have demonstrated that one of the most effective of these organoselenium compounds is 1,4-phenylenebis(methylene)selenocyanate (p-XSC). This agent is capable of inhibiting tumors in the mammary glands, colon, and lung of laboratory animals. The structural feature of X–CH_2SeCN (where X represents aromatic phenyl groups of agents in this series) for generating X-CH_2SeH may in part explain their many similarities with methylselenol precursors. In addition, the aromatic Se activates phase I and II drug metabolism enzymes, potentiating experimental carcinogen detoxification in a lung cancer model [56]. While these agents are better tolerated in rodent models than selenite [55,56], their applicability for human cancer chemoprevention requires more investigation. For sake of brevity, in this review we will focus on selenoamino acids and their analogs or derivatives as well as the inorganic Se forms in the subsequent sections.

11.4 SELENIUM METABOLITE POOLS IN CELL CULTURE MODELS

In analyzing the Se literature, it will be important to keep in mind the chemical form of Se used, the levels of Se exposure, and the serum levels in the cell culture media (e.g., 10% fetal bovine serum usually provides 100 nM Se in the final medium). In general, cell culture models using selenite as the source of Se have shown biphasic responses of cancer cell proliferation to incremental Se supplement: a modest stimulatory effect in the nanomolar to submicromolar range, and a strong suppression effect at higher Se concentrations. While the small growth stimulatory response is likely related to the nutritional actions of Se through changing the balance of SeGPXs, TRs, and other selenoproteins to achieve the optimal redox tone for growth in cell culture, studies by us and others focusing on the higher levels of Se exposure have shown that monomethyl Se compounds that are precursors to the methylselenol pool induce numerous cellular, biochemical, and gene expression responses that are distinct from those induced by the forms of Se that enter the hydrogen selenide pool [4]. These major cellular and biochemical effects are schematically summarized in Figure 11.3.

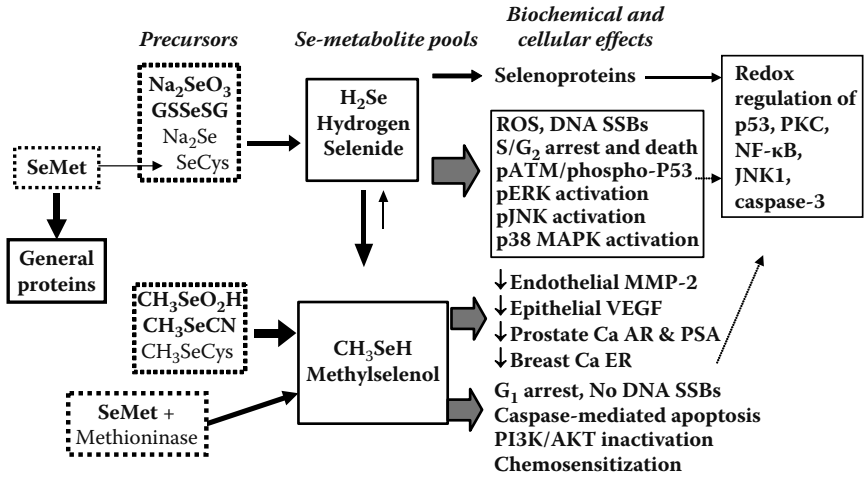

FIGURE 11.3 Distinct biochemical and cellular effects of Se precursors feeding into the genotoxic hydrogen selenide pool or nongenotoxic methylselenol pool of metabolites in cell culture models. Selenoproteins may be critical for modulating the activity of nuclear transcriptional factors in the nutritional Se intake range. Redox-active Se metabolites at supranutritional or pharmacological levels of Se intake may further contribute to global protein redox modification. Abbreviations: SSBs, DNA single-strand breaks; CH_3SeO_2H, methylseleninic acid; CH_3SeCN, methylselenocyanate; CH_3SeCys, Se-methylselenocysteine; SeCys, selenocyst(e)ine; MMP-2, matrix metalloproteinase-2; VEGF, vascular endothelial growth factor; PSA, prostate specific antigen; AR, androgen receptor.

11.4.1 Hydrogen Selenide Pool

Sodium selenite and sodium selenide, which feed into the hydrogen selenide (H_2Se) pool, rapidly (within minutes to a few hours of Se exposure) induce DNA single-strand breaks (SSBs), S phase, or G2/M cell cycle arrest, and lead to subsequent cell death by apoptosis and necrosis [4,57–61]. Sodium selenide and SeCys could recapitulate the DNA SSB induction and the apoptosis effects of selenite in the model system [58]. A superoxide dismutase mimetic compound, copper dipropylsalicylate, blocked DNA SSBs and apoptosis, indicating that selenite *per se* did not trigger these events [27]. Recent studies have provided further support for ROS (superoxide generation) as intermediates for activating p53 Ser phosphorylation in apoptosis induced by selenite in the LNCaP prostate cancer cell model [62,63].

However, little is known of whether the hydrogen selenide pool could reach the pharmacological levels used in these *in vitro* studies to affect DNA integrity (genotoxicity) *in vivo*. Our recent data indicate that selenite given by daily oral dosages of 3 mg per kg body weight to tumor-bearing nude mice increased DNA SSBs in peripheral lymphocytes, whereas the same dosage of MSeA or MSeC lacked this effect [148]. Further studies in animal models and in humans are necessary to confirm the *in vivo* genotoxicity of selenite.

11.4.2 Methylselenol Pool

We and others have shown that methylselenol precursors such as methylselenocyanate (MSeCN) and Se-methylselenocysteine (MSeC) induced apoptosis of mammary tumor epithelial cells and leukemia cells without the induction of DNA SSBs [58–60]. Furthermore, we and others have reported that methyl Se-induced cancer cell apoptosis was caspase-dependent, whereas selenite-induced cell death was independent of these death proteases in prostate cancer and leukemia cells lacking functional p53 [64,65]. The methyl Se led to G_1 arrest [59–61,66–68]. Inhibitory effects on cyclin dependent kinases [68,69] and protein kinase C (PKC) [70] have been attributed to the methyl Se pool. In terms of genotoxicity implications, our unpublished data show that at a daily oral dosage of 3 mg per kg body weight, MSeA and MSeC significantly suppressed human DU145 xenograft growth without increasing DNA SSBs in the peripheral lymphocytes of the host mice, whereas the same dosage of selenite caused increased DNA SSBs and was much less effective for suppressing xenograft growth.

In addition to these cellular effects, methylselenol precursors exert a rapid inhibitory effect on the expression of key molecules involved in angiogenesis regulation. For example, we have shown that subapoptotic doses of methylseleninic acid (MSeA) inhibited the expression and secretion of the angiogenic factor vascular endothelial growth factor (VEGF) in several cancer cell lines [25]. Methyl Se also inhibited the expression of matrix metalloproteinase (MMP)-2 in the vascular endothelial cells [24,25]. These effects plus a potent inhibitory effect on the cell cycle progression of vascular endothelial cells [66,67] indicate that methylselenol can be a key inhibitor of angiogenic switch regulation in early lesions and tumors [4]. Furthermore, we and others have recently shown that MSeA and methylselenol released by methioninase from SeMet inhibit androgen receptor expression and its signaling to prostate specific antigen (PSA) [71–73] as well as PSA stability [71]. MSeA has been also shown to inhibit estrogen receptor signaling in breast and endometrial cancer cells [74–76].

In the following sections, we review the cellular effects of Se on cancer cells and vascular endothelial cells, which are a crucial partner for cancer angiogenesis. We then will examine the effects of Se on selected signaling molecules from the cell surface, to the intracellular and nuclear locales, and to secreted proteins important for angiogenesis regulation and invasion and will attempt to relate these changes to the observed cellular consequences (e.g., cell cycle arrest, apoptosis, angiogenesis, DNA repair).

11.5 EFFECTS OF SELENIUM ON CELL CYCLE AND APOPTOSIS

11.5.1 Cell Cycle

We and others have shown that upon exposure to dose levels greater than human serum Se found in U.S. residents, a variety of tumor cells and vascular endothelial cells undergo cell cycle arrests, but in Se metabolite specific manners [59–61,66,67,77]. Selenite in general induces an S or G_2/M arrest. Superoxide

production and DNA SSBs may underlie the S or S/G_2-M phase arrest effect induced by selenite [27,61–63].

In contrast, MSSeA, MSeCN, and MSeC have been shown to induce G_1 arrest in many cell types [59–61,66,68,77]. We have shown that methylselenol released from SeMet by methioninase induced G_1 arrest in both cancer and vascular endothelial cells [67]. In terms of specific cell cycle stage action, we have shown that in human umbilical vein endothelial cells (HUVEC) deprived of endothelial cell growth supplement (ECGS), resumption of ECGS stimulation resulted in a marked mitogenic response as indicated by [^{3}H]thymidine incorporation into DNA [66]. The ECGS-stimulated DNA synthesis was inhibited in a dose-dependent manner by MSeA exposure with a IC_{50} approximately 1 μM and a complete blockage at 3 μM. MSeA resembled the action of PI3K inhibitors for targeting a common mechanism(s) controlling G_1 progression to S phase. The results support a potent inhibitory activity at achievable serum levels of Se on ECGS-stimulated mitogenesis in the mid- to late-G_1 phase.

In a mouse mammary hyperplastic epithelial cell line, TM6, Zhu et al. [68] showed that MSeA-induced G_1 arrest was accompanied by a reduction in total cellular levels of cyclin D1. Whereas MSeA had no effect on total levels of the cyclin-dependent kinase CDK4, the amount of CDK4 immunoprecipitated with cyclin D1 in MSeA-treated cells was decreased as was the kinase activity of the immunoprecipitated complex. MSeA did not significantly affect cyclin E or associated regulatory molecules. Treatment with MSeA suppressed the hyperphosphorylated form of retinoblastoma (Rb) with a commensurate increase in the hypophosphorylated form. Levels of E2F-1 bound to Rb also were elevated. Levels of insulin-like growth factor-I receptor (IGFR) and phosphorylated AKT were decreased by MSeA. They concluded that the G_1 arrest might be attributed to modulation of IGF-I-mediated signal transduction leading to inhibition of AKT activation and limitation of cyclin D1-CDK4-mediated phosphorylation of Rb in mammary cells, which have a strong dependence on insulin and IGF-I signaling.

Methyl Se-induced induction of p27 or p21 CDKI and inhibition of cyclin-dependent kinases and specific PKCs, PI3K have been reported in other cell types [61,68–70,78]. Altered expressions of cell cycle regulatory gene networks have also been described using microarray profiling techniques in various cancer cell lines after MSeA treatment [73,79,80]. Much remains to be done to delineate the signaling pathways and the target protein molecules involved in the metabolite-specific antimitogenic effects in the cancer epithelial cell as well as in vascular endothelial cells.

11.5.2 Caspase-Mediated and Caspase-Independent Apoptosis

When the Se exposure level is increased further, cell death is usually observed in addition to the cell cycle arrest effects. In terms of the pathways of apoptosis execution, caspases have been shown to be specifically activated by methyl Se exposure in all cell types examined [61,64–66,79,80]. We were among the first to report that MSeA (≥4 μM) induced DU145 human prostate carcinoma cell apoptosis

through caspase-dependent execution [64]. Specifically, apoptosis induced by MSeA involved cell detachment (resembling anoikis), the activation of multiple caspases, mitochondrial release of cytochrome c, cleavage of poly(ADP-ribose) polymerase (PARP), and DNA nucleosomal fragmentation. The last three biochemical actions were shown to require the activation of caspases. Caspase-8 contributed more than caspase-9 to the overall execution.

Independent of and complementing our work with MSeA, MSeC has been shown to cause caspase-dependent apoptosis of HL-60 human leukemia cells [65], which grow in suspension culture and do not require the cell attachment for survival and mitogenesis. In PC-3 human prostate cancer cells, the endoplasmic reticulum stress-activated caspase-12 appears to also make a sizable contribution to the caspase activation cascade [80]. Because DU145, PC-3, and HL-60 cells do not contain functional p53, these results suggest that methylselenol induces apoptosis in a p53-independent manner. In contrast, selenite induced cell death did not involve caspases in DU145 cells [61,64] or in HL-60 cells [65]. However, we and others have recently shown that in p53-wild type LNCaP cells, selenite does induce both caspase-dependent and -independent cell death execution [62,63,81,82]. See Section 11.8 for p53 modifications and p53 dependent caspase-mediated apoptosis by selenite.

In addition to the cancer cells, we have also investigated the induction of vascular endothelial cell apoptosis by Se [83]. We have shown that MSeA caused p38 MAPK hyperphosphorylation and ERK1/2 dephosphorylation before the cleavage of procaspase-3 and PARP, whereas AKT phosphorylation decreased after caspase activation. The p38 MAPK inhibitor SB202190 attenuated the MSeA-induced morphological changes, and decreased DNA fragmentation and the cleavage of procaspase-3 and PARP in concordant proportions. The general caspase inhibitor zVADfmk completely blocked the MSeA-induced PARP cleavage and DNA fragmentation, whereas zDEVDfmk, an inhibitor for caspase-3-like activities, was nearly as effective for inhibiting apoptosis. In comparison, apoptosis induced by selenite in HUVECs was observed in the complete absence of an activation of the major caspases. The data support p38 MAPK as a key upstream mediator for the methyl Se-specific induction of vascular endothelial caspase-dependent apoptosis, which is principally executed by caspase-3-like activities. In DU145 or LNCaP cells, MSeA-induced apoptosis did not require the activation of p38 MAPK or JNK [61,64,84]. These data highlight differences between cell types for the signaling pathways induced to mediate apoptosis by methyl Se.

11.5.3 Special Analysis of the Cellular Actions of SeMet

Since SeMet is being tested in the SELECT trial for prostate cancer chemoprevention [15] and other studies, it merits a closer examination of the cellular actions attributed to this Se form. In cell culture models, exposure levels of 1–2 orders of magnitude higher than serum Se level were needed to show growth inhibitory effects in cancer cell lines. For example, SeMet exposure inhibited the growth of

A549 lung cancer cells with IC_{50} of 65 μM and HT29 colon cancer cells with IC_{50} of 130 μM [85]. In prostate cancer cells, 100–500 μM SeMet was needed to induce substantial growth suppression effects and apoptosis [86]. SeMet treatment of the lung and colon cancer cell lines increased the number of cells in metaphase [85]. In colon and prostate cancer cells, such high levels of SeMet induced G_2/M arrest. In PC-3 prostate cancer cells, SeMet (500 μM) and selenite (10 μM) induced the phosphorylation of p34/cdc2 on Tyr15, consistent with G_2/M arrest [86].

The cell cycle effects of SeMet were further examined in synchronized HCT 116 colon cancer cells [87]. Treatment with 100 μM SeMet caused a transient delay in G_2/M phase of the cell cycle at 18 and 24 h after treatment. Coincidentally with this delay was a decrease in mitotic cyclin B RNA expression at 18 h after treatment. In addition, the cdc2 kinase activity of HCT 116 cells was decreased at 18 h. Morphological studies indicate an increase in the number of treated cells (45%) undergoing apoptosis at 66 h compared to control cells (27%). These studies suggest that modulation of mitotic cyclin expression and cdc2 kinase activity play a role in the ability of SeMet to inhibit tumor cell growth. The same group reported that SeMet-treated cells showed increased phosphorylation of ERK1/2 in a dose-dependent manner [88]. They also demonstrated phosphorylation of ribosomal S6 kinase (p90RSK) and histone H3 (its phosphorylation is a mitosis marker), which were both antagonized by the MEK inhibitor U0126, implying at least in part an ERK-pathway dependence for these biochemical events.

Recently, high levels of SeMet have been shown to induce p53-mediated cell cycle arrest and apoptosis in human colon cancer cells [89]. Four human colon cancer cell lines including HCT116 and RKO (wild-type p53), HCT116-p53KO (isogenic control of HCT116 cells with p53 knocked out), and Caco-2 (mutant p53) were treated with up to 100 μM of SeMet. All cell lines showed concentration and time-dependent growth inhibition with SeMet, although HCT116 and RKO cells were the most sensitive to such treatments. Interestingly, although HCT116 and HCT116-p53KO are isogenic cell lines, SeMet caused a G_2/M cell cycle arrest in HCT116 and RKO cells, but not in HCT116-p53KO cells. Similarly, both HCT116 and RKO demonstrated a significant increase in apoptosis (1–1.7-fold; $p < 0.01$) with 50–100 μM SeMet. Cell cycle arrest and apoptosis observed in HCT116 and RKO cell lines were accompanied by a marked increase in p53 protein expression following SeMet treatment.

While these results suggest that SeMet or its metabolites exert p53-dependent and -independent growth inhibitory effects in colon cancer cells by inducing G_2/M cell cycle arrest as well as apoptosis, the dosages needed were much higher than serum achievable levels in humans and higher than selenite. Whether colonic luminal SeMet concentrations can reach such high levels should be evaluated to assess the relevance of these observed effects. For nonalimentary tract cancers, the only likely route of exposure to SeMet is through the vascular delivery. It would be very unlikely for such extreme high levels to be achievable through oral SeMet supplement as shown in a recent phase I trial with 11-folds higher daily intake of SeMet than the Clark study [90]. Since the patterns of ERK activation

[88], S-G_2/M arrest [85,86], and p53 induction actions [89] of SeMet resemble those of selenite exposure [61,62,64,81], we speculate that the transselenation metabolism of SeMet to SeCys then to hydrogen selenide may be responsible for the observed effects instead of a direct action of SeMet (Figure 11.1). The inefficiency in these conversion reactions may account for the high doses of SeMet required to induce the cellular and biochemical effects.

11.6 EFFECT OF SELENIUM ON PROTEIN TYROSINE KINASE-MEDIATED SIGNALING

Protein tyrosine kinases (PTK) are enzymes that catalyze the transfer of phosphate from ATP to tyrosine residues in polypeptides. PTKs are divided into two main classes. Receptor PTKs (RPTK) are transmembrane proteins with a ligand-binding extracellular domain and a catalytic intracellular kinase domain, whereas nonreceptor PTKs lack transmembrane domains and are found in the cytosol, the nucleus, and the inner surface of the plasma membrane. They work in a variety of complementary ways to regulate cellular proliferation, survival, differentiation, function, and motility. Considerable evidence points to the involvement of PTKs in a variety of solid tumors. For example, the EGFR, IGF-1R, two receptors for VEGF (VEGFR-1 and VEGFR-2) and the nonreceptor PTK FAK, are overexpressed in many solid tumors [91]. Moreover, in prostate cancers, increased expression of EGFR correlated with prostate cancer androgen independent progression [92]. Given their established role in cancer, PTKs are now regarded as excellent targets for cancer chemotherapy and chemoprevention. Various potential chemopreventive agents, including silibinin, resveratrol, grape seed extract, curcumin, and EGCG, have been reported to block either the activation or downstream effects of EGFR in human prostate cancer cells [93]. EGFR kinase inhibitors are in phase I, II, and III testing in a wide range of solid tumors [94].

As discussed earlier, Zhu et al. [68] have reported a downregulation of IGF-1R in mouse mammary hyperplastic epithelial cell line by exposure to an MSeA level that caused G_1 arrest. We have shown that Se compounds, especially methyl Se, suppress VEGF expression both *in vitro* and *in vivo* [25,81], consistent with a potential antiangiogenesis activity through inhibiting VEGF/VEGFR-mediated signaling. However, whether and how Se compounds affect the key PTKs-mediated signaling have not been systematically addressed yet.

11.7 EFFECTS OF SELENIUM ON PI3K/AKT, MAPKs, AND PKC

11.7.1 PI3K AND MAPK PATHWAYS

PI3K is a major signaling component downstream of RPTKs [95]. The serine-threonine protein kinase AKT (also known as protein kinase B) is one of the key downstream targets of PI3K. The PI3K signaling pathway regulates many cellular processes, including cell proliferation and survival, cell size and response to nutrient availability, intermediary metabolism, angiogenesis, and tissue invasion [96].

All these processes represent hallmarks of cancer. PI3K/AKT pathway is hyperactivated in a wide range of tumor types. For example, in approximately 50% of prostate tumors, this pathway is constitutively upregulated owing to mutations or the deletion of the tumor suppressor PTEN [97], which functions as a negative regulator of PI3K in part through lipid phosphatase activity. Therefore, this pathway presents a promising target for molecular therapeutic and chemopreventive intervention.

The MAPKs relay extracellular stimuli (e.g., growth factors, cellular stress) into intracellular and nuclear events, and have been implicated in many physiological processes, including cell proliferation, differentiation, and death [98]. Three major types of MAPKs exist in mammalian cells: ERKs, p38 MAPKs, and JNKs. The activities of these enzymes are largely regulated by phosphorylation/dephosphorylation. In general, the ERKs function in the control of cell mitogenesis, and inhibitors of these enzymes are being explored as anticancer agents. JNKs and p38 MAPKs are deemed stress responsive and thus, in general, involved in apoptosis.

Reports from our group were first to document differential effects of MSeA versus selenite on AKT and MAPKs in DU145 cells [61,64]. In DU145 cells, exposure of DU145 cells to 3 μM MSeA led to a profound G_1 arrest at 24 h, and exposure to greater concentrations led to not only G_1 arrest, but also caspase-mediated apoptosis. Immunoblot analyses indicated that G_1 arrest induced by the subapoptogenic doses of MSeA was associated with increased expression of $p27^{kip1}$ and $p21^{cip1}$, but apoptosis was accompanied by dose-dependent decreases of phosphorylation of AKT and ERK1/2 in the absence of any phosphorylation change in p38 MAPK and JNK1/2. In contrast, selenite exposure caused S-phase arrest and caspase-independent apoptotic DNA fragmentation, which were associated with decreased expression of p27kip1 and p21cip1, and increased phosphorylation of AKT, JNK1/2, and p38 MAPK.

In the LNCaP cells, which have a mutant PTEN and high basal AKT activity, higher doses of MSeA (10 μM) were required to induce apoptosis than in DU145 cells, although the LNCaP cells were slightly more sensitive than the DU145 cells to selenite-induced apoptosis [84]. Treatment by MSeA modestly decreased AKT phosphorylation and paradoxically increased phospho-ERK1/2 in LNCaP cells. Selenite treatment increased the phosphorylation of p53 Ser15 and both kinases, but the selenite-induced apoptosis was not influenced by chemical inhibitors of either kinase. In contrast, PI3K/AKT inhibitors greatly sensitized LNCaP cells to apoptosis induced by MSeA, accompanied by increased mitochondrial release of cytochrome c and multiple caspase activation without changing p53 Ser15 phosphorylation. The apoptosis was further accentuated by ERK1/2 inhibition without further increase in cytochrome c release. The general caspase inhibitor z-VAD-fmk completely blocked MSeA-induced apoptosis when both kinases were inhibited, whereas a caspase-8 inhibitor exerted a greater protection than did a caspase-9 inhibitor. Transfection of DU145 cells with a constitutively active AKT increased their resistance to MSeA-induced apoptosis. These results support AKT playing an important role in regulating apoptosis sensitivity of LNCaP cells to MSeA.

An MSeA-induced activation of ERK1/2 in LNCaP cells also contributed to resistance to apoptosis. These findings support the differential involvement of these protein kinase pathways in regulating apoptosis induction by different forms of Se.

In further support of cell type specificity of signaling actions of MSeA, we have shown [66] that treatment of asynchronous endothelial cells HUVECs to 3–5 μM MSeA led to a profound G_1 arrest, and exposure to higher levels of MSeA not only led to G_1 arrest but also to DNA fragmentation and caspase-mediated apoptosis. Immunoblot analyses indicated that G_1 arrest induced by the sublethal doses of MSeA was associated with dose-dependent reductions of the levels of phospho-AKT, phospho-ERK1/2, and phospho-JNK1/2 in the absence of any change in p38 MAPK phosphorylation. Apoptosis induced by MSeA was associated with an increased phosphorylation of p38 MAPK in addition to the dephosphorylation of the above kinases. Inhibition of MSeA-induced p38 MAPK phosphorylation significantly attenuates apoptosis, suggesting that MSeA-induced apoptosis in HUVEC is principally mediated by the p38 MAPK pathway [83].

Ip and coworkers [99] have examined whether MSeA primarily modulates the PI3K-phosphoinositide-dependent kinase 1 (PDK1) side of AKT phosphorylation or the phosphatase side of AKT dephosphorylation in PC-3 prostate cancer cells, which are PTEN-null. They showed that MSeA (10 μM) decreased AKT phosphorylation at Thr308 (by PDK1) more than at Ser473 (by an unidentified kinase) site. The protein levels of PI3K and phospho-PDK1 were not affected by MSeA. However, the activity of PI3K was reduced by 30% in MSeA-treated cells, thus could discourage the recruitment of PDK1 and AKT to the membrane due to low phosphatidylinositol-3,4,5-trisphosphate formation by PI3K. Consistent with the above interpretation, they reported that the membrane localization of PDK1 and AKT was significantly diminished. In the presence of a calcium chelator or a specific inhibitor of calcineurin (a calcium-dependent phosphatase), the suppressive effect of MSeA on phospho-Akt (Ser473) was greatly reduced. The finding suggests that MSeA-mediated dephosphorylation of AKT via calcineurin is likely to be an additional mechanism in regulating the status of phospho-AKT.

In mouse mammary epithelial tumor cell model (TM6 cells) *in vitro*, the effect of MSeC (50 μM) was examined for the involvement of the PI3K pathway [78]. Synchronized TM6 cells treated with MSeC and collected at different time points were examined for PI3K activity and AKT phosphorylation along with phosphorylations of Raf, MAPK/ERK kinase (MEK), ERK, and p38 MAPK. PI3K activity was inhibited by MSeC followed by a dephosphorylation of AKT. The phosphorylation of p38 MAPK was also downregulated after these cells were treated with MSeC. In parallel experiments, MSeC inhibited the Raf–MEK–ERK signaling pathway as well.

These studies suggest that MSeA, MSeC, and selenite modulate multiple intracellular kinase signaling pathways in cancer cells and vascular endothelial cells *in vitro*, with the cell type and Se form specificities. *In vivo* validation studies are necessary to assess the significance of these findings.

11.7.2 Redox Inactivation of JNK-1 and Caspase-3 in Embryonic Kidney Cells

In contrast to the JNK and p38 MPAK phosphorylation activation observed in cancer cells exposed to apoptotic levels of selenite [61,64], Park et al. [100] have shown that pretreatment with nanomolar level selenite (≤100 nM) suppresses both UV-activated JNK/SAPK and the p38 MAPK pathway in 293T human embryonic kidney cells while having no effect on TPA-PKC activation of the ERK pathway. Furthermore, such low levels of selenite directly inhibited JNK/SAPK activity in the test tube assay but not the p38 activity. They showed that cysteine 116 in JNK1 was the redox-sensitive site.

They have also shown that low levels of selenite protected HEK293 human embryonic kidney cells from cell death induced by ultraviolet B radiation (UVB) [101]. Exposure of HEK293 cells to UVB radiation resulted in the activation of caspase-3-like activity, and pretreatment of the cells with caspase-3 chemical inhibitor prevented UVB-induced cell death. Pretreatment with selenite suppressed the caspase-3-like enzymatic activity of UVB-exposed cells. Selenite also inhibited the activity of purified recombinant caspase-3 in the test tube. The inhibitory action of selenite on a recombinant active caspase-3 could be reversed by sulfhydryl reducing agents, such as dithiothreitol and beta-mercaptoethanol.

These studies suggest that nutritional low levels of selenite treatment can inhibit the JNK/SAPK-caspase apoptosis signaling pathway through thiol-redox mechanisms in non-transformed cells. It was not clear how much effects were mediated by selenite or its metabolites directly in cells, or by increasing the activity of the selenoproteins such as SeGPXs or TRs. It was not known whether low levels of methyl Se metabolites exert the same type of redox modifying effects.

11.7.3 Protein Kinase C

The redox modifying action of low levels of selenite was also reported even earlier with PKC [102,103]. The PKC family of kinases are intracellular receptors for the phobol ester-type of tumor promoters and some members play a crucial role in events related to tumor progression. Gopalakrishna has shown in test tube assays that redox-active selenocompounds, such as selenite in nM range, can inactivate PKC, particularly the Ca^{2+}-dependent isozymes, by reacting with the critical cysteine-rich regions present within the catalytic domain, while, in some cases with higher selenite, also reacting with the cysteine residues present within the zinc-fingers of the regulatory domain. Furthermore, he postulates that TR, through a direct interaction involving its selenosulfur center with the zinc-thiolates of PKC, can reverse the redox modification of this kinase induced by redox-active Se metabolites, which can form redox cycle [104]. Therefore, he proposes an interrelationship exists between these two mechanisms of Se actions to explain how resistance to Se develops in advanced tumor cells probably due to an overexpression of functional TR.

Since the JB6 epidermal epithelial transformation model used by this group is PKC-driven, the Se-induced inactivation of PKC may at least in part be responsible for the Se-induced inhibition of tumor promotion and cell growth. How applicable this pathway is to other organ sites requires further investigation, especially considering that in the Clark study nonmelanoma skin cancer risk was not decreased and was modestly but statistically increased by Se-yeast [5,9]. In addition to selenite, Sinha et al. [70] have reported an inhibition of PKC by high levels of MSeC in a mammary preneoplastic epithelial model.

11.8 EFFECTS OF SELENIUM ON p53, NF-κB, AND AP-1 NUCLEAR TRANSCRIPTION FACTORS

11.8.1 p53 Tumor Suppressor

The tumor suppressor gene p53 encodes a nuclear transcriptional factor with multiple activities in guarding the genomic integrity, cell cycle checkpoints, and apoptosis [105]. DNA damage by UV or ionizing radiation, as well as many DNA damaging drugs, activate p53 by a number of mechanisms: increased p53 protein stability by blocking degradation; increased p53 gene transcription, which has recently been linked to a responsive element to itself [106]; and p53 posttranslational covalent modifications, such as phosphorylation, acetylation, and sumoylation [107]. Among these modifications, phosphorylation of several Ser sites by ATM/ATR kinases, as well as by MAPKs, have been the best studied [107]. Redox modification of p53 cysteinyl thiols by Ref-1, a dual-function protein that can both regulate the redox state of a number of proteins and function as a DNA repair (A/P) endonuclease, is another important mechanism for regulating p53 transactivational activity [108].

11.8.1.1 Redox Regulation of p53 Transcriptional Activity and DNA Repair by Selenium

In a series of papers, Smith and coworkers have shown a potentially important redox-modification effect of Se on p53 activity and DNA repair pathways. First, they showed that pretreatment with SeMet (20 μM or higher, nonapoptotic) induced a DNA repair response in normal human fibroblasts in cell culture, and increased their long-term survival from DNA damage [109]. They showed enhanced excision repair complex formation in SeMet-treated cells as a possible mechanism for the inducible DNA repair response. Next, they established that SeMet exposure activated the p53 protein by a redox mechanism that requires the redox factor Ref-1 [110], which is potentiated by TR/thioredoxin [111]. Measurement of reduced thiol levels in p53 showed a SeMet-dependent increase that was blocked by a dominant-negative Ref-1. By using a C-terminal peptide containing only p53 cysteine residues 275 and 277, they demonstrated the importance of these residues in the SeMet-induced response. SeMet treatment of cells increased the sequence-specific DNA binding and transactivation by p53. They showed that

the long-term survival after DNA damage with UV or IR was increased by SeMet treatment in mouse embryo fibroblasts wild type for p53 genes, but not in p53-null cells. In both studies, SeMet was used at levels 10–100 times higher than physiologically achieved in serum.

Subsequently, Smith et al. [112] compared SeMet with selenite and MSeA. Low levels (1 μM) of selenite and MSeA can increase p53 activity, defined as transactivation of a p53-dependent reporter gene, as well as did the high levels of SeMet (20 μM). Both selenite and MSeA increased elevated the reduced cysteinyl thiol content of p53 as did SeMet. They also reported p53 phosphorylation was affected in cells treated with selenite and MSeA at apoptotic doses (10 μM). MSeA treatment caused phosphorylation of Thr residue(s), but did not affect any known Ser phosphorylation sites. By contrast, selenite treatment caused phosphorylation of p53 Ser20, 37, and 46 known to mediate apoptosis. Nonapoptotic doses of SeMet did not cause detectable phosphorylation of p53 Ser or Thr. These data suggest a common mechanism for p53 redox modulation by all three Se forms at low levels (likely upstream candidates are TR, thioredoxin, and Ref-1), but their actions on p53 phosphoactivation differ among Se forms.

The same group has recently linked the breast cancer suppressor gene product Brca1 to Ref-1 and p53 [113]. They showed that the two proteins interact concurrently with p53 in targeting a SeMet-induced DNA repair response. Moreover, like p53 and Ref-1, Brca1 was required for SeMet-mediated DNA damage protection, as brca1$^{-/-}$ mouse fibroblasts were not protected from UV-radiation by SeMet treatment. These findings indicate that besides p53 and Ref-1, Brca1 is required for Se protection of normal fibroblast cells from DNA damage.

While the authors initially suggested selective induction of the DNA repair branch of the p53 pathway by SeMet, it is likely that the effects are not specific to SeMet, but through a common mechanism involving either improving the activities of selenoproteins SeGPX or TRs through the generation of hydrogen selenide or the direct redox action of the redox-active Se species. The high levels of SeMet needed to achieve the redox modulation of p53 as compared to the low levels of selenite and MSeA may reflect the inefficient transselenation reaction from SeMet, as discussed earlier, because of its weak cell cycle arrest and apoptosis actions. An enhancement of p53-mediated DNA excision repair response by nutritional Se may provide a mechanism to account for the cancer risk reduction due to the prevention and repair of DNA oxidative damage. It may also help to account for the selective protective effects against genotoxic drug-induced side effects in normal cells/tissues [114]. The applicability of these findings to epithelial cells and whether a differential protective effect exists between cancer and normal cells will need to be examined.

11.8.1.2 p53 Phosphorylation and Apoptosis

In recent studies, we and others have shown that selenite (>2 μM) induces caspase-dependent and -independent apoptosis in p53 wild-type human LNCaP prostate cancer cells involving p53 Ser-15 phosphorylation [62,63,81]. Because selenite

induces DNA SSBs, we investigated whether these cells execute selenite-induced apoptosis through caspase pathways in a p53-dependent manner. The results showed that exposure of LNCaP cells for 24 h led to DNA laddering, and to the cleavage of PARP and several procaspases. Selenite treatment led to a significant increase in p53 phosphorylation on Ser-15 ($Ser^{15}P$). Time course experiments showed that p53 $Ser^{15}P$ occurred several hours prior to caspase activation and PARP cleavage. The general caspase inhibitor zVADfmk completely blocked PARP cleavage, but only partially decreased DNA laddering and did not affect p53 $Ser^{15}P$. Attenuating p53 by a chemical inhibitor, pifithrin-α, decreased the selenite-induced p53 $Ser^{15}P$ and led to concordant reductions of PARP cleavage and apoptosis. Therefore, selenite-induced p53 $Ser^{15}P$ appeared to be important for activating the caspase-mediated apoptosis in the LNCaP cells. In contrast to selenite, we showed that LNCaP cells exposed to apoptotic levels of MSeA (10 μM) did not show p53 $Ser^{15}P$ [81,84].

Smith and coworkers confirmed the phosphorylation of P53 on additional Ser residues (Ser20, Ser37, and Ser46) by selenite (10 μM) exposure in H1299 colon cancer cells (p53 null) transiently transfected with a wild-type P53 expression vector [112]. They showed that MSeA (10 μM) did not induce phosphorylation of any of the above Ser sites, but instead caused phosphorylation of Thr residue(s), which was not observed for selenite. SeMet at the same concentration did not affect p53 phosphorylation or induce apoptosis. Selenite has been shown to activate ATM [115], which is a known kinase for p53 $Ser^{15}P$ [107]. It is very probable that the ATM is one of the kinases responsible for the selenite-induced p53 $Ser^{15}P$.

The requirement of p53 and its transcriptional target Bax and the mitochondria/caspases-9 pathway for selenite-induced apoptosis in LNCaP cells was affirmed by Zhao et al. [62] and by our own work [63]. The results support that apoptotic levels of selenite induce a rapid superoxide burst, DNA damage, and p53 phosphorylative activation, leading to Bax upregulation and translocation into mitochondria, transducing signaling to the caspases.

11.8.2 NF-κB

NF-κB is a major transcription factor consisting of five members of the mammalian NF-κB family: RelA(p65), RelB, c-Rel, p105/p50, and p100/p52, all of which contain homologous N-terminal RHDs (Rel homology domains) [116]. It controls the expression of various genes, including encoding cytokines, cell adhesion molecules, and inducible NO synthase (iNOS), and COX-2. A key to NF-κB regulation is the inhibitory Bκ (IκB) proteins that in response to diverse stimuli are rapidly phosphorylated by IκB kinase complex, ubiquitinated, and undergo proteasomal degradation, releasing NF-κB factor. The essential cysteine residues of NF-κB are maintained in the active reduced state through the action of thioredoxin and TR system. NF-κB is often constitutively activated in tumors and is a key antiapoptotic factor in cancer cells and has been shown to contribute to acquired drug resistance [117].

In terms of regulation by Se, it was reported [118] that both NF-κB-dependent transactivation of a reporter gene and NF-κB activation in response to tumor necrosis factor (TNFα) or H_2O_2 treatments were decreased in human T47D breast cancer cell transfectants that overexpress SeGPX than their parental cells in Se-adequate medium. These cells contained lower basal ROS levels and lower intracellular ROS burst in response to TNFα treatment than their parental cells. In Se depleted medium that could not support SeGPX activity, the transfected and parental cells did not differ in these parameters. The cellular contents of the two NF-κB subunits (p65 and p50) and of IκB-α were unaffected by SeGPX overexpression, whereas the nuclear translocation of NF-κB as well as IκB-α degradation were inhibited in SeGPX-overexpressing cells exposed to oxidative stress (hydrogen peroxide). Moreover, in control T47D cells exposed to TNFα, a time correlation was observed between elevated ROS levels and IκB-α degradation. These results suggest that Se through SeGPX regulates intracellular ROS, which in turn are key elements that regulate the phosphorylation of IκB-α, an event that precedes and controls the degradation of this protein, and then NF-κB activation.

In addition to the inhibitory effect mediated through SeGPX in a nutritional context, Kim and Stadtman [119] have shown that in human T cells and lung adenocarcinoma cells after bacterial lipopolysaccharide treatment, the DNA binding of NF-κB in the nuclear extract was decreased progressively by added selenite levels and, at 7 μM selenite, DNA-binding activity was completely inhibited. The direct selenite inhibitory effect was reversed by addition of a dithiol, DTT. As a target of NF-κB, the iNOS activity as measured by NO products in the medium (NO_2^- and NO_3^-) was decreased by selenite addition to cell suspensions. This loss of iNOS activity was due to decreased synthesis of NO synthase protein. They speculated that the toxic levels (>5–10 μM) of selenite can react with essential thiol groups on enzymes to form RS-Se-SR adducts with resultant inhibition of enzyme activity.

We have shown [120] that selenite and MSeA, at the concentrations that induced apoptosis, inhibited NF-κB DNA binding induced by TNF-α and lipopolysaccharide in DU145 and JCA1 prostate cells with different kinetics. Both compounds also inhibited the κB-luciferase reporter activity in prostate cells. We showed that selenite and MSeA inhibited IκB kinase activation and IκB-α phosphorylation and degradation induced by TNF-α and lipopolysaccharide in prostate cells. NF-κB blockage by IκB-α dn. mutant resulted in the sensitization of prostate carcinoma cells to apoptosis induced by these Se compounds.

These results support the multiple targeting actions of Se through selenoproteins as well as possible direct adduct formation by redox-active Se metabolites to suppress the NF-κB pathway in cancer cells. A number of studies have shown the association of an inhibition of NF-κB by nutritional Se levels with a protection against heart damage [121], diabetic complications [122], and possibly HIV [123]. Readers interested in those diseases are referred to the original literature for specifics.

11.8.3 Activating Protein-1 Family

The AP-1 (*jun* and *fos* protooncogenes) signaling pathway is critical for carcinogenic transformation, among its numerous cellular functions [124]. The binding of Jun–Jun

and Jun–Fos dimers to the AP-1 DNA binding site is known to be regulated by a redox process involving conserved cysteine residues [125]. Handel et al. [126] have shown that by electrophoretic mobility-shift analyses, AP-1 DNA binding was inhibited by added selenite with 50% inhibition occurring at approximately 1 μM. Cysteine-to-serine mutants demonstrated that the effect of selenite required Cys272 and Cys154 in the DNA-binding domains of Jun and Fos, respectively. In terms of targeting specificity, 10 μM selenite added to cultured cells inhibited expression of an AP-1-dependent reporter gene, but not an AP-2-dependent reporter gene.

A different group came to similar conclusions using a different cell type [127]. In addition to selenite, its glutathione reduction product, selenodiglutathione (GS-Se-SG), efficiently inhibited AP-1 DNA binding in nuclear extracts from Se-treated 3B6 lymphocytes. Exposure of cells to a GS-Se-SG concentration of 0.75 μM resulted in 50% inhibition of AP-1 DNA binding, whereas the same effect was achieved with 7.5 μM selenite. Nuclear extracts prepared from human 3B6 lymphocytes exposed for 4 h to 10 μM selenite showed a 50% reduction of AP-1 binding. These data show nonphysiologically high levels of selenite and selenodiglutathione inactivate the AP-1 factor probably through formation of mixed disulfides as was for NF-κB [119]. The physiological relevance of these actions in the nutritional range of Se intake is not clear.

11.8.4 Differential Effects on Liver AP-1 and NF-κB by Selenium Deficiency

Christensen and Pusey [128] showed that feeding rats a Se-deficient diet versus a supplemented diet (0.5 ppm Se as selenite) for 180 days increased the liver nuclear extract DNA binding ability to NF-κB, Oct-1, and NF-1/CTF, but decreased the binding to AP-1 and HiNF-D, which controls histone 4 transcription. The liver SeGPX activity in the Se-deficient rats was less than 0.5% of the Se-supplemented rats. The results showed that in spite of redox-sensitive thiols present in NF-κB and AP-1 that could be inhibited by high levels of selenite or its glutathione metabolites, their DNA binding activity (which presumably reflects expression level differences as well as binding affinity differences) was affected by nutritional Se supplement in opposite directions. The nutritional Se status did not affect the binding activity for SP-1, HSE (heat shock element). Whether methyl Se will have the same type of differential effects *in vivo* on normal tissues and tumors has not been examined and it will be very interesting as to how they may be related to the antitumor efficacy.

11.9 EFFECTS OF SELENIUM ON STEROID HORMONE RECEPTOR SIGNALING PATHWAYS

11.9.1 Androgen Receptor (AR) Expression and Signaling

AR signaling has been extensively documented as playing an important role in the development of both androgen-dependent and -independent prostate cancer [129]. PSA is an androgen-dependent gene product of the normal prostate epithelial cells and is widely used clinically for prostate cancer diagnostics and as an indicator of

therapeutic efficacy and recurrence [130]. In the androgen-responsive LNCaP prostate cancer cell model, we have found that exposure to subapoptotic concentrations of MSeA or methylselenol inhibited PSA protein expression and secretion, whereas selenite and SeMet lacked any inhibitory effect [71]. The inhibition was detectable at 3 h of exposure and required a threshold level of MSeA to sustain. Turnover experiments showed that MSeA caused a rapid PSA degradation, which was partially blocked by lysosomal inhibitors but not by a proteasomal inhibitor. Dong et al. [72] showed that MSeA treatment reduced the PSA mRNA level, downregulated AR protein expression, and inhibited the androgen-stimulated PSA promoter transcription, which were confirmed in our study [71]. They further demonstrated the universality of MSeA suppression of the AR and PSA in five human prostate cancer cell lines (LNCaP, LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3), irrespective of their AR genotype (wild type vs. mutant) or sensitivity to androgen-stimulated growth [131]. By using the androgen response element-luciferase reporter gene assay, they found that MSeA suppression of AR transactivation is accounted for primarily by the reduction of the androgen receptor protein level. In addition to PSA, MSeA inhibited other AR-regulated genes implicated in prostate carcinogenesis and the inhibitory effects were significantly attenuated by AR overexpression. In terms of cell fate, transfection of AR in LNCaP cells weakened the inhibitory effect of MSeA on cell growth. Very recently published work by Gao and coworkers [132] showed that MSeA decreased the stability of AR mRNA after 8 h and increased AR protein degradation in LNCaP cells. Chromatin immunoprecipitation analyses showed that DHT increased the recruitment of AR and coactivators, such as SRC-1 and TIF-2, to the promoter of the PSA gene, and that recruitment was greatly diminished in the presence of 5 μM MSeA. On the other hand, MSeA enhanced the recruitment of corepressors, such as SMRT, to the promoter of the PSA gene. In a nude mouse xenograft study with LNCaP cells, Gao's group [133] showed that MSeC given by i.p. injection significantly inhibited LNCaP tumor growth ($P < 0.05$) and decreased AR expression in tumor tissues. In contrast, SeMet has been shown not to decrease PSA expression by a number of groups [71,134,135]. The above findings imply a unique mechanism to account for the prostate specific cancer chemopreventive action of methyl Se to control prostate cancer genesis and progression. The methyl-Se specificity is of particular importance for the choice of Se agents for future clinical investigations.

11.9.2 Estrogen Receptor Signaling

Estrogen is critical to the development and differentiation of estrogen target tissues, including the breast, and is a significant risk factor for breast cancer. In spite of the fact that *in vivo* cancer chemoprevention studies supporting the methylselenol hypothesis were carried out exclusively in rodent mammary cancer models [2,49–51,136], the impact of methyl Se on estrogen receptor signaling has not been critically evaluated until recently. Gao and associates [74] examined the effect of MSeA on estrogen receptor expression and activation. MSeA decreased the levels of expression of estrogen receptor-α mRNA and protein; reduced the

binding of labeled estradiol to estrogen receptor in MCF-7 cells; and inhibited the transactivating activity of estrogen receptor in MCF-7 cells using a luciferase reporter construct linked to estrogen responsive element. MSeA treatment decreased the DNA binding of the estrogen receptor to the estrogen responsive element site using an electrophoretic mobility gel shift assay and suppressed estrogen induction of the endogenous target gene c-myc. In contrast to the effect on estrogen receptor-α in MCF-7 cells, MSeA increased growth inhibitory estrogen receptor-β mRNA expression in estrogen-independent MDA-MB231 human breast cancer cells. Similar findings of MSeA effects on estrogen receptor-α signaling in MCF-7 cells were reported by Shah et al. [75].

Furthermore, Shah et al. [76] showed that MSeA at 2.5 μM potentiated growth inhibition of 4-hydroxytamoxifen (100 nM) in tamoxifen-sensitive MCF-7 and T47D breast cancer cell lines. Remarkably, in tamoxifen-resistant MCF-7-LCC2 and MCF7-H2Delta16 breast cancer cell lines and endometrial-derived HEC1A and Ishikawa cells, coincubation of 4-hydroxytamoxifen with MSeA resulted in a marked growth inhibition that was substantially greater than MSeA alone. Growth inhibition by MSeA and MSeA + 4-hydroxytamoxifen in all cell lines was preceded by a specific decrease in estrogen receptor-α mRNA and protein without an effect on estrogen receptor-β levels. Estradiol and 4-hydroxytamoxifen induction of endogenous estrogen-dependent gene expression (pS2 and c-myc) as well as estrogen response element driven reporter (ERE(2)e1b-luciferase) were also attenuated by MSeA in all cell lines before an effect on growth inhibition. Their data suggest that a specific decrease in estrogen receptor-α levels by MSeA was required for both MSeA potentiation of the growth inhibitory effects of 4-hydroxytamoxifen and resensitization of tamoxifen-resistant cell lines.

The differential regulation of estrogen receptor α versus β in breast cancer cells of different estrogen dependency statuses may represent a novel mechanism of Se action against breast cancer and other estrogen-dependent cancers. These findings have yet to be validated in preclinical models. A specificity of methyl Se against estrogen signaling, if confirmed, plus their demonstrated efficacy against chemically-induced mammary carcinogenesis in rodents [2,49–51,136] suggest these second-generation Se agents should be more useful and efficacious for the prevention of breast cancer in women than SeMet or Se-yeast. It is a shame that the enthusiasm for breast cancer studies with Se has been deeply dampened since the publication of the Clark study [5], in which numerically more women developed breast cancer in the Se-yeast group than the placebo, although the result was not statistically significant due to too few female subjects in that cohort.

11.10 EFFECTS OF SELENIUM ON SECRETORY PROTEINS IMPORTANT FOR ANGIOGENESIS AND INVASION

11.10.1 Methylselenium-Specific Inhibitory Effect of VEGF Expression

Transformed epithelial cells contribute to angiogenic switching by upregulating the expression and secretion of angiogenic stimulatory factors and/or downregulating

the expression of angiogenesis inhibitors [137]. Because of the central role that VEGF plays in neoangiogenesis [138], an inhibitory effect on VEGF expression by Se can be expected to repress the angiogenic switch for the early lesions. We have found that Se treatment, whether given in a chemoprevention setting or in an acute therapy setting, was associated with a significant inhibition of VEGF expression in some but not all mammary carcinomas induced by 1-methyl-1-nitroso urea (MNU) in the rat [24]. In cell culture, we have reported a methyl Se specificity of the inhibition of tumor epithelial VEGF expression [25]. In human prostate (DU-145) and breast (MCF-7 and MDA-MB-468) carcinoma cell lines, exposure to MSeA led to a rapid and sustained decrease of the cellular and the secreted VEGF protein levels. The concentration of MSeA required for suppressing VEGF expression was much lower than that needed for apoptosis induction. Selenite lacked any inhibitory activity in either acute or chronic exposure in these cells [25]. Taken together, the data support the hypothesis that the methyl Se pool inhibits the expression of VEGF in the transformed epithelial cells.

11.10.2 MMP-2 and Other MMPs

Expression, recruitment, and activation of MMP-2 and/or other MMPs by the stimulated endothelial cells are necessary to break down the adjacent extracellular matrix for the endothelial cells to invade through during sprouting. A crucial role of MMP-2 or MMP-9 in setting angiogenic switch has been shown in several models [139,140]. We have used a human umbilical vein endothelial cell (HUVEC) model to examine the effects of Se exposure on the expression of matrix metalloproteinase (MMP)-2 and endothelial proliferation and survival to identify Se metabolite-specific activities on these critical components of an angiogenic response [24,25,66,67,83]. We have shown a methyl Se-specific inhibitory activity on MMP-2 expression [24,25]. This was supported by a clear contrast of the inhibitory effects of MSeA and methylselenocyanate (MSeCN) with forms of Se that feed into the hydrogen selenide pool, for example, sodium selenite and sodium selenide. The MMP-2 inhibitory activity occurred at methyl Se levels (IC_{50} of ~2 μM) that were within human plasma Se range and occurred rapidly (0.5–1 h) and required cellular activation. Our results support the methyl Se pool for inhibiting the extracellular matrix degradation potential of vascular endothelial cells.

It will be important to characterize the biochemical and molecular mechanisms for methyl Se to inhibit MMP-2 expression in the endothelial cells and to determine whether such mechanisms are applicable to other MMPs and secretory proteins that are involved in angiogenesis and in tumor invasiveness, growth, and survival. It was found that nonapoptotic levels of selenite inhibited the invasion of HT1080 human fibrosarcoma cells [141]. Adhesion of HT1080 cells to the collagen matrix was also inhibited by treatment with selenite, but cell–cell interaction and cell motility were not affected by selenite. Moreover, prolonged selenite treatment (3 days) reduced expression of MMP-2 and -9 and urokinase-type plasminogen activator, but increased tissue inhibitor of metalloproteinase-1. The inhibitory effect of selenite on the protease expressions was likely mediated by the suppression of transcription

factors, NF-κB and AP-1. This study demonstrated that selenite in the supranutritional range could exert transcriptional control of MMPs upon prolonged exposure, in contrast and in addition to the mechanism of rapid decrease of VEGF and MMP-2 proteins upon MSeA exposure.

11.11 POSSIBLE CHEMICAL BASIS FOR SELENIUM EFFECTS

11.11.1 Covalent and Noncovalent Modulation on Thiol Redox

A number of years ago, Ganther [48] proposed several chemical reactions through which Se metabolites may directly modify the redox-sensitive enzymes or transcriptional factors to alter their functional activities: formation of selenotrisulfide bonds (–S–Se–S–); formation of selenylsulfide bonds (–S–Se–); catalysis of disulfide bond formation or its reversal (–SH ← Se → –S–S–); and formation of diselenide bonds (–Se–Se–). The first three reactions would affect the activities of many enzymes and proteins with critical sulfhydryl groups, while the last reaction would specifically affect activities of selenoproteins, which have SeCys residues at these active centers. Confirmation of the existence of these reaction products remains a major technical challenge.

In preceding sections, we discussed redox modification of PKC [102–104], JNK1 [100], caspase-3 [101], and p53 [110,112]. In each of these cases, the target proteins are intracellular and contain redox-sensitive cysteinyl thiols. Exposure of cells to low levels of Se as selenite was sufficient to exert redox-modification effects [100,102,103,112]. In the case of SeMet, which required 10–20 times more than selenite or MSeA [110,112], it is most likely that the redox modifying effect on p53 was not the direct effect of SeMet, but a reflection of its inefficient conversion to a Se form or forms that are redox active.

In spite of test tube assays showing a direct inhibitory effect of low levels of selenite on PKC, JNK, and caspase-3 activities, it was not clear how much of the redox modifying effects noted for these proteins in the cells can be attributed to the Se metabolites *per se* or to the improved selenoproteins and general redox tone of the cell. Genetic models with specific knocking down of one or more selenoproteins [37,40] provide tools to address this question in the future. In case of NF-κB and AP-1, high levels of selenite can directly affect their binding affinity to their respective DNA *cis* elements and mix-disulfide adduct formation was postulated as a likely chemical basis. However, nutritional Se supplement affected their DNA binding affinity in the rat liver extract in opposite directions [128], supporting additional cellular and *in vivo* mechanisms to regulate the specificity and direction of response than possibly explained by the adduct formation reaction.

In terms of extracellular targeted proteins, we have shown a rapid inhibition by methyl Se of MMP-2 and VEGF, which are secretory proteins containing disulfide bonds (S–S) to maintain their structural integrity [25]. We have speculated that the formation of mixed disulfides with the strongly reducing methylselenol CH_3SeH (–S–S– → –S–Se–CH_3) may disrupt the protein folding and cause the rapid degradation of the nascent proteins synthesized and processed by endoplasmic reticulum

(ER) and Golgi. Our recent finding of MSeA-induced rapid degradation of PSA [71], which is a secretory protein with several bisulfide bonds, adds further support. The global redox modification of cellular proteins by MSeA treatment [142] and the unfold protein response (UPR) [143] phenomena described recently by Ip's group and collaborators highlight the prevalence of thiol redox targeting.

11.11.2 Global Protein Redox Modification and ER Stress Response Induced by MSeA

Lee et al. [142] recently described the use of a neural network-based analysis to identify proteins sensitive to MSeA-induced redox modification using the reactive thiol specific reagent, biotin-conjugated iodoacetylethylenediamine BIAM, to monitor thiol proteome changes on 2D gel. The patterns identified support a global redox modification in the multitude of proteins from all compartments of the cell, consistent with the pervasive nature of a small Se metabolite(s) to strike many sensitive cellular and subcellular protein targets. At least some of these modifications will affect the folding and integrity of the proteins, triggering the UPR [143]. The accumulation of aberrantly folded proteins in the ER triggers a defined set of transducers to correct the defects or commit the cells to apoptosis if the rescue effort is exhausted [143].

Along this line of interpretation, Ip's group [144] has shown that in PC-3 human prostate cancer cells treated with MSeA, a number of signature endoplasmic reticulum stress markers were induced: (1) the survival/rescue molecules, such as phosphorylated protein kinase-like endoplasmic reticulum-resident kinase (phospho-PERK), phosphorylated eukaryotic initiation factor-2α (phospho-eIF2α), glucose-regulated protein (GRP)-78, and GRP94; and (2) the apoptotic molecules, such as caspase-12, caspase-7, and CAAT/enhancer binding protein homologous protein or growth arrest DNA damage-inducible gene 153 (CHOP/GADD153). Additional evidence suggested that CHOP/GADD153 might be an important transcription factor in apoptosis induction by MSeA *in vitro*. In general, a higher concentration of MSeA was required to elicit the apoptotic markers compared with the rescue markers. The apoptotic markers increased proportionally with the dose of MSeA, whereas the rescue markers failed to keep pace with the increasing challenge from MSeA. GRP78 is a rheostat of the ER stress transducers. In GRP78-overexpressing cells, the ability of MSeA to upregulate phospho-PERK, phospho-eIF2α, GRP94, caspase-12, caspase-7, and CHOP/GADD153 was significantly muted. A generous supply of GRP78 would allow cells to cope better with ER stress, thereby improving the odds for survival and negating the commitment to apoptotic death.

Ip's group provided further evidence by knocking down GRP78 induction by small interference RNA [145]. In the presence of MSeA, CHOP/GADD153 expression was raised even higher by GRP78 knockdown. Under this condition, the MSeA effect on $p21^{cip1}$, CDK1, and CDK2 was also magnified in a manner consistent with enhanced cell growth arrest. Additional experiments with CHOP/GADD153 siRNA knockdown strongly suggested that CHOP/GADD153 may play a positive role in

upregulating the expression of p21cip1 in a p53-independent manner (PC-3 cells are p53 null).

These findings support the idea that MSeA, through modifying thiol redox in cellular proteins, can induce UPR, which activates a compensatory survival response to balance with the apoptotic signaling, which may in part be mediated by the select UPR molecules. We have reported a similar phenomenon of upregulation of ERK1/2 in LNCaP cells by MSeA, decreasing the apoptosis signaling induced by MSeA [84]. It is hopeful that the combination of proteomic approaches with expression genomics may provide comprehensive signature action profiles of the molecular targets and pathways of the different Se pools.

11.12 SELENIUM IN CANCER THERAPY

11.12.1 Reduction of Side Effects of Drugs and Enhancement of Drug Efficacy

In addition to the extensive work focusing on chemoprevention by Se, recent studies have brought a renewed interest in the therapeutic potential of Se as either a monotherapy or an enhancer of existing treatment modalities. Rustum and coworkers [114] have used athymic nude mice bearing human squamous cell carcinoma of the head and neck (FaDu and A253) and colon carcinoma (HCT-8 and HT-29) xenografts to evaluate the potential role of Se compounds as selective modulators of the toxicity and antitumor activity of selected anticancer drugs, with particular emphasis on irinotecan, a topoisomerase I poison. They showed that a sublethal dose of Se either as MSeC or SeMet was highly protective against toxicity induced by a variety of chemotherapeutic agents. Furthermore, MSeC increased significantly the cure rate of xenografts bearing human tumors that are sensitive (HCT-8 and FaDu) and resistant (HT-29 and A253) to irinotecan. The high cure rate (100%) was achieved in nude mice bearing HCT-8 and FaDu xenografts treated with the MTD of irinotecan (100 mg/kg/week×4) when combined with Se. Administration of higher doses of irinotecan (200 and 300 mg/kg/week × 4) was required to achieve high cure rate for HT-29 and A253 xenografts. Administration of these higher doses was possible due to selective protection of normal tissues by Se. The observed *in vivo* protective action against drug toxicity was highly dependent on the schedule of Se, which required a minimum of 3 days ahead of the first drug treatment. The afore discussed redox regulation of p53 and DNA repair function (Section 11.8) may provide an explanation of the reduction of side effects observed here. A better understanding of the mechanisms of the enhancement action on drug efficacy and protective action against drug side effects is essential to clinical translation of these findings into patient benefit.

11.12.2 A Phase I Study with SeMet and Irinotecan

Based on the animal data, Rustum and coworkers [90] conducted a phase I study to determine the impact of a fixed, nontoxic high dose of SeMet on the maximum

tolerated dose (MTD) of irinotecan. SeMet was given orally as a single daily dose containing 2.2 mg of Se starting 1 week before the first dose of irinotecan. The Se dosage was 11 times higher than that used in the Clark study [5]. Irinotecan was given intravenously once a week for 4 weeks every 6 weeks (one cycle). The starting dose of irinotecan was 125 mg/m^2/wk. Escalation occurred in cohorts of three patients until the MTD was defined. Pharmacokinetic studies were done for selenium, and irinotecan and its metabolites. The results showed that three of four evaluable patients at dose level 2 of irinotecan (160 mg/m^2/wk) had a dose-limiting diarrhea. None of the six evaluable patients at dose level 1 (125 mg/m^2/wk irinotecan) had a dose-limiting toxicity. One patient with a history of irinotecan-refractory colon cancer achieved a partial response. SeMet displayed a long half-life of prolonged accumulation toward steady-state concentrations. SeMet did not significantly change the pharmacokinetics of CPT-11, SN-38, or SN-38G; however, the coadministration of SeMet significantly reduced the irinotecan biliary index, which has been associated with gastrointestinal toxicity. It was concluded that SeMet at the dose and schedule used did not allow the safe escalation of irinotecan beyond the previously defined MTD of 125 mg/m^2. Disease stabilizations were noted in this highly refractory population. Further escalation of SeMet and longer pretreatment with SeMet are recommended in future trials to achieve defined protective serum concentrations of Se. Considering the better action profiles of methyl Se than SeMet, it will be very interesting to consider MSeC or MSeA for future trials.

11.12.3 Selenium as an Enhancer of Drug-Induced Apoptosis

Using androgen-independent and p53 nonfunctional prostate cancer cell culture models, we have investigated the Se specificity and signaling pathways underlying the enhancement action on apoptosis-induced by different classes of chemotherapeutic drugs [146]. DU145 and PC3 human androgen-independent prostate cancer cells were exposed to minimal apoptotic doses of Se and/or the topoisomerase I inhibitor 7-ethyl-10-hydroxycamptothecin (SN38), the topoisomerase II inhibitor etoposide, or the microtubule inhibitor paclitaxel/taxol. Our results showed that MSeA increased the apoptosis potency of SN38, etoposide, or paclitaxel several folds higher than the expected sum of the apoptosis induced by MSeA and each drug alone. The combination treatment did not further enhance JNK1/2 phosphorylation that was induced by each drug in DU145 cells. The JNK inhibitor SP600125 substantially decreased the activation of caspases and apoptosis induced by MSeA combined with SN38 or etoposide, and completely blocked these events induced by MSeA/paclitaxel. The caspase-8 inhibitor zIETDfmk completely abolished apoptosis and caspase-9 and caspase-3 cleavage, whereas the caspase-9 inhibitor zLEHDfmk significantly decreased caspase-3 cleavage and apoptosis but had no effect on caspase-8 cleavage. None of these caspase inhibitors abolished JNK1/2 phosphorylation. In contrast to MSeA, selenite did not show any enhancing effect on the apoptosis induced by these drugs. Our results show that the enhancing effect was primarily through interactions between MSeA and JNK-dependent targets to amplify the caspase-8-initiated activation cascades in a p53-defective background.

The MSeA-specific enhancement action on drug-induced apoptosis was also found with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Yamaguchi et al. [147] demonstrated that concomitant treatment with TRAIL and MSeA produced synergistic effects on the induction of apoptosis in androgen-dependent LNCaP and androgen-independent DU-145 prostate cancer cells. MSeA rapidly downregulated expression of the cellular FLICE inhibitory protein, a negative regulator of death receptor signaling. In addition, they demonstrated that the synergistic effects of MSeA and TRAIL resulted from the activation of the mitochondrial pathway-mediated amplification loop. The addition of MSeA effectively blocked TRAIL-mediated BAD phosphorylation at Ser112 and Ser136 in DU-145 cells and was accompanied by induction of the mitochondrial permeability transition and release of apoptogenic cytochrome c and Smac/DIABLO proteins from the mitochondria and into the cytosol.

These results suggest that MSeA may help to enhance efficacy of and overcome resistance to drug-induced or TRAIL-mediated apoptosis in prostate cancer cells. Whereas p53 was not required for the enhancement effect of MSeA on apoptosis induced by drugs or TRAIL as discussed earlier [146,147], we have shown a critical role of the p53 and Bax/mitochondria pathway of caspases to mediate selenite's ability to enhance apoptosis induced by TRAIL in the LNCaP cells [63]. Selenite induced a rapid generation of superoxide and p53 Ser-15 phosphorylation, and increased Bax abundance and translocation into the mitochondria. A combined treatment of selenite and TRAIL led to synergistic increases of Bax abundance and translocation into mitochondria, loss of mitochondrial membrane potential, cytochrome c release, and the cleavage activation of caspases-9 and -3. Inactivating p53 with a dominant negative mutant abolished apoptosis without affecting superoxide generation, whereas a superoxide dismutase mimetic agent blocked p53 activation, Bax translocation to mitochondria, cytochrome *c* release and apoptosis induced by selenite/TRAIL. In support of Bax as a crucial target for cross talk between selenite and TRAIL pathways, introduction of Bax into p53-mutant DU145 cells enabled selenite to sensitize these cells for TRAIL-induced apoptosis. The results indicate that selenite induces a rapid superoxide burst and p53 activation, leading to Bax upregulation and translocation into mitochondria, which restores the cross talk with stalled TRAIL signaling for a synergistic caspase-9/-3 cascade-mediated apoptosis execution.

It is therefore possible that the p53 functional status of the cancer may influence the choice of Se forms to provide the most enhancement of efficacy to be balanced with an optimal reduction of side effects. Since the risk for selenite genotoxicity in the treatment of a cancer patient is less of a concern than for primary prevention use, the combined use of selenite and methyl Se with chemotherapeutic drugs may target a broader spectrum of cancers.

11.13 SUMMARY AND IMPLICATIONS

The mechanistic studies reviewed in this chapter have indicated that the Se forms and doses are critical determinant factors for regulating a multitude of

cell signaling pathways from the cell surface to the nucleus. At low dose range (nM), selenoproteins as well as redox-active Se metabolites may confer modest protection against ROS-driven carcinogenesis by genetic and epigenetic processes, and enhance DNA repair through redox modulation of p53 and its targets. At chemopreventive and therapeutic doses, two distinct Se metabolite pools exert diverse and differential effects on these signaling pathways, leading to cell cycle arrests and apoptosis. The methylselenol metabolite pool has many desirable attributes of cancer chemoprevention and therapy, targeting PI3K/AKT signaling pathways and angiogenic switch regulators in general cancers, as well as sex hormone signaling in gender-specific cancers. This pool of Se appears not to induce ROS or DNA SSBs, and in general does not activate JNK stress pathways. The hydrogen selenide pool in excess of selenoprotein synthesis can lead to DNA SSBs and genotoxicity to normal cells. The cell cycle and apoptosis responses induced by this type of Se can be greatly dependent on the functional status of the p53 and associated with the activation of the JNK/p38MAPK pathways and ROS generation. We speculate that the redox regulation of p53 and DNA repair pathways and the weak cell cycle and apoptosis actions of SeMet may be attributable to its inefficient metabolism to enhance selenoprotein synthesis and redox-active Se form(s), rather than its direct action per se. Available data support the methylselenol precursors, such as MSeC and MSeA, as more meritorious candidates than SeMet or selenite for future clinical investigations of cancer preventive efficacy and as chemosensitizers of cancer therapy.

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ABBREVIATIONS

AKT/PKB	protein kinase B
AR	androgen receptor
ATM	ataxia tetangiectasia mutated
CH_3SeCH_3	dimethylselenide
$(CH_3)_3Se+$	trimethylslenonium
CH_3SeCys (or MSeC)	Se-methylselenocysteine
CH_3SeH	methylselenol
ERK	extracellular signal regulated kinase
GSSeH	glutathioneselenol
GSSeSG	selenodiglutathione

H_2Se	hydrogen selenide
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MSeA	methylseleninic acid
p38 MAPK	also known as stress-activated protein kinase 2
PARP	poly(ADP-ribose)polymerase
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PSA	prostate specific antigen
PTK	protein tyrosine kinase
ROS	reactive oxygen species
RPTK	receptor protein tyrosine kinase
SAPK	stress-activated protein kinase
Se	selenium
SeCys	selenocysteine
SeMet	selenomethionine
SeO_2	selenium dioxide
VEGF	vascular endothelial growth factor

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12 Modulation of Gene Expression by Dietary Carotenoids and Retinoids: Role in Cancer Prevention

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CONTENTS

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12.1 INTRODUCTION

From a biological perspective, carotenoids can be most easily classified as those that possess provitamin A activity and those that do not. From a chemical perspective, carotenoids are generally classified as the hydrocarbon carotenoids, of which the prototype would be beta-carotene, a $C_{40}H_{56}$ hydrocarbon containing 2 beta-ionone rings and 11 conjugated double bonds; and the xanthophylls, oxygenated carotenoids such as lutein ($C_{40}H_{56}O_2$), found in all green leaves, with hydroxyl groups on each of the rings. Both compounds can be found at micromole amounts in human serum and are obtained from consumption of carotenoid-containing fruits and vegetables [1]. Carotenoids also include straight-chain molecules, such as lycopene ($C_{40}H_{56}$), the red pigment found in tomatoes. The principal structural features of carotenoids are the possession of a methyl-substituted, conjugated double-bond system, reflecting their synthesis from isoprene units in the plant [2]. Until recently, carotenoids were considered to have two major biological functions: first, the possession of a beta-ionone ring by the provitamin A carotenoids allows their bioconversion in mammals to retinoids—compounds with essential roles in vision and in normal growth and differentiation [3]; and second, the conjugated double-bond system possessed by all carotenoids allows them to be effective lipid-phase antioxidants that can protect cell organelles from free-radical mediated damage. As to be discussed later, there is now evidence that carotenoids can directly regulate the expression of genes protective against carcinogenesis. Retinoids are those compounds capable of activating the retinoic acid nuclear receptors (RAR and RXRs); the natural ligands for these receptors are all-*trans* retinoic acid and 9-*cis* retinoic acid, respectively. Structures of representative carotenoids and retinoids are shown in Figure 12.1. The role of carotenoids in the biosynthesis of retinaldehyde, the visual pigment in the retina, is a separate and discrete function that will not be further considered here. Instead, we will concentrate on the role of carotenoids and retinoids as modulators of expression of genes directly or indirectly involved in carcinogenesis.

12.2 CAROTENOIDS MODULATE OXIDATIVE DAMAGE AND REDOX STATE OF CELLS: CHEMICAL OXIDATIVE DAMAGE

Cell constituents are confronted with multiple sources of highly reactive oxidative species capable of causing lipid peroxidation and DNA damage. In purely chemical systems, carotenoids can be shown to be effective quenchers of reactive oxygen species such as singlet oxygen, as well as radical chain breaking agents. However, as first described by Burton and Ingold, the situation is complicated by the ability of beta-carotene to act as a pro-oxidant under conditions of high partial pressure of oxygen (pO_2) [4]. Under these conditions, the initial reaction with an oxidant produces an unstable intermediate that can itself react with, for example, unsaturated lipids to produce a chain reaction and the formation of additional reactive species

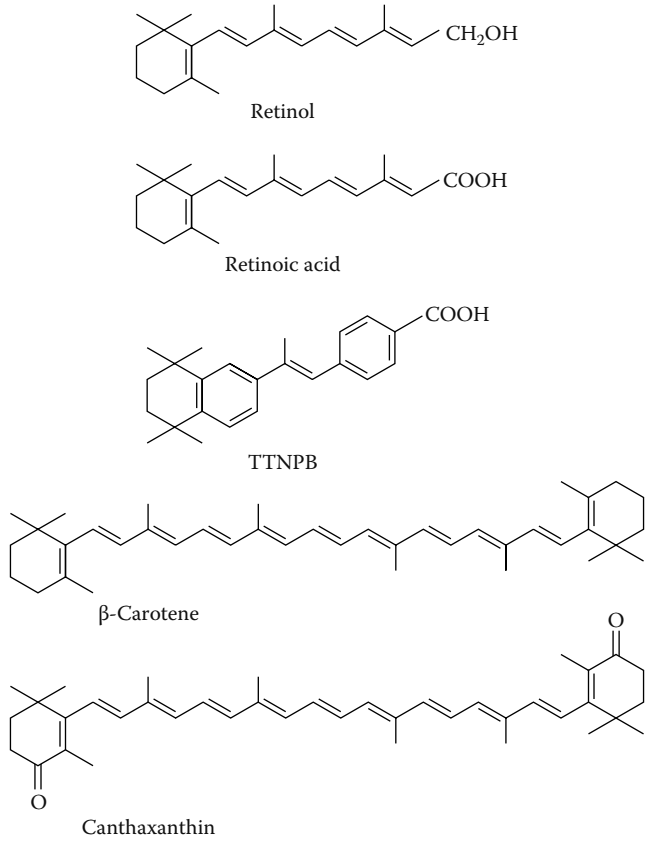

FIGURE 12.1 Structures of the key retinoids and carotenoids discussed in this chapter.

such as epoxides and carbonyl compounds. The biological significance of this phenomenon is unclear, but may be in part responsible for the increased lung cancer rates observed in intervention trials conducted with high-dose beta-carotene in smokers and asbestos-exposed individuals [5,6]. Fortunately, not all carotenoids become pro-oxidants under these conditions; astaxanthin (3,3′-dihydro-4,4′-diketo-beta-carotene) has been described as a more potent antioxidant than beta-carotene yet does not act as a pro-oxidant under high partial pressure of oxygen [7]. This would imply that astaxanthin and possibly other keto-carotenoids may be inherently safer antioxidants in any context of high oxygen concentration, such as the lung or in patients receiving supplemental oxygen.

There are many examples where oxidative stress has pathological consequences in humans. For example, chronic inflammation—either as a consequence of autoimmune disorders or unresolved infection—leads to the generation by

immune cells of large amounts of diverse reactive oxygen species. These reactive species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl), have physiological functions in being cytotoxic to invading pathogens, while the production of nitric oxide (NO) by macrophages leads to vasodilation and increased blood flow to infected tissues [8,9]. In conditions of chronic inflammation, however, this production of reactive oxygen species may continue for months or years and be damaging to local tissue and surrounding stroma. For example, chronic viral hepatitis leads to progressive loss of the liver parenchyma and replacement by fibrotic lesions—a process known as cirrhosis. Similar tissue damage occurs in conditions of chronic gastritis and colitis, atherosclerosis, and rheumatoid arthritis. Unfortunately, damage occurs not just to the architecture of the tissue but also to the genome. These same reactive oxygen species are known to be capable of causing DNA lesions such as 8-hydroxy deoxyguanosine and 8-oxo-guanosine, both promutagenic lesions. They also can deaminate DNA, leading again to potential mutagenic changes if unrepaired [10]. The increased cell division caused by reactive hyperplasia in chronically inflamed tissues, together with the increased rate of DNA damage, almost certainly results in the increased rate of malignancy seen in these conditions [11]. Indeed, chronic infection of gastric epithelium with *H. pylori* is now known to cause most cases of chronic gastritis in the West, a condition that can lead to stomach cancer in a significant proportion of cases [12].

Preliminary data from the group of Nishino in Japan have suggested that supplemental lycopene (10 mg/day) and α-tocopherol administered orally to patients with chronic hepatitis C result in a dramatic decrease in the incidence of hepatocellular carcinoma in comparison to controls beginning about 1 year after initiating treatment [13]. These data are consistent with reports from the same group that liver fibrosis in rats with copper overload can be suppressed by lycopene alone [14]. Globally, liver cancer is the most prevalent cancer resulting most frequently from chronic viral infection and consumption of aflatoxin-contaminated foods; therefore, this observation is of enormous potential significance. It remains to be determined whether the chemopreventive action of this antioxidant mixture of lycopene and α-tocopherol is mediated solely through its antioxidant/anti-inflammatory properties or whether lycopene is exerting more specific effects on gene regulation, as will be discussed later.

Astaxanthin, a xanthophyll predominantly found in marine organisms and responsible for the pink pigmentation of shrimp and lobster and of birds like the flamingo who feed on marine organisms, might also possess potent anti-inflammatory activity. In studies in cell culture and in mice, astaxanthin was shown to inhibit the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), prostaglandins, and nitric oxide (NO). This activity was the result of inhibited activation of the nuclear transcription factor NF-κB, probably a result of scavenging reactive oxygen species known to activate this inflammation pathway [15].

12.3 CAROTENOIDS MODULATE EXPRESSION OF DRUG METABOLIZING ENZYMES

12.3.1 Effects in the Liver

The phase I and II detoxifying enzymes exist for the purpose of making more water-soluble xenobiotic compounds so that they may be ultimately excreted by the kidneys. The phase I enzymes perform initial modifications on the xenobiotics typically by the addition of hydroxyl groups; in contrast, the phase II enzymes catalyze the addition of large hydrophilic molecules, such as glutathione or modified sugars, to the xenobiotics allowing urinary excretion. Unfortunately, many chemical carcinogens become activated by phase I enzymes to their ultimate carcinogenic form [16]. As initially reported by Astorg et al., the effects of carotenoids on phase I enzymes are complex and appear to be both carotenoid and species specific. For example, in rats the CYP11A-inducing carotenoids, beta-apo-8′-carotenal, astaxanthin and canthaxanthin, decreased *in vivo* aflatoxin B1 (AFB1)-induced DNA single strand breaks (SSB) and the binding of AFB1 to liver DNA and plasma albumin; and increased *in vitro* AFB1 metabolism to aflatoxin M-1, a less genotoxic metabolite. However, lycopene had no effect [17]. Because of the multiplicity of phase I enzymes with defined substrate specificities, modulation of these enzymes may both activate and inactivate chemical carcinogens depending upon the site of chemical modification. It is recommended that in order to avoid these interpretational problems, experimental studies probing mechanisms should utilize carotenoids administered after carcinogen administration or utilize carcinogens that do not require metabolic activation. For example, in my studies with 10T1/2 cells, carotenoids were added only 7 days after removal of carcinogen or, where simultaneous treatment was required, X-irradiation was utilized as carcinogenic stimulus.

The situation with phase II enzymes is somewhat more simple because induction of all enzymes in this response pathway is believed to be protective against oxidative stress and/or carcinogen damage [18]. As elegantly demonstrated by Talalay's group in their studies of the protective role conveyed by consumption of cruciferous vegetables, it is now known that the nuclear transcription factor Nrf2 becomes activated by changes in the redox state of the cell and transcriptionally activates genes controlled by the antioxidant response element (ARE). Under normal circumstances Nrf2 is cytoplasmic and bound to the inhibitory protein Keap1. This protein contains multiple sulfydryl, residues that act as sensors for the redox state of the cell [19]. Upon modification of these residues, Nrf2 is released, translocates to the nucleus, and activates genes such as glutathione *S*-transferase and glutathione synthetase [19].

Although there was some evidence that carotenoids could activate phase II enzymes [20], until recently their influence on genes activated by the ARE had not been demonstrated. Using an ARE-luciferase reporter system, lycopene in particular has now been shown to cause reporter activation together with the induction of

the phase II enzymes NAD(P)H: quinone oxidoreductase and γ-glutamylcysteine synthetase together with an increase in intracellular glutathione. The role of Nrf2 in mediating these responses was demonstrated using a dominant-negative Nrf2 construct [21]. These results must be viewed as potentially highly significant to the protective role of carotenoids against oxidative stress; a role that does not directly depend upon their interaction with reactive oxygen species (ROS). Interestingly, this activity of lycopene could be duplicated by ethanol extraction products of lycopene, suggesting that degradation products of lycopene may be in part or totally responsible for this biological activity. Similar findings have been reported for the ability of oxidation products of lycopene to enhance gap junctional communication (GJC) [22].

The significance of stimulating the ARE system is highlighted TM experiments conducted by others who have demonstrated that inhibition of this response by targeted knockout of Nrf2 enhances the susceptibility of mice to carcinogenesis [23]; conversely, the use of siRNA to downregulate expression of Keap1, thus activating Nrf2, dramatically increased the levels of phase II enzymes in cultured human keratinocytes [24].

12.3.2 Modulation of Signaling Pathways in the Lung

The early association of the beta-carotene content of diet with decreased risk of lung cancer [25] led to three large clinical trials of synthetic beta-carotene: two trials were conducted in tobacco-exposed or tobacco- and asbestos-exposed individuals, while a third was conducted in much lower risk, predominantly non-smoking, American physicians. The results were, to say the least, disappointing. In studies conducted with the high-risk smokers, lung cancer rates actually increased by approximately 20% in the intervention group [26,27], while no effect was seen in the low-risk physicians [28]. Needless to say, these results have severely dampened enthusiasm for other large trials in healthy individuals. However, it should be noted that supplemental levels of beta-carotene were approximately 10-fold higher than those normally consumed in a healthy diet; moreover, the deleterious effects of supplementation seem restricted to the lung. In a separate study, comparable high doses of beta-carotene were shown capable of inducing a small degree of protection for smokers against head and neck cancer, although again moderately increasing lung cancer rates [29].

In an attempt to explain these disturbing and confusing results, Wang, Russell, and coworkers began studies in the ferret, an animal that unlike rats and mice, readily absorbs dietary carotenoids. Utilizing ferrets fed low- and high-dose beta-carotene at levels designed to mimic dietary exposure and supplemental exposure of humans, respectively, they were able to convincingly reproduce the results obtained in the human intervention studies. Not only did cigarette smoke-exposed ferrets supplemented with high-dose beta-carotene develop more severe lung lesions than smoke-only exposed ferrets, but high-dose beta-carotene alone induced squamous metaplasia in the lungs of these animals. This was found to be most likely a consequence of the induction by high-dose beta-carotene of enzymes CYP1A1

and 1A2, which are in part responsible for the catabolism of retinoic acid. This reduction in retinoic acid concentrations in turn led to elevated levels of the transcription factor activator protein 1 AP-1 and elevated levels of c-Jun and cyclin-D, which would be expected to increase the proliferation rate in affected tissue [30]. In contrast, low-dose beta-carotene was able to weakly attenuate the pathological effects induced by smoke exposure of the ferret lung [31]. More recently, this group has demonstrated that protection is not limited to beta-carotene but also extends to lycopene; here, both low- and high-dose lycopene were able to protect ferrets against lung metaplasia induced by cigarette smoke exposure. In this situation, protection was reported to be unrelated to retinoic acid metabolism, but instead involved the insulin-like growth factor-1 (IGF-I) system. While IGF-I levels themselves were not modified by lycopene treatment, levels of an IGF-binding protein (IGFBP-3) were elevated by approximately 50% and smoke-induced decreases in this binding protein were abrogated by lycopene treatment [32]. At present it is not known whether lycopene may have deleterious effects in the smoke-exposed lung, since the concentrations of lycopene in lung tissue attained in these experiments, even with a high oral dosage, were approximately 10-fold lower than those obtained by beta-carotene at toxic doses. It must be mentioned on a cautionary note that while these studies were conducted with purified lycopene from synthetic or tomato origin, tomatoes contain other bioactive compounds. For example, the polyphenol quercetin found in tomatoes and related compounds, such as genestein found in soy products, have been shown to influence the IGF-I signaling system leading to increased apoptosis in cultured prostate carcinoma cells [33]. Interactions between carotenoids and the IGF-I system are discussed in more detail elsewhere in this volume.

Of additional interest is the observation that peroxisome proliferator-activated receptor (PPAR) agonists will also induce expression of the beta-carotene 15,15′ dioxygenase by activating a PPAR responsive element within the 5′ promoter region of this gene [34]. As will be discussed later, we have evidence that carotenoids activate PPAR-γ and directly modulate expression of connexin (Cx43) [35], implying that carotenoids may modulate their own metabolism in addition to the metabolism of retinoic acid. A surprising variety of chemicals are capable of activating PPAR-γ; these include fatty acids and prostaglandins, as well as fibrates and thiazolidinediones—drugs utilized clinically in obesity and diabetes [36,37]. It is at present unclear if carotenoids activate PPAR-γ directly or if activity is due to degradation products of carotenoids produced by chemical or enzymatic oxidation, which have structural similarities to fatty acids and prostaglandins.

12.4 RETINOIDS AND DIETARY CAROTENOIDS AS CANCER PREVENTIVE AGENTS

There is abundant epidemiological evidence that consumption of dietary carotenoids, in contrast to the high-dose intervention studies discussed earlier, is associated with protection against cancer in humans [38]. Interest in retinoids as cancer preventive

agents was first stimulated by observations that the symptoms of retinoid deficiency in skin were pathologically similar to early changes caused by chemical-carcinogen exposure, that is, squamous metaplasia. It was demonstrated that vitamin A compounds could reverse this pathology and effectively prevent the development of skin cancer in experimental animals [39]. These observations have been translated clinically, however, with little practical success. Retinoic acid has been demonstrated to be active in preventing cervical carcinoma [40] and carcinoma of the head and neck [41], however, their use has been severely restricted by cutaneous toxicity. In other organ sites, in spite of demonstrating activity in experimental animals, retinoids have not shown activity in bladder cancer prevention or in prevention of breast cancer [42].

12.5 RETINOIDS AND CAROTENOIDS INHIBIT CARCINOGEN-INDUCED NEOPLASTIC TRANSFORMATION IN CULTURE

In order to probe the structure–activity requirements for chemopreventive activity and to enable the conduct of more mechanistic studies, my colleagues and I began studies in transformable C3H/10T1/2 cells (10T1/2). We had previously shown these cells to respond to chemical and physical carcinogens by the quantitative formation of neoplastically transformed foci (Figure 12.2). These foci when cloned produced progressively growing sarcomas in immunosuppressed mice [43]. Of interest to our future study of retinoids and carotenoids in this system, we had shown that the ability of carcinogen-initiated cells to progress to those with the full neoplastic phenotype was strongly influenced by the population density of cells in culture [44] and by the colony size attained by initiated cells prior to the population entering post confluent growth arrest [45,46]. We hypothesized that these effects could best be explained by the existence of communicating pathways mediated via gap junctions, between nontransformed cells and their carcinogen-initiated counterparts [47]. As will be discussed shortly, we later demonstrated that the ability of retinoids and carotenoids to suppress neoplastic transformation could be explained by their ability to increase cell–cell communication through gap junctions.

Addition of retinoids to carcinogen-exposed cultures resulted in complete inhibition of neoplastic transformation in these cells [48]; this activity correlated with their potency as vitamin A analogs [49]. The exception to this statement was the lack of activity of all-*trans* retinoic acid, the natural ligand for retinoic acid receptors (RARs). This discrepancy was later discovered a consequence of the high rate of metabolic degradation of this molecule by 10T1/2 cells [50]. Carotenoids were also shown effective in this system. The first carotenoids to be tested were those available in a "beadlet" formulation because of the problems of drug delivery of these lipophilic molecules to cells in culture. This limited our studies to beta-carotene and canthaxanthin [51]. In order to extend studies to a more diverse series of dietary carotenoids, we developed tetrahydrofuran (THF) as a delivery

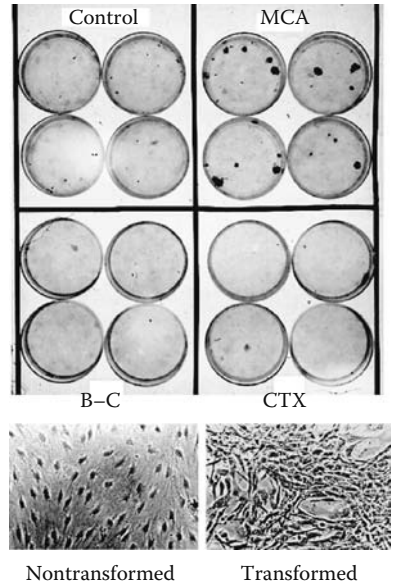

FIGURE 12.2 Top panel: Photomicrograph of 60 mm dishes after 5 weeks in culture; top left, acetone treated control; top right, 5 μg/ml methylcholanthrene (MCA) for 24 h; bottom left, 5 μg/ml methylcholanthrene (MCA) for 24 h followed after 7 days by beta-carotene 1 μM for 4 weeks; bottom right, 5 μg/ml (MCA) for 24 h followed after 7 days by canthaxanthin 1 μM for 4 weeks. Bottom panel: left, photomicrograph of a confluent monolayer of nontransformed 10T1/2 cells; right, transformed cells produced by treatment with 5 μg/ml MCA for 24 h. All images are of cells stained with Giemsa after 5 weeks in culture.

solvent. Use of THF results in the formation of a pseudosolution of carotenoids in cell culture medium—a form that is highly bioavailable. When carotenoids were added 1 week after removal of the chemical carcinogen, all carotenoids tested, regardless of their provitamin A activity, were capable of inhibiting the development of neoplastic transformation. Moreover, just as in our earlier studies with retinoids, removal of the carotenoid led to emergence of neoplastic transformed foci some 3–4 weeks later [52]. This indicated that we were not dealing with selective cytotoxicity, but with a reversible inhibition of the process of neoplastic transformation. These studies demonstrated that, at least in the model cell culture system employed, retinoids and dietary carotenoids had the following properties:

1. They were active when added at least 1 week after removal of carcinogen, and thus it was clear they inhibited neoplastic transformation in the postinitiation phase of carcinogenesis.
2. If treatment was stopped, neoplastic foci appeared in cultures 3–4 weeks after cessation indicating their action was reversible and thus not a consequence of selective cytotoxicity to carcinogen-initiated cells.

3. Once neoplastic transformation occurred, treatment did not inhibit the growth of transformed cells either alone or in confrontation with nontransformed cells. Activity could not, therefore, be the result of selective growth inhibition of transformed cells.
4. For the carotenoids, action appeared independent of conversion to chemopreventive retinoids, since activity was observed even with straight-chain hydrocarbons such as lycopene.
5. Although all carotenoids exhibited antioxidant activity in cultures, as demonstrated by decreased thiobarbituric acid reactive-substances (TBAR) formation, their chemopreventive activity did not correlate with their antioxidant properties; indeed, alpha-tocopherol, the most potent antioxidant tested in this system, was only a very weak inhibitor of neoplastic transformation [53].
6. For both retinoids and carotenoids, activity as inhibitors of neoplastic transformation strongly correlated with their ability to upregulate gap junctional communication in nontransformed cells.

Gap junctions and their role in carcinogenesis are discussed in some detail in the next section.

12.6 GAP JUNCTIONAL COMMUNICATION AND CARCINOGENESIS

Gap junctions are water-filled pores called connexons that connect adjacent cells in most organs of the body. These pores allow direct cytoplasmic-to-cytoplasmic communication of water-soluble molecules and ions. Because of the limiting size of the pore, only molecules below 1000 Da can pass, excluding molecules, such as mRNA and protein, thus maintaining genetic identity of the cells. The existence of this network of communication creates a syncytium through which cells can exchange nutrients, waste products, and signaling molecules such as cAMP, Ca^{++}, and so on [54]. The structural element of a gap junction is a transmembrane protein called a connexin; six of these connexin molecules are known to radially assemble to enclose the central pore. This structure can then dock with a similar structure on a contacting adjacent cell to form a complete connexon. Thus, the structural unit of the gap junction is composed of 12 connexin molecules contributed equally by each of the communicating partners [55]. This arrangement is shown diagrammatically in Figure 12.3. Passage of molecules or ions through the central pore appears to be via passive diffusion down concentration gradients. At present, over 20 connexin family members have been recognized, which are differentially expressed according to cell type and state of differentiation [56]. Cx43 is the most widely expressed connexin and is the family member induced by retinoids and, as we later discovered, carotenoids. Studies in connexin knockout mice have revealed a growing list of biological functions of connexins and a number of human diseases are now linked to connexin mutations [57].

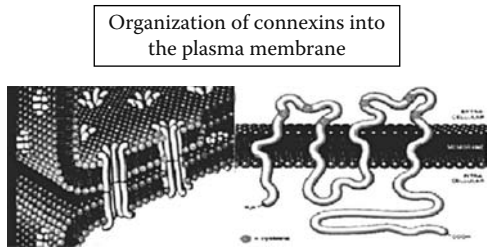

FIGURE 12.3 Organization of connexins into the plasma membrane. Left panel: Diagrammatic cross section through an area of cell–cell contact containing gap junctions. Connexin proteins are shown traversing the phospholipid bilayer in the plasma membrane. Each cell contributes six connexins to form a cylinder enclosing a central water-filled pore seen in cross section (foreground). Right panel: A single connexin molecule traverses the plasma membrane four times with both N- and C-terminal ends in the cytoplasm. Connexins assemble to form a connexon by forming three sulfhydryl bonds between the highly conserved cysteine, which resides in each opposing loop (C). Thus, each connexon is bound by 18 sulfhydryl bonds to produce a tight seal blocking the entry of extracellular ions such as Ca^{++}. (Reprinted from Bertram, J. S., *Sci. Med*., 7(2), 18, 2000. With permission.)

A consistent finding in studies of human or animal tumor cell lines and in studies of neoplastic transformation *in vitro* is that tumor cells communicate poorly, if at all, with their normal counterparts [58]. Decreased GJC seems not a consequence of connexin mutations, but either of altered trafficking or assembly of connexin molecules into functional connexons, or alternatively to downregulated expression via epigenetic pathways. The original hypothesis of growth control through junctional communication, so eloquently proposed by Loewenstein and Rose [59], was originally derived from studies of transformed cells in culture. In this model, communication-competent normal cells induce gradients of growth-controlling signal molecule(s); when a critical cell density is achieved the gradient intensity reaches a level sufficient to inhibit growth of all cells within a communicating compartment. Cells that fail to receive the signal, that is, those with impaired GJC, would be at a proliferative advantage and able to progress through the carcinogenic process. This model is illustrated in Figure 12.4. Consistent with this model, we have shown that carcinogen-initiated 10T1/2 cells communicate poorly with their nontransformed counterparts [60]. Furthermore, in clinical studies we have demonstrated that preneoplastic cells of the uterine cervix (cervical dysplasia) are severely impaired in their expression of Cx43 in comparison with pathologically normal tissue indicating that decreased gap junctional communication is an early event in carcinogenesis [61]. Studies in connexin deficient mice have also confirmed the predictions of enhanced susceptibility to carcinogenesis. Homozygous knockout of Cx32, which is expressed predominantly but not exclusively in the liver, resulted in mice with enhanced susceptibility to chemically induced lung carcinogenesis and physically induced liver carcinogenesis [62,63]. In the case of Cx43 expression, the connexin expressed in 10T1/2 cells, it has been determined

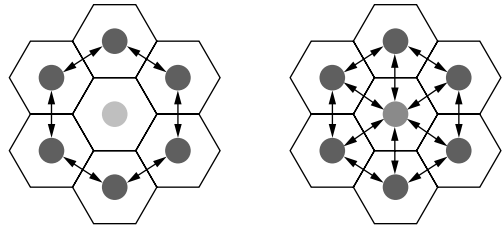

FIGURE 12.4 Hypothetical model of growth control by junctional communication. A central carcinogen-initiated cell is surrounded by growth-inhibited normal cells. In the left panel, the initiated cell is not in communication with normal cells and undergoes inappropriate proliferation. This will allow clonal expansion and the progressive accumulation of additional mutations resulting in malignancy. In the right panel, junctional communication of growth inhibitory signals from surrounding cells is upregulated by retinoids or carotenoids; the initiated cell becomes itself growth arrested and progression to malignancy is delayed. (Reprinted from Bertram, J. S., *Sci. Med.*, 7, 18, 2000. With permission.)

that heterozygous knockout mice (homozygous animals die shortly after birth) have increased susceptibility to urethane, a lung carcinogen [64]. In both types of knockout mice there were no reports of increased spontaneous incidence of cancer, strongly supporting the role of connexins in the postinitiation process of carcinogenesis. It is of interest that many classes of tumor promoters—agents that accelerate the process of carcinogenesis, but are not themselves carcinogenic—inhibit communication through gap junctions [65].

As a note of caution in interpreting these studies it should be noted that other investigators have shown in other systems that growth control by connexin expression does not appear to be dependent upon the formation of functional gap junctions, and have suggested that protein–protein interactions may be responsible [66]. Others have shown that in the case of Cx43, expression of the C-terminal region of the protein, which contains multiple phosphorylation sites, but does not integrate into the plasma membrane, is also sufficient to induce growth arrest [67]. However, our studies principally conducted in 10T1/2 cells have clearly demonstrated the requirement for cell–cell contact [45] and the formation of functional gap junctions [47].

12.7 CAROTENOIDS INDUCE Cx43 IRRESPECTIVE OF THEIR PROVITAMIN A OR ANTIOXIDANT PROPERTIES

The ability of carotenoids to inhibit neoplastic transformation or to upregulate expression of connexin43 did not correlate with their provitamin A activity, as might be expected from the similar actions of retinoids and carotenoids on Cx43

expression. Indeed, the non-provitamin A carotenoids canthaxanthin and astaxanthin, both oxygenated carotenoids, were more potent than the provitamin A carotenoid beta-carotene [52,68]. Recent studies have shown that these two classes of chemopreventive agents, that is, carotenoids and retinoids, induce Cx43 expression by engaging different nuclear receptors. Cx43 induction at the level of protein and mRNA by retinoids, but not carotenoids, is inhibited by pharmacological antagonists of the nuclear retinoic acid receptors (RARs), while induction by non-provitamin A carotenoids is inhibited by antagonists of PPAR-γ [35].

12.8 FORCED EXPRESSION OF Cx43 IN HUMAN CARCINOMA CELLS REDUCES MARKERS OF MALIGNANCY

The studies discussed earlier relating connexin expression and increased GJC with growth control and inhibition of neoplastic transformation relied on correlations to prove the association. However, these correlations do not prove a cause-and-effect relationship. For example, the actions of carotenoids on Cx43 gene expression may go hand-in-hand with actions of carotenoids on growth control but be functionally unrelated to these actions. To more firmly establish the role of upregulated Cx43 expression and enhanced junctional communication as central to the role of retinoids and carotenoids as antiproliferative and cancer preventive agents, we embarked upon the development of cells in which Cx43 was inducible, not by carotenoids or retinoids, but by using a bacterial-promoter system in which activity of the artificially introduced gene is controllable by picogram amounts of doxycycline, which at these concentrations is not known to produce other effects in mammalian cells. The major advantage of an inducible system is that Cx43 induction is rapid, occurring within hours, allowing cells in the noninduced situation to serve as their own controls. This is an advantage that cannot be overstated as we discovered extensive heterogeneity in growth control among cells recently cloned from established cell lines. Moreover, constitutive expression of a growth-suppressing gene would be expected to act as a negative selective pressure on transfected cells [69]. Unfortunately, because of technical difficulties these studies have been limited to the genetic engineering of established human tumor cell lines. Three such lines have now been created: one from a cervical carcinoma [61], one from a fibrosarcoma [70], and one from a breast adenocarcinoma (Chen and Bertram, in preparation). In all cell lines, Cx43 has been shown to be rapidly inducible from the bacterial promoter, to be integrated into the plasma membrane, and to form functional gap junctions with adjacent induced tumor cells. The consequences of Cx43 induction have also been consistent: all three engineered cell lines exhibited reduced anchorage-independent growth—that is, growth as spheroids suspended in a semisolid medium. The ability of neoplastic cells to grow in suspension has long been used to distinguish neoplastic cells from their normal counterparts. In normal epithelial and fibroblastic cells, contact with an extracellular matrix leads to the formation of focal contacts that allow for cell replication after

mitogenic stimulus. In the absence of such contacts, normal cells will not proliferate and frequently undergo apoptosis. The lack of this requirement in tumor cells presumably reflects their acquired ability to migrate through and proliferate in inappropriate locations [71]. The ability of forced expression of Cx43 to restore this requirement for cell proliferation was unexpected but fits into some recently generated clinical data. Here, administration of high doses of supplemental lycopene to patients with prostate cancer for 3 weeks prior to radical prostatectomy was found to be associated with increased expression of Cx43 and decreased pathological severity of treated versus control tumors—a finding most easily explained by induction of apoptosis in these tumors [72]. Similar clinical studies of lycopene conducted by others have also showed evidence for induction of apoptosis in prostate tissue [73]. If confirmed in a larger group of patients, these studies would indicate that at least in the case of lycopene and prostate cancer, carotenoids could have therapeutic potential. It is at present unknown how Cx43 expression influences anchorage-independent growth. One possibility is that the plasma membrane-associated connexins act as a focus for the reassembly of cytoskeletal elements known to be disrupted during carcinogenesis. In this context, Cx43 is known to associate with microtubules and with Z0-1, a constituent of epithelial tight junctions that can interact with cytoskeletal elements [74,75]. Thus, Cx43 expression strongly inhibits a major *in vitro* marker of malignancy. A more direct test for malignancy is the ability to grow as a tumor when injected as a xenograft into immuno-compromised mice. The result for one such study with human cervical carcinoma cells is shown in Figure 12.5. It can be clearly seen that in mice administered doxycycline in the drinking water in order to induce Cx43 expression in the injected cells, subcutaneous tumors grew much more slowly than in control animals [61]. Thus, both *in vivo* and *in vitro* Cx43 expression reduce indices of neoplasia in human carcinoma cells.

In monolayer culture, where cells grow as a two-dimensional layer on plastic, Cx43 induction did not cause changes in growth rate or saturation density. This was in contrast to the results obtained with nontransformed 10T1/2 cells, where increased junctional communication after retinoid or carotenoid treatment led to decreased proliferation. The lack of response of cells growing in monolayer culture to Cx43 induction may be attributed to the fact that these cells, derived from human tumors and which had been extensively passaged *in vitro*, had lost the ability to either transmit or respond to junctionally mediated cell signaling. None of these cell lines could be induced to junctionally communicate with growth-inhibited 10T1/2 cells, and in co-culture with these cells, neoplastic growth was not inhibited. However, the breast carcinoma cells formed functional junctions with a growth-inhibited epitheloid rat kidney cell line (NRK), which constitutively expresses Cx43. When induced to express Cx43 the human breast carcinoma cells became growth inhibited. The implication of these studies is that the breast carcinoma cells can no longer generate growth inhibitory signals but can still respond to signals supplied by the growth-inhibited NRK cells (Chen and Bertram, in preparation).

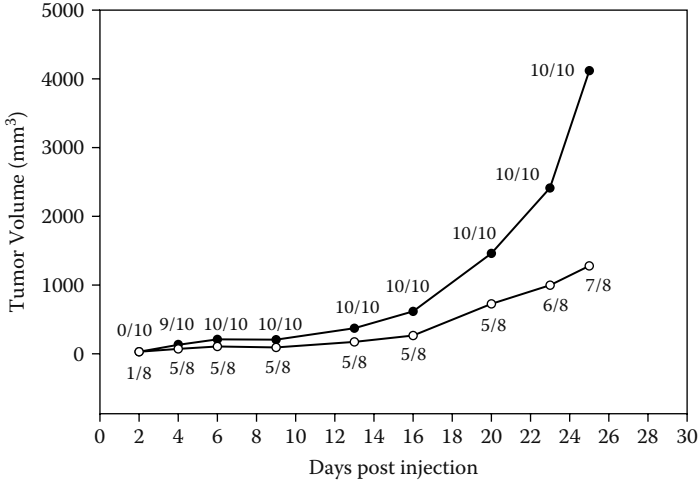

FIGURE 12.5 Expression of Cx43 reduces the growth rate of human cervical carcinoma cells in the "nude" mouse. HeLa cells were engineered to express Cx43 under the influence of a bacterial promoter driven by doxycycline. Immunocompromised nude mice were injected subcutaneously with HeLa cells, then randomized to receive doxycycline (0.2 mg/ml in 5% sucrose) in the drinking water, or sucrose alone as controls. Tumor volumes were measured by calipers at the indicated times. (○-○), doxycycline treated; (●-●), sucrose controls. Numbers by each data point represent total number of tumors/number of tumor injections. (Reprinted from King, T. J., Fukushima, L. H., Hieber, A. D., Shimabukuro, K. A., Sakr, W. A., Bertram, J. S., *Carcinogenesis*, 21, 1097, 2004. With permission.)

As discussed earlier, downregulated expression of Cx43 is an early event, being observed even in dysplasia, a pathology known to predispose to malignancy but which is not yet malignant [61]. Our data demonstrating that upregulated expression of Cx43 achieved by pharmacological or molecular means results in decreased proliferation of normal and malignant cells suggest that if the observed downregulated expression of Cx43 in dysplasia can be corrected, progression to malignancy may be delayed. Indeed, in clinical intervention studies, retinoids in the case of cervical dysplasia [40] and oral leukoplakia [76], and carotenoids in the case of oral leukoplakia [77] have been shown to significantly retard carcinogenic progression. The role of gap junctional communication in these responses has not been evaluated but the data are strongly suggestive that agents capable of normalizing junctional communication would have cancer preventive properties. That GJC can indeed be upregulated *in vivo* is indicated by clinical trials of retinoic acid after topical application to normal human skin for 2 weeks prior to excision during cosmetic surgery. Here, Cx43 expression was shown to be strongly upregulated in suprabasal cells at the protein level by immunofluorescence and Western blotting [78]. Though not yet tested clinically, carotenoids have also been shown to induce Cx43 expression in human epidermis grown in organotypic culture; again expression was observed in suprabasal cells [79].

12.9 MECHANISTIC STUDIES OF Cx43 INDUCTION BY CAROTENOIDS

To further examine the similarities and differences between carotenoid versus retinoid induction of Cx43 at the molecular level, we recently conducted studies utilizing the Cx43 promoter fused to a luciferase reporter gene. While others have suggested that gene regulation may be exhibited at the level of translation [80] or as a consequence of altered mRNA stability [81], we have reached an opposite conclusion: the increased expression of this gene is a direct consequence of transcriptional activation. We arrived at this conclusion as a result of the following experiments: in the 10T1/2 model system, Cx43 mRNA expression was induced by treatment with retinoids or carotenoids, then additional mRNA synthesis was blocked by treatment with actinomycin D. In such cultures, total cellular abundance of Cx43 mRNA decreased at the same rate in treated cells as in cells treated only with solvent control [82]. The half-life of this message was approximately 5.5 h, which is consistent with the short half-life of this message reported in other systems. Unfortunately, we were unable to get a specific mRNA signal utilizing the nuclear runoff technique. In a second series of experiments, both retinoids and carotenoids were shown to increase the activity of a luciferase reporter gene construct [82]. Taken together, the increased abundance of Cx43 mRNA, its similar stability in treated and control conditions, and the responsiveness of a luciferase reporter all provide strong evidence for direct transcriptional activation of this gene. To further evaluate which regions of the promoter conveyed retinoid and/or carotenoid responsiveness, we examined the promoter sequence for binding sites to known transcriptional proteins. As has been previously reported, the promoter region contains no canonical binding sites for nuclear receptors, such as RAR, PPAR, or the vitamin D receptor (VDR). It does contain however a number of Sp1 binding sites, one of which has been previously associated with transcriptional repression. Using an electromobility gel shift assay system, we were able to show that this sequence was capable of binding both Sp1 and Sp3 extracted from nuclei of treated 10T1/2 cells. We were not able to detect quantitative differences in binding between treated and control cells. To further probe the significance of this sequence to the regulation of Cx43 mRNA expression, we performed site-directed mutagenesis of this Sp1 site and performed luciferase reporter gene assays to compare activity of this mutated versus the wild-type sequence. The mutated sequence was found to have lost responsiveness to both retinoids and carotenoids. However, the mutated sequence had higher promoter activity than did the wild-type sequence, suggesting binding of a transcriptional repressor (Sp3) that can be removed by retinoid or carotenoids [82].

In summary, we have evidence that both retinoids and carotenoids increase GJC by increasing transcription of the Cx43 promoter. This is supported by two lines of evidence: first, both retinoid and carotenoid treatment result in upregulated expression of luciferase Cx43 promoter constructs in F9 cells; second, that the increased steady-state levels of Cx43 mRNA as a result of retinoid or carotenoid treatment are not a consequence of an increased half-life of Cx43 mRNA.

12.10 LYCOPENE MODULATES THE IGF-I SIGNALING PATHWAY

The influence of lycopene on proliferation of carcinoma cells appears not limited to its ability to modulate Cx43 expression. In studies conducted by others, growth stimulation of MCF7 mammary cancer cells by IGF-I was markedly reduced by physiological concentrations of lycopene [83]. Lycopene treatment markedly reduced the IGF-I stimulation of tyrosine phosphorylation of insulin receptor substrate 1 and the binding capacity of the AP-1 transcription complex, suggesting that effects on proliferation were due to interference in IGF-I receptor signaling [83]. Interactions appear not limited to cell culture studies, as epidemiologic investigations have shown a strong inverse correlation between plasma lycopene and circulating IGF-I [84] and, as discussed earlier, lycopene modulated IGF signaling in the ferret lung [32]. Interactions between IGF-I signaling pathways and carotenoids are discussed in more detail elsewhere in this book.

12.11 CONCLUSION

Carotenoids can impact several stages of the carcinogenic process: they can modify carcinogen activation; they can suppress aberrant proliferation of preneoplastic cells via upregulated expression of Cx43; finally, they can reduce the malignant potential of fully transformed cells by suppressing anchorage independent growth, by inducing apoptosis and by reducing mitogenic signaling by the IGF-I pathway (Figure 12.6). The low intrinsic toxicity of carotenoids makes them

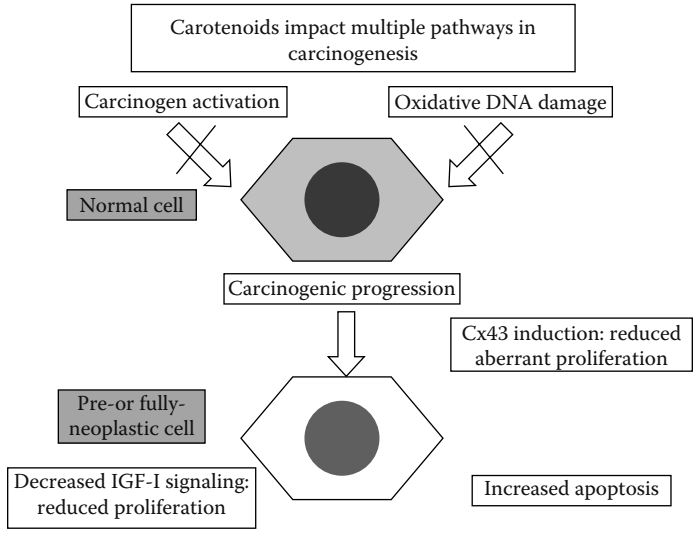

FIGURE 12.6 Potential sites of interactions of carotenoids with the process of chemical carcinogenesis.

potentially useful agents with which to suppress carcinogenesis and counter chronic or acute pathology caused by ROS.

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13 Targeting the Epigenome with Dietary Agents

*Barbara Delage and Roderick H. Dashwood**

CONTENTS

13.1 INTRODUCTION

There is much interest in the field of gene–diet interactions and the mechanisms by which nutritional factors modulate gene expression. Historically, most work has focused on the role of dietary micronutrients and vitamins in the maintenance of genomic integrity, and on specific deficiencies that result in DNA damage, micronucleus formation, or chromosomal abnormalities. Dietary factors are critical substrates and cofactors in DNA metabolic pathways regulating the genome machinery. As such, they are capable of influencing multiple stages in carcinogenesis, including DNA repair, cell cycle regulation, apoptosis, differentiation, angiogenesis, and inflammation. The ability of food components to alter gene expression, without actually changing the primary genetic sequence, is an important and exciting avenue of research centered on so-called epigenetic mechanisms. The term

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epigenetics refers to the various processes that alter gene activity without altering the primary DNA code, including modifications that can be transmitted to daughter cells. This review provides an overview of epigenetics and the ways in which dietary factors can modulate the epigenome.

13.2 BASIC PARADIGM OF METHYLATION EVENTS AND EPIGENETICS

DNA methylation has received much attention as a mechanism for gene silencing and unsilencing, in both prokaryotes and eukaryotes. In mammals, this process consists of the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the 5′ position of cytosine adjacent to guanine in the DNA sequence (cytosine-phosphoguanosine, or CpG dinucleotide), resulting in 5-methyl-2′-deoxycytidine. In general, CpG dinucleotides are underrepresented throughout the genome, but can be found in CpG dinucleotide-rich regions called *islands* within ~75% of human gene promoter regions [1]. CpG dinucleotides that are not associated in clusters are mostly methylated, whereas CpG islands in gene promoters are usually protected from methylation, which facilitates transcription [2]. Cytosine methylation is known to be important for the monoallelic expression of about 100 imprinted genes [3,4], for X-chromosome inactivation early in development of female mammals [5], for host defense against the activation of transposons [6,7], and as a mechanism for regulating testis-specific genes [8]. Demethylation and remethylation processes are critical during embryonic development. Indeed, in mammals, almost all genomic methylation is erased during the preimplantation stage of development and then reestablished by waves of global *de novo* methylation that exclude only CpG islands [9]. Somatic cell nuclear transfer and other assisted reproduction technologies can result in incomplete epigenetic reprogramming of chromosome methylation in embryos, with loss of imprinting and abnormal gene expression [10–12]. Although the methylation of CpG islands was thought to explain tissue- and cell-specific gene expression [13], patterns of methylation are similar in the majority of adult somatic cells [14]. Examination of a panel of genes for evidence of tissue-specific methylation revealed a critical role of CpG island methylation for at least some genes involved in development and differentiation, including *CD43* [15], *embryonic globin* [16], *cyclin D1* [17], *t1alpha* [18], *maspin* [19], *HOXa5* [20], *MCJ* [21], and *14-3-3sigma* [22]. However, there is much to be learned about methylation as a physiological mechanism of gene regulation during normal development, and in controlling cell-specific expression in adult somatic tissues. In 1999, Walsh and Bestor [23] hypothesized that the vicinity of transposable elements or retroviral DNA, which represent >35% of the mammalian genome and are heavily methylated [6], can increase the probability of *de novo* methylation in flanking promoter sequences. Furthermore, some specific transcription factors, insensitive to cytosine methylation, can prevent *de novo* methylation [24,25] or induce the demethylation of local CpG sites in a replication-dependent manner [26], giving the impression of regulated tissue-specific methylation.

13.3 DNA METHYLATION AND GENE SILENCING

There is convincing evidence for a role of promoter methylation in gene silencing [2]. The DNA methylation mechanism in mammals is carried out by four DNA methyltransferase enzymes (DNMT): DNMT1, DNMT2, DNMT3a, and DNMT3b. DNMT1 exhibits a high affinity for hemi-methylated DNA and is thought to be responsible for maintaining preexisting DNA methylation patterns during cell division [27,28]. DNMT2 is a newly discovered enzyme that exhibits methyltransferase activity *in vitro* [29], but little is known about its role *in vivo*. The *de novo* enzymes DNMT3a and DNMT3b control addition of methyl groups at previously unmethylated CpG sites [27,28]. Abnormal DNA methylation has been related to developmental defects during embryogenesis following DNA methyltransferase disruption in murine models [30–33]. In general, DNA methylation favors compact and inactive chromatin, resulting in transcriptional silencing [2,34], whereas the undermethylated genome of DNMT-deficient mice is associated with gene activation [35].

Several hypotheses have been proposed to explain CpG island-associated gene silencing. Methylated cytosines are thought to limit accessibility to DNA and prevent the binding of transcriptional activators to their DNA recognition sites in the promoters of target genes [36,37]. Methylation at CpG islands may sterically interfere with the binding of specific transcription factors as well as the basal transcriptional machinery. Indeed, cytosine methylation occurs not only in promoter sequences but also within the body of genes, and methyl CpG sites in intronic and exonic regions may influence RNA polymerase II occupancy and chromatin accessibility, thereby repressing initiation as well as transcription elongation [38,39]. However, some transcription factors, such as Sp1, appear to be indifferent to the DNA methylation status in some circumstances [40], but not in others [41,42].

There is also growing interest in the chromatin structure, and the recruitment of specific repressor complexes containing histone deacetylases (HDAC), which remove acetyl groups [43], as well as histone methyltransferases (HMT), which add methyl groups to lysines and arginines on histone tails [44]. Specifically, the posttranslational acetylation of histones is controlled by the opposing activities of HDAC and histone acetyltransferases (HAT). There are six families of HAT [45], and four broad classes of HDAC, based on their homology to yeast HDAC. Class I HDAC (1, 2, 3, 8), homologous to yeast RPD3 protein, are expressed in all tissue types and are found almost exclusively in the nucleus. Class II HDAC (4–7, 9, 10), homologous to yeast Had 1 protein, exhibit a more restricted expression pattern and can shuttle between the nucleus and the cytoplasm. One HDAC, HDAC 11, shares features with both class I and II HDAC. HDAC II now belongs to class IV HDAC. Class III HDAC include human SIRT (also called sirtuins) 1–7, homologous to yeast Sir2, which are NAD-dependent and generally nonresponsive to inhibitors of class I and II HDAC. HDAC, HAT, and HMT govern a large and complex array of histone modifications. A noncovalent linkage between positively charged histones and the negatively charged DNA may result in a condensed or "restricted" conformation

that reduces access to DNA. The mechanism involves the active participation of the DNA methylation machinery, not restricted to various DNMT enzymes. Indeed, some methylation-dependent DNA-binding domain proteins (MBP family: MBD1-4, methyl-CpG-binding protein 2 [MeCP2], and Kaiso) can recognize and bind methylated DNA and then in turn recruit transcriptional corepressor molecules to modify the surrounding chromatin and repress gene expression [46–48]. The ability of *de novo* DNA methylation to recruit protein complexes able to modify chromatin structure was originally viewed as a "nucleation" event that altered histones in the local vicinity, suggesting that transcription cannot occur without first inhibiting DNA methylation. However, more recent studies revealed that histone modifications can initiate the process of DNA methylation.

13.4 DNA METHYLATION STATUS AND CANCER

The major hallmarks of cancer cells are their unlimited replication potential, independence toward growth factors, resistance against drugs, evasion of programmed cell death, angiogenic potential, and ability to metastasize and invade other tissues. In principle, each of these characteristics represents a potential target for intervention with chemopreventive or therapeutic agents. Knudson [49] developed the "two-hit" model to explain constitutive gene induction or repression in the context of mutations that arise, respectively, within proto-oncogenes or tumor suppressor genes, but the model has been expanded to include consideration of epigenetic modifications [50]. Indeed, epigenetic alterations can influence the initiation of cancers in the absence of mutations, and in all probability are as common as mutational events in the development of cancer. It is now clear that tumorigenesis is characterized by epigenetic deregulation, specifically by the coexistence of global genome hypomethylation and gene-specific hypermethylation [51]. Methylation of tumor suppressor gene promoters increases cancer susceptibility [52] and contributes directly to the progression of some cancers [53,54]. Silencing-associated hypermethylation alters the expression of genes involved in cell cycle regulation (*Rb, $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, RASSF1A*), DNA repair (*hMLH1, BRCA1*), apoptosis (*DAPK*), carcinogen detoxification (*GSTP1*), hormone response (*ERα, RARβ*), and cell communication and adherence (*E-cadherin*). In addition to hypermethylation, genome-wide global hypomethylation occurs early during tumor development and increases progressively with grade of malignancy, and a decrease in methylcytosines is observed in repeated DNA sequences, dispersed retrotransposons, and endogenous retroviral elements. Hypomethylation is suggested to augment DNA rearrangements and chromosome instability [55]. A mouse model with a hypomorphic mutation in *DNMT1*, reducing DNMT1 expression to 10% of normal, has genome-wide hypomethylation in all tissues; these mice develop aggressive T cell lymphomas associated with an increase of chromosome 15 trisomy [56,57]. Hypomethylation also may influence the activation of retrotransposons [58] and proto-oncogenes [59], as observed

with the consumption of methyl-deficient diet [60]. Genome-wide demethylation in the *Apc*$^{Min/+}$ mouse protected against intestinal tumors, but promoted liver tumors in the same model [61].

13.5 GENE SILENCING AND THE HISTONE CODE

As mentioned earlier, one mechanism of gene silencing involves posttranslational modifications to histones, known collectively as the *histone code*. N-terminal "tails" of the core histones, particularly of histones H3 and H4, are targets for multiple modifications including acetylation, methylation, phosphorylation, ubiquitination, poly ADP ribosylation, and sumoylation [62]. Among these changes, the best characterized modifications include histone deacetylation and methylation of histone H3 at lysine 9 (H3K9). However, the significance of each histone modification is far from clear, and may not always correlate with predicted nuclear functions. Recently, Vakoc et al. reported recruitment of H3K9 methyltransferase by RNA polymerase II, suggesting a role of methyl H3K9 during the elongation phase of transcription [63,64], while the absence of this specific methylating enzyme resulted in loss of gene silencing in plants and flies [65,66]. Histone acetylation was shown to be generally correlated with transcriptionally active chromatin, as was the phosphorylation of serine 10 of histone H3, the monoubiquitination of lysine 123 of histone H2B, and the methylation of lysines 4, 36, or 79 of histone H3. In contrast, histone sumoylation, deacetylation, demethylation of lysine 20 of histone H4, and methylation of lysines 9 or 27 of histone H3 are correlated with transcriptionally silent regions. Histone modification patterns can have differential effects on chromatin structure, such that histone deacetylation, H3K9 methylation, and DNA methylation are not functionally independent. Epigenetic "marking" systems have been shown to mutually and dynamically reinforce transcription states, and the prerequisite enzymes and epigenetic marks have been observed to colocalize [67–69]. Methylated CpG is associated with low levels of histone acetylation and H3K9 methylation within specific gene promoters in human cancers [70], and within inactive X-chromosome-associated silent genes [71,72], whereas unmethylated CpG islands are enriched in acetylated histones [43,73].

There is debate over the relative importance of DNA methylation versus histone modification as starting points for gene silencing and unsilencing. Recruitment of HDAC-containing repressor complexes by DNMT and MBD [46–48] has been taken as evidence for histone deacetylation as a secondary event, and methyl-CpG-binding proteins MeCP2 and MBD1 associate with HMT and facilitate subsequent methylation at lysine 9 of histone H3 [44,74]. *DNMT1*-knockout colorectal cancer cells display DNA demethylation at centromeric regions associated with acetylation and trimethylation at lysine 9 of histone H3 [75]. Double genetic disruption of *DNMT1* and *DNMT3b* reduced global genomic DNA methylation, and was accompanied by derepression of $p16^{INK4a}$ and growth suppression [76], whereas loss of *DNMT1* in mouse fibroblasts was associated with unsilencing of $p21^{WAF1/CIP}$ [77]. These findings support a role for DNMT in

the silencing of certain tumor suppressor genes, but whether chromatin remodeling occurred subsequent to DNA methylation remains an open question.

Indeed, one paradigm suggests that changes in chromatin structure and histone modification must first occur to enable subsequent alterations in DNA methylation. Mutations in H3K9 methyltransferase genes *dim-5* and *kryptonite*, in *Neurospora crassa* and *Arabidopsis thaliana*, respectively, were related to a reduction in DNA methylation, suggesting that methylation of H3K9 occurs prior to DNA methylation [78–80]. In vertebrates, it has been reported that mouse embryonic stem cells lacking Suv39h H3K9 methyltransferase exhibit an altered DNA methylation profile at pericentromeric satellite repeats [81]. Additionally, serial passage of colorectal cancer cells containing knockout of both *DNMT1* and *DNMT3b* leads to resilencing of the tumor suppressor gene $p16^{INK4a}$ associated with the methylation of H3K9, preceding DNA methylation changes [82]. In breast cancer cell lines, deacetylation and H3K9 methylation events also seem to direct *de novo* DNA methylation during the progressive silencing of the tumor suppressor gene *RASSF1A* [83]. Another study revealed that the repression of a transgene integrated in chicken erythroid cells required the loss of H3K4 demethylation and histone deacetylation prior to H3K9 methylation and DNA methylation [84].

A more likely model (Figure 13.1) is that cooperation exists between histone modification and DNA methylation, leading to a common repressive pathway on gene transcription. In mammals, an "adaptor molecule" called heterochromatin protein 1 (HP1α,β,γ) recognizes and binds with high affinity to methylated histone tails [85]. HP1 may recruit HDAC proteins that deacetylate histone H3, creating substrate sites for methylation by HMT, which in some experiments has been coimmunoprecipitated with HP1 [86]. Methylation of H3K9 could then create HP1-binding sites and extend a repressive state of chromatin. Data from *N. crassa* and *A. thaliana* revealed that HMT enzymes can recruit DNMT (CMT3) via an adaptor protein LPH1 (an HP1 homologue), which binds the methyl H3K9 modification [79]. The association between DNMT1 and DNMT3a with HP1 and HMT enzymes also was demonstrated in mammals [87]. This suggests that HMT and HP1 may recruit DNMT to methylate DNA, as well as the reverse scenario in which the recruitment of MeCP2 by DNMT-methylated CpG may enroll HMT and HDAC [44]. A direct connection between DNMT3b and the ATP-dependent chromatin remodeling factor hSNF2H, as well as with some HDAC proteins, was recently shown as part of the mechanism leading to highly organized chromatin [88,89]. Collectively, these studies establish clear interplay between DNA methylation, histone methylation, and histone deacetylation in gene silencing, but the model proposed in Figure 13.1 still regards chromatin as an essentially passive structure punctuated by periods of active remodeling. A new paradigm suggests that binding sites are continually being scanned by nuclear proteins in a random, undirected fashion, and whether a gene will be selected for transcription depends on a sequence of dynamic events, each with a given probability. This "scanning chromatin" model is perhaps more realistic in that it considers chromatin as a highly dynamic structure [90].

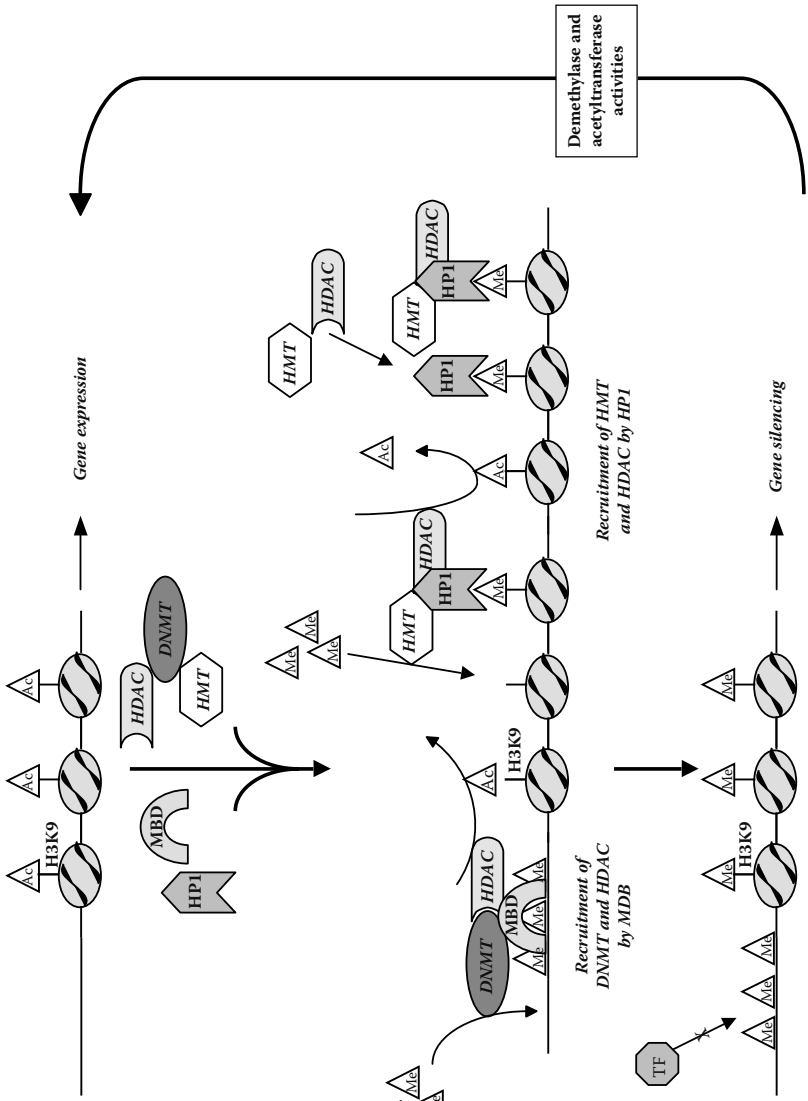

FIGURE 13.1 Molecular mechanisms involved in the regulation of gene expression. Ac, acetyl group; DNMT, DNA methyltransferase; HDAC, histone deacetylase; H3K9, Lys 9 of histone H3; HMT, histone methyltransferase; HP1, heterochromatin protein 1; MBD, methyl-CpG binding domain protein; Me, methyl group; TF, transcription factor.

13.6 DEREPRESSION OF EPIGENETICALLY SILENCED GENES BY DRUGS

An avenue of research that has evolved alongside studies of DNA methylation and chromatin remodeling considers the events involved in reactivation of epigenetically silenced genes. This is a major area of interest due to the potential for chemopreventive and therapeutic agents to derepress genes in cancer cells. Epigenetically silenced tumor suppressor genes are successfully derepressed by synthetic DNA methyltransferase inhibitors, such as 5-azacytidine (5-azaC), 5-aza-2′-deoxycytidine (decitabine, ADC), zebularine, and procainamide [91–94]. There are examples of 5-azaC being more effective than the potent HDAC inhibitor trichostatin A (TSA) in reversing the silencing of proviral transgenes, endogenous genes, episomal, and integrated reporter constructs [95–98]. Moreover, the efficiency of transcriptional activation by HDAC inhibitors was shown to be inversely correlated with the methylation density [38,96], implicating DNA methylation as being critical in the maintenance of gene repression. As corroborating evidence, TSA efficiently upregulated $p15^{INK4b}$ transcribed at a basal level in the colorectal cancer cell line RKO, but failed to reactivate the same gene in acute myelogenous leukemia KG1a, due to silencing associated with a hypermethylated promoter region [95]. Weak changes were detected for methyl H3K9 and methyl H3K4 in the promoter of a hypermethylated and silenced *hMLH1* gene in RKO cells treated with TSA [99]. However, decitabine reinduced *hMLH1* and completely reversed histone modifications, supporting the idea that methylation density at CpG islands and/or DNMT activities helps maintain a particular combination or pattern of histone modifications [99,100].

Interestingly, some data support the reverse situation, in which DNA demethylation may depend on the covalent histone modifications. In plants, reduction of DNA methylation in *DNMT* mutants *MET1* and *CMT3* fails to affect the methylation of H3K9 [101]. Hyperacetylation of histones after treatment of *N. crassa* with TSA induces demethylation of specific regions of the genome [102], suggesting that acetylation of histones can control DNA methylation status. In cancer cells, HDAC inhibitor TSA alone can alter DNA methylation status, although other examples indicate it does so even in the absence of DNMT inhibitors [103–105]. In addition to the induction of histone hyperacetylation through inhibition of HDAC, TSA may downregulate DNMT when it is overexpressed in tumors. Indeed, TSA was shown to reactivate gene expression in certain cancer cells by reducing the stability of DNMT1 [106] and DNMT3b [107] mRNA transcripts, and by affecting the recruitment of methyl-CpG binding proteins [108]. These findings illustrated the ability of HDAC inhibitors to influence DNA hypomethylation. Together, the data show that the predominance of one mechanism over another may depend on cell type, promoter arrangement, transcription factors bound, and type of epigenetic mechanisms leading to silencing. Furthermore, numerous studies support a synergistic impact of DNMT and HDAC inhibitors on gene derepression [100,109–111]. This avenue of research has gained further attention with evidence for the involvement of specific dietary constituents affecting DNA methylation status and histone modifications.

13.7 TARGETING DNMT ACTIVITY WITH DIETARY CONSTITUENTS

As alluded to earlier, neoplasia is associated with epigenetic changes consisting mainly of global DNA hypomethylation, regional DNA hypermethylation, and overexpression of DNMT [112,113]. Several food and beverage components modulate cancer susceptibility by modifying DNA methylation, including those that act as direct DNMT inhibitors.

The major tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) (Figure 13.2) has been shown to prevent tumorigenesis in multiple organ sites, including skin, oral cavity, lung, esophagus, stomach, small intestine, colon, liver, pancreas, bladder, breast, and prostate [114]. Tea polyphenols such as EGCG are strong antioxidants *in vitro*, and in cultured cells they affect a multitude of signal transduction pathways. Recent studies suggested that EGCG operated as a DNA demethylating agent, either via direct inhibition of DNMT or through indirect mechanisms involving catechol-*O*-methyltransferase (COMT) and dihydrofolate reductase (DHFR) activities.

Fang et al. first demonstrated the inhibition of DNMT by EGCG in a cell-free assay *in vitro* [115], with K_I of 7 μM and an IC_{50} of 20 μM in the absence of Mg^{2+}, or IC_{50} 0.5 μM in the presence of Mg^{2+} [116]. Computational modeling of the interaction between EGCG and human DNMT1 identified a catalytic site residue (Glu 1265) potentially interacting with EGCG via H-bonding [116]. *In vitro* data revealed that other catechol-containing dietary flavonoids, namely (+)-catechin and (−)-epicatechin, as well as noncatechin-flavonoids quercetin, fisetin, and myricetin, were inhibitors of DNMT, although less potent than EGCG [116].

Dietary flavonoids also might inhibit DNMT activity through a noncompetitive mechanism. They are good substrates for COMT, which belongs to the same S-adenosylmethionine-dependent methyltransferase superfamily as DNMT [117]. COMT is a phase II enzyme involved in the inactivation of many catechol substrates, by transferring a methyl group from the universal methyl donor SAM to the substrate. The methylation of dietary catechol-containing phytochemicals by COMT may reduce the bioavailability of SAM and increase the pool of demethylated SAM (*S*-adenosyl-L-homocysteine, SAH), a feedback inhibitor of SAM-dependent methyltransferases, including COMT and DNMT (Figure 13.3). Thus, dietary catechol-containing flavonoids may affect the overall rate of DNA methylation by favoring the formation of a noncompetitive inhibitor of DNMT, SAH. In the same manner, caffeic acid and chlorogenic acid, which are found in soybeans, coffee beans, and many fruits, can inhibit DNMT through an increase of SAH formation and subsequent reductions in DNA methylation [118].

Another potential mechanism for DNMT inhibition by EGCG comes from its ability to attenuate dihydrofolate reductase (DHFR) [119,120]. The SAM utilized by DNMT as a methyl donor is exclusively provided by folate-mediated one-carbon metabolism. Briefly (Figure 13.4), DHFR catalyzes the conversion of dietary folate (vitamin B9) successively to 7,8-dihydrofolate (7,8-DHF) and 5,6,7,8-tetrahydrofolate (5,6,7,8-THF), which is then converted by glycine hydroxymethyltransferase

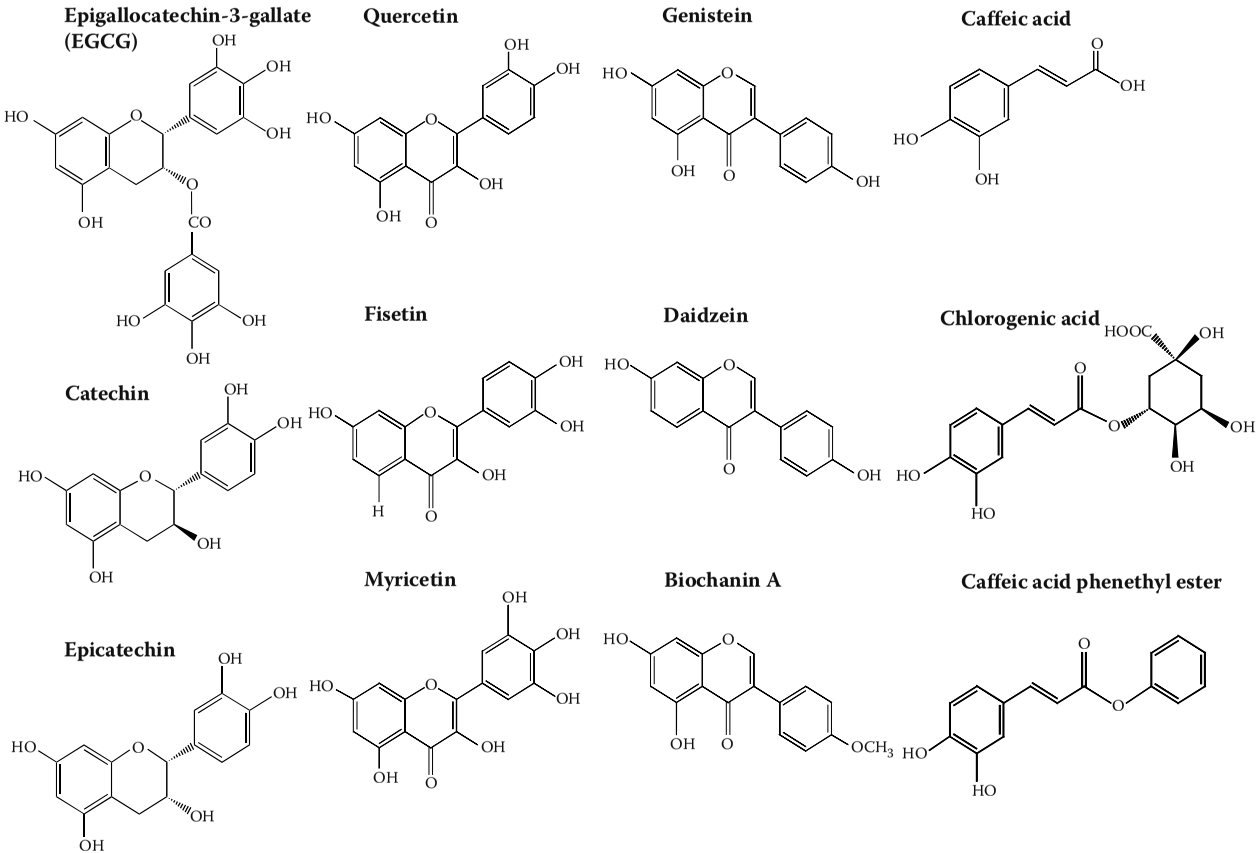

FIGURE 13.2 Chemical structures of dietary DNA methylytransferase inhibitors.

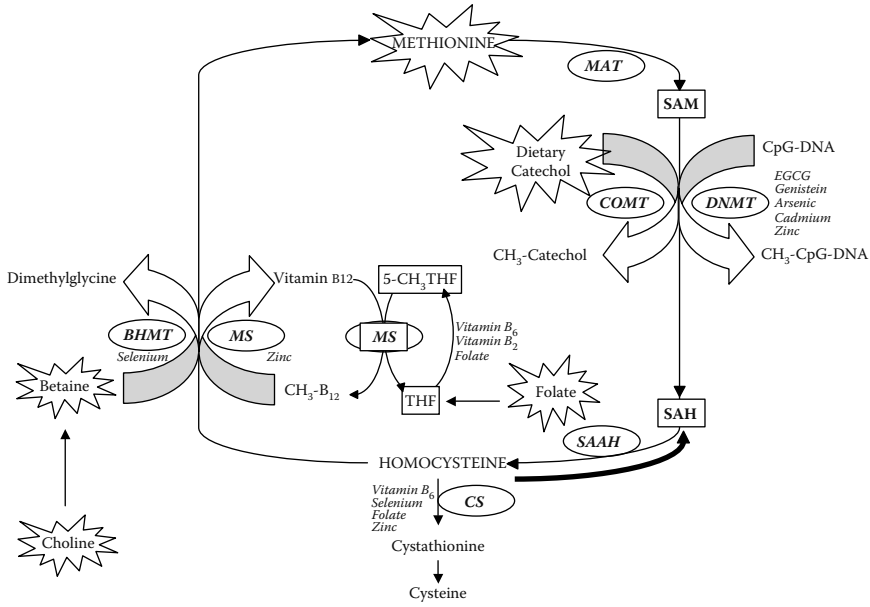

FIGURE 13.3 Overview of methionine-homocysteine cycle and dietary regulations. BHMT, betaine homocysteine methyltransferase; CH_3, methyl group; COMT, catechol-*O*-methyltransferase; CpG, cytosine-guanine dinucleotide island; CS, cystathionine-β-synthase; DNMT, DNA methyltransferase; EGCG, (–)-epigallocatechin-3-gallate; MAT, methionine adenosyl transferase; MS, methionine synthase; SAAH, S-adenosyl-L-homocysteine hydrolase; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine; THF, tetrahydrofolate; CH_2THF, methylenetetrahydrofolate; CH_3THF, methyltetrahydrofolate.

(GHMT) to 5,10-methylenetetrahydrofolate (5,10-CH_2THF). The latter intermediate is converted by methylenetetrahydrofolate reductase (MTHFR) to 5-methyltetrahydrofolate (5-CH_3THF), which donates a methyl group to convert homocysteine to methionine. Methionine adenosyl transferase (MAT) converts methionine into SAM, but this pathway is attenuated in dietary folate deficiency and in certain polymorphisms of the *MTHFR* gene that increase cancer susceptibility through modulation of the SAM pool and of DNA methylation. Inhibition of *Stenotrophomonas maltophilia* (a nocosomial pathogen) DHFR by EGCG provides a mechanism for the antibacterial activity of the tea flavonoid [119]. The results imply that EGCG might induce a deficiency in folate, but no such demonstration has been reported *in vivo* based on studies of SAM depletion or changes in DNA methylation. It should be noted that other authors failed to observe direct DNMT inhibition or gene derepression by EGCG [94,121].

Genistein is a major isoflavone found in soybeans (Figure 13.2). This compound has been shown to inhibit the proliferation of hormone-dependent cancer cell lines and the growth of breast and prostate tumors in rodent models. Many

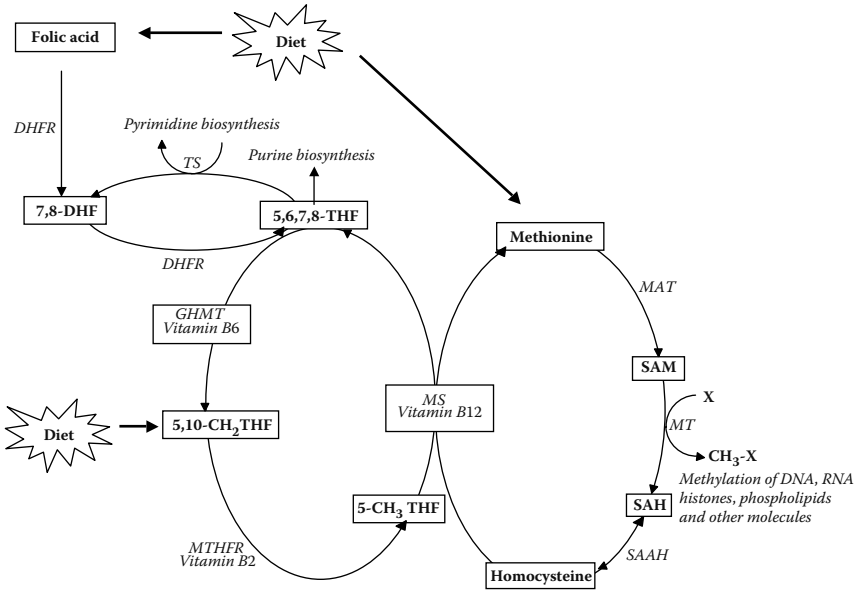

FIGURE 13.4 Folate-mediated one-carbon metabolism. CH_3, methyl group; DHF, dihydrofolate; DHFR, dihydrofolate reductase; GHMT, glycine hydroxymethyltransferase; MAT, methionine adenosyl transferase; MS, methionine synthase; MT, methyl transferase; MTHFR, methylenetetrahydrofolate reductase; SAAH, S-adenosyl-L-homocysteine hydrolase; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine; THF, tetrahydrofolate; CH_2THF, methylenetetrahydrofolate; CH_3THF, methyltetrahydrofolate; TS, thymidylate synthase.

studies have demonstrated its estrogenic activity, explaining why genistein is regarded as a dietary "phytoestrogen," since it can compete with estradiol and alter cell growth through actions on the estrogen receptor (ER). Mice treated with an genistein-enriched diet for 2 or 4 weeks had changes in the DNA methylation pattern in prostate, but not liver [122]. Recently, Fang et al. induced a specific demethylation in the promoter of several tumor suppressor genes (*RARβ, p16^{INK4a}*, and *MGMT*) associated with growth inhibition in genistein-treated cancer cell lines [123]. Kinetic analysis revealed an inhibition of DNA methyltransferase activity by genistein in a substrate- and methyl donor-dependent manner. Contrary to EGCG, genistein does not exhibit a catechol residue, excluding SAH negative feedback due to COMT activity. Other soy isoflavones, daidzein, and biochanin A (Figure 13.2) seem less effective than genistein as inhibitors of DNMT, but still reactivate *RARβ* transcription [123].

In addition to phytochemicals, certain metals found in our environment as contaminants (mainly in air and diet) have been described as direct inhibitors of DNMT activity. Arsenic in fish and seafood was identified as a putative DNA demethylating agent, since its biotransformation requires methylation steps catalyzed

by methyltransferases, which may favor depletion of the SAM pool and accumulation of SAH, as described earlier for COMT substrates [124,125]. A recent study investigating the effect of arsenic trioxide in human liver cancer cell lines reported DNA hypomethylation, which restored the expression of silenced tumor suppressor genes (*p16^{INK4a}, RASSF1A, E cadherin*, and *GSTP1*). Specifically, arsenic affected the transcription of the *DNMT1* gene and inhibited DNMT1 activity in a dose-dependent manner *in vitro* [126]. Cadmium and zinc also were identified as inhibitors of methyltransferase activities [127]. One mechanism mediating cadmium-induced alterations in gene expression is related to hypomethylation of proto-oncogene promoters. Takiguchi et al. observed hyperproliferation and invasiveness of cadmium-treated rat liver cells that was correlated with inhibition of DNMT activity and a decrease in genome methylation. An *in vitro* DNA methyltransferase assay demonstrated that cadmium acts as an uncompetitive inhibitor, dependent on SAM concentration [128]. The correct structure and catalytic activity of many proteins requires zinc as an essential trace element, and zinc-dependent enzymes involved in one-carbon metabolism may influence the bioavailability of folate and ultimately the pool of SAM [129]. However, contrary to zinc, arsenic and cadmium strongly affect human health. Indeed, they exhibit genotoxicity and are considered as human carcinogens. Among the epigenetic mechanisms invoked for these heavy metals, there is evidence for hypermethylation of the *p53* gene promoter in arsenic-treated cells [130] and DNMT activation after chronic exposure to cadmium [128].

Selenium is a nonmetal trace mineral found mainly in muscle meats and seafood. Selenium is methylated to monomethylated, dimethylated, and trimethylated metabolites that use SAM as a methyl donor. The hypothesis suggesting that selenium can compete with DNMT for methyl supply from SAM was invalidated by the observation that dietary selenium supplementation failed to affect the store of SAM and SAM/SAH ratios [131]. Moreover, in the same study, rodents fed selenium-deficient diets displayed global DNA hypomethylation in both liver and colon [131], which was associated with an increased number of carcinogen-induced colonic aberrant crypts [132]. In contrast, Fiala et al. demonstrated that different organic and inorganic selenium compounds may affect DNA methylation by inhibiting DNMT in human colonic carcinoma [113], whereas selenium deficiency failed to affect DNMT activity in rodent liver and normal colon mucosa [133]. *In vivo* studies suggested that selenium may affect specific one-carbon metabolism steps, such as the remethylation of homocysteine, by increasing betaine homocysteine methyltransferase (BHMT) activity [134].

The conversion of homocysteine to methionine is of high importance for SAM production, as well as homocysteine detoxification. The transition requires a methyl group provided by either 5-CH_3THF or betaine and is catalyzed by methionine synthase (MS) and BHMT, respectively (Figure 13.3). Thus, dietary status for betaine, its precursor choline, and folate can impact upon DNA methylation status, and inadequate dietary folate intake increases cancer susceptibility [135,136] due to its central role in SAM production (Figure 13.4). Dietary folate deficiency is associated with increased plasma homocysteine as a consequence of decreased

MS activity, and an alteration of cystathionine-β-synthase (CS) activity, catalyzing the first step of homocysteine transsulfuration (requiring vitamin B6) [134]. This may lead not only to decreased methionine levels, but also to increased SAH, because the equilibrium in the homocysteine-SAH interconversion actually favors SAH synthesis, which inhibits MS and DNMT activities. Moreover, MS is a vitamin B12-dependent enzyme catalyzing the methyl transfer from 5-CH3THF to homocysteine using the cofactor vitamin B12 (cobalamin) as a transient acceptor for the methyl group. Thus, deficiency in coenzyme B12 limits homocysteine remethylation and affects DNA methylation [137]. The alternative pathway involving BHMT is also limited by the availability of methyl donor choline [138] and is regulated by betaine and selenium [139]. Animals fed methyl-deficient diets (no folate and choline, plus low in methionine) exhibit global DNA hypomethylated and regional hypermethylation associated with upregulation of MBP and DNMT enzymes [140,141]. Such diets induce tumor formation, without the need for any carcinogen treatment or genetic manipulation *in vivo*. Collectively, these studies indicate that the critical methylating step involving homocysteine interconnects different metabolic pathways that can be altered by several nutritional components, including choline, betaine, folate, and methionine, as well as cofactors B2, B6, B12, and zinc (Table 13.1; Figures 13.3 and 13.4). Detrimental effects of alcohol also may be related, in part, to alterations in folate metabolism [142,143]. Chronic alcohol consumption was associated with modifications of DNA methylation via impaired MS activity, decreased SAM, and increased SAH levels [144,145].

This area is complicated because methylated histones are found in both condensed and conformationally open chromatin; hypermethylated promoters contain methylated H3K9, whereas undermethylated promoters have methylated H3K4. Nonetheless, methyltransferases such as HMT are clearly affected by alterations in one-carbon metabolism, and it would be interesting to test for nutrients that modulate SAM as a mechanism for epigenetic gene regulation, as seen with drugs such as 5-azaC [100,146]. An additional avenue of research includes the unsilencing of genes via cooperating activities between DNMT and HDAC inhibitors.

13.8 COOPERATING ACTIVITIES BETWEEN DNMT AND HDAC INHIBITORS

The transcriptional regulation model (Figure 13.1) predicts that DNMT inhibitors might synergize with other agents to augment gene transcription. There is evidence that targeting DNA methyltransferases alone may be necessary, but not sufficient, to affect gene derepression [147]. Obvious candidates to enhance gene activation would be HDAC inhibitors, which cooperate with other cancer therapeutic agents such as proteasome inhibitors, apoptotic agents, and all-*trans* retinoic acid (RA) to induce cell death in cancer cells [148,149]. An important consideration for strategies that seek to reactivate gene expression is the phenomenon of resilencing, as observed for $p16^{INK4a}$ in colorectal cancer cells disrupted

TABLE 13.1
Dietary Components Known to Influence DNA Methylation

Dietary Component	References
Alcohol	[145]
Arsenic	[124,126,131]
Betaine	[138,206]
Biochanin A	[123]
Cadmium	[128]
Caffeic acid	[118]
Catechin	[116]
Chlorogenic acid	[118]
Choline	[138,140,141]
Dadzein	[123]
Epigallocatechin-3-gallate (EGCG)	[115,116]
Fisetin	[116]
Folate (folic acid, vitamin B9)	[135–137,140,141]
Genistein	[122,123]
Methionine	[140,141]
Myricetin	[116]
Quercetin	[116]
Retinoic acid (vitamin A)	[207]
Selenium	[113,131]
Vitamin C (ascorbic acid)	[208–210]
Vitamin B2 (riboflavin)	[211]
Vitamin B6 (pyridoxine)	[212]
Vitamin B12 (cobalamin)	[137]
Zinc	[213]

for both *DNMT1* and *DNMT3b*. Bender et al. similarly detected remethylation of the $p16^{INK4a}$ promoter following initial decitabine-induced demethylation [150]. The latter findings clearly establish that cancer cells possess an innate ability to resilence tumor suppressor genes, and that abrogation of DNMT activity alone might not suffice as a strategy for cancer therapy or prevention. There is the related issue of drug resistance versus overt toxicity in response to single agents, such as 5-azaC and TSA. To circumvent this dilemma, such agents might be used at lower effective doses if they are combined with food components that possess demethylating activities or anti-HDAC properties.

Classical HDAC inhibitors, such as TSA, have been reviewed extensively in terms of their activity in cell and animal models [151,152], and microarray analyses identified genes reactivated after treatment with 5-azaC, HDAC inhibitors, or their combination [153,154]. Several classes of HDAC inhibitors exist, including

TABLE 13.2
Main Histone Deacetylase Inhibitors

Class	Compound Name
Hydroxamic acid-derived compounds	trichostatin A (TSA)
	suberoylanilide hydroxamic acid (SAHA)
	m-carboxycinnamic acid bis-hydroxamide (CBHA)
	azelaic bishydroxamic acid (ABHA)
	Oxamflatin
	Scriptaid
	Pyroxamide
	NVP-LAQ824
	PXD101
	suberic bishydroxamic acid (SBHA)
	LBH589
Cyclic peptides	cyclic hydroxamic acid-containing peptides (CHAPs)
	depsipeptide (FK-228, FR901228)
	apicidin
	trapoxin
Short-chain fatty acids	butyrate, phenylbutyrate
	valproic acid
Benzamides	MS-275
	CI-994 (N-acetyl-dinaline)
Ketones	trifluoromethyl ketone
	a-ketoamides

short-chain fatty acids, hydroxamic acid-derived compounds, cyclic peptides, benzamides, and ketones (Table 13.2). HDAC inhibitors as single agents are currently used in clinical trials since they show promising antineoplastic activity [151,152].

HDAC inhibitors also occur in the human diet and are gaining attention due to their ability to epigenetically influence gene expression (Table 13.3, Figure 13.5). Butyrate, formed in the digestive tract by fermentation of dietary fibers, was one of the first identified modulators of HDAC activity [155]. Subsequently, organosulfur compounds in garlic, such as diallyl disulfide (DADS), and their metabolites (allyl mercaptan, *S*-allylmercaptocysteine), as well as sulforaphane and other isothiocyanates from cruciferous vegetables, were seen to alter histone acetylation status and/or HDAC activity *in vitro* and *in vivo* [156–160]. Alterations in cell growth, induction of apoptosis, and inhibition of invasion and metastasis are common features observed with the use of these natural compounds. For example, as with TSA, butyrate, DADS, and sulforaphane were all reported to reactivate the tumor suppressor $p21^{WAF1}$ in cancer cells [161–163], leading to cell cycle arrest and/or apoptosis. Curcumin, a polyphenol derived from the plant *Curcuma longa*, also exerts anticancer activities associated with the modulation of histone acetylation status. However, curcumin was associated with decreased levels of HDAC

TABLE 13.3
Dietary (and Medicinal) Compounds Modulating Histone Acetylation and/or HDAC/HAT Activities

Dietary Component	Examples of Food/Plant Sources	References
Allyl compounds		
Allyl mercaptan	Garlic (*Allium sativum* L.)	[157]
S-allylmercaptocysteine	Garlic (*Allium sativum* L.)	[158]
Diallyl disulfide (DADS)	Garlic (*Allium sativum* L.)	[156,157,162]
Isothiocyanates		
Sulforaphane	Broccoli, broccoli sprouts	[163]
6-Methylsulfinylhexyl isothiocyanate	Japanese horseradish (wasabi)	[214]
Short-Chain Fatty Acids		
Butyrate	Dietary fiber fermentation	[161]
Metals		
Copper		[215,216]
Nickel		[217,218]
Methylxanthines		
Theophylline	Black and green tea	[219–221]
Coumarin derivatives		
Dihydrocoumarin	*Melilotus officinalis* (sweet clover)	[222]
Stilbene derivatives		
Piceatannol	Blueberries	[223]
Resveratrol	Red grapes, wines, eucalyptus, spruce, berries, peanuts	[223,224]
Chalcone derivatives		
Butein	*Rhus verniciflua* (stems)	[223,224]
Isoliquiritigenin	*Glycyrrhiza glabra* (licorice)	[223,224]
Flavone derivatives		
Fisetin	*Rhus toxicodendron* (leaves)	[223,224]
Luteolin	Sweet red pepper, celery, parsley	[223]
Quercetin	Apple, tea, onion, nuts, berries, cauliflower, cabbage	[223,224]
Others		
Curcumin	*Curcuma longa* (tumeric roots)	[164–166]
Garcinol	*Garcina indica* fruit	[225]
Anacardic acid	Cashew nut	[226]

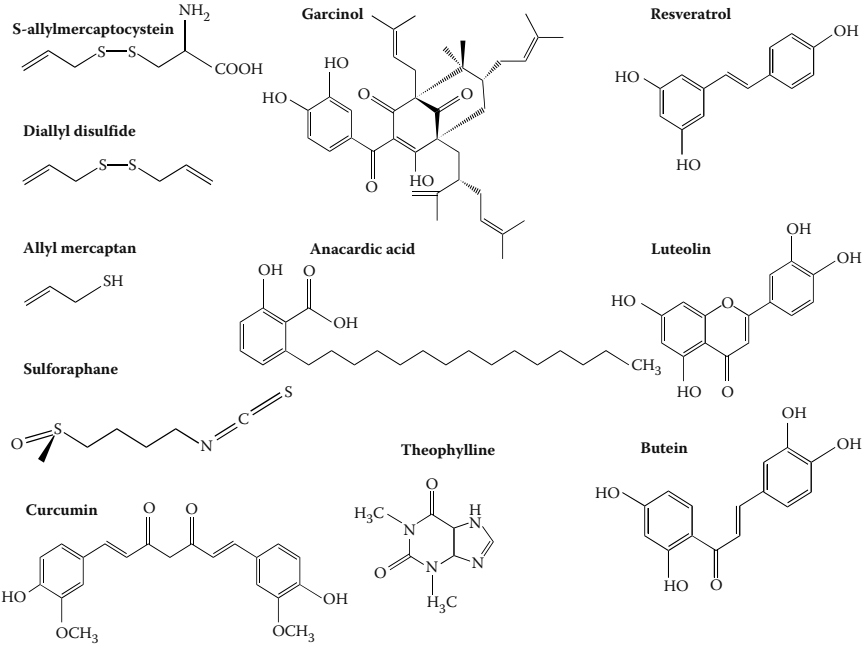

FIGURE 13.5 Structures of phytochemical HAT/HDAC modulators.

and histone hyperacetylation in Burkitt lymphoma cells [164], whereas HAT inhibition and histone hypoacetylation were observed in HeLa cells [165], prostate cancer cells [166], and hepatoma cells [167]. Aberrant HAT function has been detected in a number of cancers [168,169], and since HAT inhibition has antiproliferative effects [170], there is growing interest in the development of HAT inhibitors as antineoplasic agents [171,172]. Among natural dietary compounds, only butyrate and its derivatives have been included in clinical trials; despite initial success in inducing remission in patients with leukemia [173], other therapeutic interventions have not been as promising. However, optimization of the route and length of administration of butyrate may increase its therapeutic effects, as well as using such compounds in combination with other agents that might provide cooperative mechanisms.

One well-documented example of cooperation between agents targeting epigenetic changes is the treatment of hematopoietic neoplasia. Acute promyelocytic leukemia (APL) is characterized by the absence of myeloid cell maturation, resulting from the inhibition of gene expression governed by vitamin A active metabolites called retinoic acids (RA). APL provides a powerful example of HDAC dysregulation that may be targeted by inhibitors of HDAC. Indeed, the expression of a PML/RARα fusion protein, caused by reciprocal chromosomal translocations, induces the abnormal recruitment of HDAC proteins at the RARE (retinoic acid-activated receptor response element) in RA responsive gene promoters. Aberrant

histone deacetylation induced-silencing of genes regulating promyelocyte differentiation is responsible for neoplastic transformation. The oncoprotein also induces DNA and histone methylation of PML/RARα target genes through recruitment of DNMT [174] and Suv39h [175], respectively. Complete remission of some patients with APL is achieved by treatment with RA. Indeed, RA binding to the RAR moiety leads to the release of HDAC proteins, and transcriptional coactivators associated with HAT may then be recruited. Recently, Fazi et al. demonstrated that RA also induces DNA demethylation in the $RAR\beta_2$ promoter through downregulation of DNMT1, DNMT3a, and DNMT3b expression and activity [176]. $RAR\beta_2$ is a major tumor suppressor gene epigenetically silenced in a large variety of cancers [177], and treatment with demethylating agents successfully reactivates its expression [178]. Interestingly, both 5-azaC and HDAC inhibitors (TSA and SAHA) potentiate effects of RA on target genes in leukemia blasts from bone marrow and peripheral blood of APL patients [176,179]. However, RA was required, since DNMT and HDAC inhibitors alone failed to reactivate $RAR\beta_2$. Some RA resistant-leukemia cells failed to respond to RA alone, and need transcriptional derepression of methylated RA target genes by others agents. Treatment of RA-refractory APL blasts with RA plus the HDAC inhibitor sodium phenylbutyrate restored RA sensitivity and cell differentiation [180,181]. Combined treatments of RA with other HDAC inhibitors, namely TSA, depsipeptide, valproic acid, and phenylbutyrate, were also effective in reactivating target genes and facilitating differentiation in APL-derived cell lines and in non-APL acute myeloblasts (AML) [154,182,183]. Interestingly, arsenic trioxide is effective in the treatment of resistant or relapsed cases of APL, after treatment with RA [184], as well as newly diagnosed APL cases [185,186]. Caffeic acid and the related compound in honeybee propolis called caffeic acid phenethyl ester (Figure 13.2) also potentiated RA-induced differentiation of the promyelocytic cell line HL-60 [187,188]. The former compounds have been reported as inhibitors of arachidonic acid metabolism, and they induce differentiation and apoptosis in cancer cells, but effects on DNMT-mediated DNA methylation and increased formation of SAH by COMT have not been addressed. A recent report identifying target proteins associated with growth arrest in genistein-treated HL-60 cells [189] also supports the idea that dietary agents might improve existing cancer therapeutic drugs [190,191]. Recent studies suggested a benefit of green tea polyphenols in patients with lymphocytic leukemia [192], and will likely provide impetus for trials combining "classic" therapeutic drugs with food or beverage compounds affecting epigenetic remodeling.

Drug combinations *in vitro* and *in vivo* provide a precedent for epigenetic therapy. Synergistic interactions between DNMT and HDAC inhibitors were evaluated in leukemic blasts [193–195], as well as in breast and lung cancer cells [95,196–199]. The interaction between the powerful demethylating agent decitabine and HDAC inhibitors such as TSA, depsipeptide, NVP-LAQ824, valproic acid, and phenylbutyrate resulted in enhanced antineoplastic effects, including reactivation of a number of cancer-related genes silenced by aberrant methylation and histone deacetylation. This approach to combination therapy is now being

considered for dietary compounds. For example, genistein and sulforaphane exhibited synergistic effects on gene reactivation and cell growth [123], including derepression of *p16*^{INK4a} and *MGMT*. A combination of TSA with the flavonoid quercetin provided evidence for synergistic cytotoxicity in HL-60 cells [200]. However, in both studies, an important question was the physiological relevance of the concentrations required and the likelihood of achieving such levels in the context of normal dietary consumption or possibly in clinical trials.

Clinical trials evaluating epigenetic remodeling agents are of growing interest [201–204]. In one pilot study using phenylbutyrate plus 5-azaC in AML patients, demethylation events and acetylated chromatin were clearly observed, but no correlation was seen with clinical response [201]. In general, there is a need for improved biomarkers, which is a common theme in many or all clinical intervention trials. However, this is more complex in the area of epigenetics because of the potential for influencing multiple disease conditions, not simply cancer. One example would be the newly discovered modulators of class III HDAC called sirtuin activating compounds (STAC; Table 13.3, Figure 13.5) [205]. STAC include dietary polyphenols, such as resveratrol, that reportedly extend lifespan in yeast, worms, and flies via allosteric changes in sirtuins, thereby activating cellular defenses against stress. Most recently, resveratrol was shown to protect neurons from toxic effects of mutant huntingtin, a protein implicated in the development of Huntington's disease [205]. Most intriguing, however, is the idea that STAC might activate similar mechanism(s) as caloric restriction, which increases life expectancy in mammals by delaying cancer, cardiovascular disease, diabetes, and neurodegeneration. There is much to be done to establish these concepts in mammals and in humans, but for those interested in dietary factors and their impact on epigenetic mechanisms, these are certainly interesting times.

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14 Effects of Astaxanthin on Microarray Profiling of Gene Expression Patterns of Glomerular Cells in Diabetic Mice

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CONTENTS

14.1 INTRODUCTION

It has been postulated that increased oxidative stress by high glucose concentrations in the blood is important in the pathogenesis of diabetic nephropathy. Studies using natural and synthetic antioxidants, such as N-acetyl-L-cysteine, vitamins C and E, α-lipoic acid, taurine, and probucol, have provided convincing evidence that glomerular hypertrophy and the accumulation of collagen and transforming growth factor-β (TGF-β) due to high glucose concentrations are largely mediated

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by reactive oxygen species [1–4]. Therefore, it is thought that such studies might provide further insight into therapeutic strategies for treating patients with diabetes mellitus. In this review, we summarize the function of astaxanthin and present our recent results obtained from a nutrigenomics approach in order to investigate the beneficial effect of astaxanthin on diabetic nephropathy.

14.2 WHAT IS ASTAXANTHIN?

Astaxanthin, which is found as a common red-colored pigment in algae, fish, and birds, is a carotenoid that exerts many highly potent pharmacological effects, such as antioxidative activity [5–7], immunomodulating actions [8,9], anticancer activity [10], and anti-inflammation action [11,12]. Astaxanthin is reported to be more effective than other antioxidants such as vitamin E and β-carotene in the prevention of lipid peroxidation in solution and in various biomembrane systems [5,6]. Goto et al. [13] reported that the efficient antioxidant activity of astaxanthin could be due to the unique structure of its terminal ring moiety. Astaxanthin traps radicals not only at the conjugated polyene chain but also in its terminal ring moiety, in which the hydrogen atom at the C3 methine is suggested to be a radical trapping site. In addition to an antioxidative effect, many investigators have demonstrated anti-inflammatory properties of astaxanthin *in vivo*. We have reported that astaxanthin can attenuate exercise-induced acute inflammation with neutrophil infiltration in mouse skeletal muscle and heart [14]. Ohgami et al. [12] have also demonstrated that astaxanthin has a dose-dependent anti-inflammatory effect against endotoxin-induced uveitis, by the suppression of nitric oxide, prostaglandin E2, and tumor necrosis factor-α production, through directly blocking nitric oxide synthase enzyme activity. It was recently reported that its anti-inflammatory effect might be derived from its inhibitory effect against nuclear factor (NF)-κB activation [15]. In this study, astaxanthin reduced ocular inflammation in eyes with endotoxin-induced uveitis by downregulating proinflammatory factors and by inhibiting the NF-κB-dependent signaling pathway [15].

Some microorganisms are rich in astaxanthin; the Chlorophyte alga *Haematococcus pluvialis* is believed to accumulate the highest levels of astaxanthin in nature. Commercially grown *H. pluvialis* can accumulate >30 g of astaxanthin kg^{-1} dry biomass [16]. Fuji Chemical Industry has developed unique closed-bioreactor systems in Maui, Hawaii, and Gustavsberg, Sweden, which effectively stimulate the optimum conditions for astaxanthin production that yield the highest quality and minimize the risk of contaminants. Therefore, a market for neutraceutical astaxanthin has started to develop, as recent research has pointed to the possible functions of astaxanthin in the human body.

14.3 PREVENTION OF DIABETIC NEPHROPATHY BY ASTAXANTHIN

In 2002, we first reported the potential usefulness of astaxanthin treatment for reducing glucose toxicity using db/db mice, a rodent model of type 2 diabetes [17].

This mouse is a genetic model of type 2 diabetes mellitus, which develops hyperglycemia in association with insulin resistance and obesity beginning in the second month of age [18]. After 10–20 weeks of sustained hyperglycemia, the db/db mouse exhibits clinical and histological features of diabetic nephropathy that parallel those of human disease. The kidneys show the characteristic histological lesions of diabetic nephropathy, including mesangial matrix expansion and glomerular basement membrane thickening [19]. Therefore, the db/db mouse represents a suitable model for studying diabetic glomerulosclerosis and examining pathogenic influences and treatment strategies that may be applicable to the human disease.

In our study, the ability of islet cells to secrete insulin was determined by the glucose tolerance test, and this ability was found to be preserved in an astaxanthin-treated group, although histologic study of the pancreas revealed no significant differences in the β-cell mass between astaxanthin-treated and -untreated db/db mice [17]. Next, using the same model of diabetic mice, we recently demonstrated that astaxanthin treatment significantly ameliorates diabetic nephropathy, which is determined based on urinary albumin levels and histological findings (Table 14.1) [20]. In addition, it was clearly observed that long-term oral treatment with astaxanthin reduced not only the increased albuminuria otherwise observed in untreated diabetic mice but also ameliorated such increases in the urinary excretion of 8-hydroxydeoxyguanosine (8-OHdG) and in 8-OHdG expression in the mesangial cells, with little effect on blood glucose levels [20]. Furthermore, our data demonstrated that the esterified astaxanthin used in our previous study was effectively absorbed and was transported successfully to the kidneys [20]. Taken together, these results suggested that astaxanthin might directly attenuate diabetic oxidative damage, although a slight decrease in blood glucose levels would also be expected to contribute to the attenuation of such oxidative damage.

TABLE 14.1
Effects of Astaxanthin on the Urinary Albumin Excretion Rate, the Urinary 8-Hydroxydeoxyguanosine (8-OHdG), and the Number of 8-OHdG-Positive Cells per Glomeruli

	db/m	db/db	db/db + astaxanthin
Urinary albumin (mg/day)	79.7 ± 11.8	234.4 ± 71.5$^{\#}$	77.7 ± 13.8^{+}
Urinary 8-OHdG levels (ng/day)	78.1 ± 14.8	336.1 ± 68.6$^{\#}$	166.7 ± 44.5^{+}
8-OHdG-postive cells in (number/blomeruli)	1.7 ± 0.4	29.0 ± 3.1$^{\#}$	9.8 ± 0.8^{+}

Note: All values indicate the means ± SE of 8 mice. $^{\#}p < 0.01$ versus db/m mice and $^{+}p < 0.05$ versus db/db mice.

14.4 EFFECTS OF ASTAXANTHIN ON GENE EXPRESSION PROFILE IN DIABETIC GLOMERULAR CELLS

In patients with diabetic nephropathy, as well as in experimental animal models, various molecules associated with oxidative stress, collagen synthesis, and transforming growth factor-β (TGF-β) have been reported to play important roles in the onset and aggravation of diabetic nephropathy [21,22]. The enhanced expression of these molecules, the abnormal regulation of cell signaling, and the genetic polymorphism of these genes may all contribute to dysregulated cell proliferation and to an enhanced expansion of the extracellular matrix in the renal glomerular region. Many studies of the pathogenesis of diabetic nephropathy have been based on the analysis of the expression of a single molecule, or of a relatively limited number of these molecules in diabetic glomerular cells. Recently, DNA microarray techniques have become available that have enabled the characterization of the mRNA expression pattern of a large number of genes simultaneously. We recently identified specific gene expression profiles in the renal glomerular cells of diabetic db/db mice, and investigated the effects of astaxanthin on the expression of these genes using a comprehensive GeneChip system analysis [23].

We used a high-density oligonucleotide microarray technique for the mRNA expression profile of renal glomerular cells to investigate the mechanism of diabetic nephropathy and to clarify the effects of chronic treatment with astaxanthin on these changes in the levels of mRNA. Through laser-assisted microdissection to obtain cell-specific RNA, renal glomerular cells were identified on cryostat sections (8 μm) of specimens obtained from the kidneys of the mice, and the cells were isolated by laser-assisted microdissection using an LM200 system (Olympus, Tokyo, Japan). Although laser capture microdissection can be used to produce cell-specific RNA, this method is limited by the amount of RNA that can be realistically obtained from captured populations of cells, making it likely that the yield will be insufficient for the commonly used GeneChip assay. To overcome this obstacle, our experiments were performed according to the Affymetrix GeneChip Eukaryotic Small Sample Target Labeling Assay (Version II) protocol [24]. Using this protocol, we succeeded in obtaining a sufficient amount of biotinylated cRNA to perform the GeneChip analysis from the small amount of renal glomerular cells obtained by laser captured microdissection.

We used the GeneChip of Mouse Expression Array 430A (Affymetrix, Santa Clara, California), which contained 22,690 probes representing approximately 15,000 full-length sequences and approximately 4,000 expressed sequence tag (EST) clusters selected from the UniGene database. Comparison of the expression profiles from normal db/m mice and diabetic db/db mice, and from db/db mice and astaxanthin-treated db/db mice enables the identification of differentially regulated genes associated with diabetic-induced hyperglycemia and activity of astaxanthin, respectively. Of the 22,690 probes examined, 779 (3.4%) were upregulated (550 probes) or downregulated (229 probes) at least 1.5-fold in the diabetic mice in comparison with the db/m mice. To further refine the list of diabetes-affected genes, our next goal was to identify the genes that are known to interact

biologically. To this end, we used the Pathway Analysis tool (Ingenuity System Mountain View, California) to carry out analyses of the 550 upregulated genes. Table 14.2 shows six genetic networks affected in the glomerular cells of diabetic mice, as defined by the Pathway Analysis tool. These networks describe functional relationships between gene products based on known interactions reported in the literature. These networks, shown in Table 14.3, were associated with oxidative phosphorylation, the citrate cycle, ubiquinone biosynthesis, pyruvate metabolism, fatty acid biosynthesis, and the synthesis/degradation of ketone bodies. In particular, the results regarding networks associated with the oxidative phosphorylation pathway were found to be highly significant, since a greater number of identified genes were present in this pathway than would be expected by chance. This pathway includes 20 probe sets for genes located at the inner mitochondrial membrane, and these genes are members of the electron transport system, in particular, complexes I, III, and IV (Figure 14.1). The abnormal upregulation of these genes may be associated with the increased production of reactive oxygen species (ROS) from the mitochondrial membrane, which has also been demonstrated in previous studies. Recently, Nishikawa et al. [25] showed that the hyperglycemia-induced production of ROS is abrogated by inhibitors of mitochondrial metabolism or by the overexpression of uncoupling protein-1 (UCP-1) or manganese superoxide dismutase (MnSOD). In addition, normalization of mitochondrial ROS production by each of these agents can prevent glucose-induced activation of protein kinase C, the formation of advanced glycation end products, the accumulation of sorbitol, and the activation of nuclear factor κB (NF-κB) in bovine vascular endothelial cells, as well as in cultured human mesangial cells [25,26], all of which are known to be involved in the development of diabetic complications. The present study showed that the expression of upregulated mitochondrial genes was decreased by treatment with astaxanthin. These data may suggest that astaxanthin can reverse the abnormal function of glomerular cell mitochondria in diabetic db/db mice.

Next, in order to focus on oxidative stress in diabetic nephropathy in particular, the number of genes was narrowed to 90 probe sets, which were selected using a computer on-line soft of NetAffyx™ Analysis Center (http://www.affymetrix.com/analysis/index.affx; July 1, 2005) and the keywords *oxidative stress*. Among the 90 probes related to oxidative stress, 21 were upregulated in the diabetic db/db mice, as compared to the db/m mice. As shown in Table 14.4, three probes of tyrosine 3-monooxygenase were upregulated at least 8.0-fold in the diabetic mice in comparison with the db/m mice; however, there were no significant differences between the db/db mice and the astaxanthin-treated db/db mice (*NC* in Table 14.4 indicates no change in the average difference). The peroxiredoxin 1 gene probe (1436691_x_at) was a highly upregulated gene in diabetic mice and was significantly reduced at a ratio of 0.14 by treatment with astaxanthin. In the Mouse Expression Array 430A (Affymetrix), four probe sets were included for the peroxiredoxin gene: 1436691_x_at, 1433866_x_at, 1434731_x_at, and 1437014_x_at. The expression of all four probes was upregulated at least 8.0-fold in the diabetic mice, and was downregulated by treatment with astaxanthin (Table 14.4).

TABLE 14.2
Upregulated Genes in db/db Mice

Probe Set ID	Description	Average Difference*		Log Ratio	Fold Change**
		db/db	db/m		
1438634_x_at	LIM and SH3 protein 1	2695.1	10.3	8.0	**256.00**
1433443_a_at	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	6472.6	76.6	7.3	**157.59**
1438610_a_at	Crystallin, zeta	10955.2	95.5	7.1	**137.19**
1423684_at	—	3573.7	43.8	7.0	**128.00**
1415820_x_at	—	2286.4	18.0	6.8	**111.43**
1448382_at	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase	1482.1	27.9	6.7	**103.97**
1455039_a_at	Transcriptional regulator, SIN3B (yeast)	2383.9	23.7	6.7	**103.97**
1456176_x_at	DNA segment, Chr 11, ERATO Doi 333, expressed	6941.6	84.7	6.7	**103.97**
1450387_s_at	—	11485.4	157.3	6.6	**97.01**
1416180_a_at	Radixin	1363.3	29.0	6.5	**90.51**
1416633_a_at	RIKEN cDNA 5730536A07 gene	1771.9	14.7	6.5	**90.51**
1426385_x_at	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	3762.1	62.2	6.5	**90.51**
1437143_a_at	Thioredoxin domain containing 1	7150.4	41.7	6.5	**90.51**
1455897_x_at	High mobility group nucleosomal binding domain 1	9034.5	77.7	6.5	**90.51**
1449040_a_at	Selenophosphate synthetase 2	10394.9	103.9	6.4	**84.45**
1434099_at	—	1091.2	19.6	6.3	**78.79**
1456196_x_at	—	3454.6	47.8	6.3	**78.79**
1420037_at	—	6773.1	85.8	6.3	**78.79**
1449059_a_at	3-Oxoacid CoA transferase 1	8173.9	77.6	6.3	**78.79**
1434499_a_at	Lactate dehydrogenase 2, B chain	9177.8	58.0	6.3	**78.79**

1424827_a_at	Casein kinase 1, alpha 1	4755.4	39.8	6.2	**73.52**
1434892_x_at	Retinoblastoma binding protein 4	7714.7	59.6	6.2	**73.52**
1423890_x_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	76228.2	1190.9	6.2	**73.52**
1430542_a_at	Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5	3309.2	41.8	6.2	**73.52**
1436298_x_at	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoribosylaminoimidazole, succinocarboxamide synthetase	2049.5	23.6	6.1	**68.59**
1435446_a_at	Choline phosphotransferase 1	7782.3	153.8	6.1	**68.59**
1416143_at	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F	7867.0	116.2	6.1	**68.59**
1436900_x_at	Leptin receptor overlapping transcript	1133.7	19.8	6.0	**64.00**
1427262_at	—	1768.5	46.3	6.0	**64.00**
1456226_x_at	Discoidin domain receptor family, member 1	5273.1	57.7	6.0	**64.00**
1436691_x_at	Peroxiredoxin 1	39422.3	367.1	6.0	**64.00**
1439184_s_at	Thioredoxin-like 5	1615.9	24.4	6.0	**64.00**
1435791_x_at	Ribosomal protein L17	2858.6	45.3	6.0	**64.00**
1434056_a_at	Gene model 137, (NCBI)	2872.6	52.0	5.9	**59.71**
1456341_a_at	Basic transcription element binding protein 1	5894.2	99.7	5.9	**59.71**
1439411_a_at	Exportin 7	4260.4	40.7	5.9	**59.71**
1455815_a_at	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	14398.9	205.9	5.9	**59.71**
1425140_at	Lactamase, beta 2	9634.1	55.9	5.9	**59.71**
1433514_at	Ethanolamine kinase 1	2752.3	42.6	5.8	**55.72**
1436783_x_at	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	4090.5	94.2	5.8	**55.72**

(*continued*)

TABLE 14.2 (continued)
Upregulated Genes in db/db Mice

Probe Set ID	Description	Average Difference*		Log Ratio	Fold Change**
		db/db	db/m		
1416316_at	—	7482.6	104.7	5.8	**55.72**
1420618_at	Cytoplasmic polyadenylation element binding protein 4	6068.4	55.3	5.8	**55.72**
1430526_a_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	1456.4	21.8	5.8	**55.72**
1426326_at	Zinc finger protein 91	2156.3	39.6	5.7	**51.98**
1438625_s_at	Necdin /// PCTAIRE-motif protein kinase 1	2578.6	66.5	5.7	**51.98**
1420827_a_at	—	2694.3	44.8	5.7	**51.98**
1433704_s_at	Translocation protein 1	4317.0	112.1	5.7	**51.98**
1418503_at	Heat shock protein, A	2361.8	22.6	5.7	**51.98**
1435164_s_at	Ubiquitin-activating enzyme E1C	1787.0	14.3	5.7	**51.98**
1416166_a_at	Peroxiredoxin 4	557.7	11.3	5.7	**51.98**

Source: From Naito, Y. et al., *Int. J. Mol. Med.* 18, 685, 2006. With permission.

* Average difference indicates the level of expression of the gene.

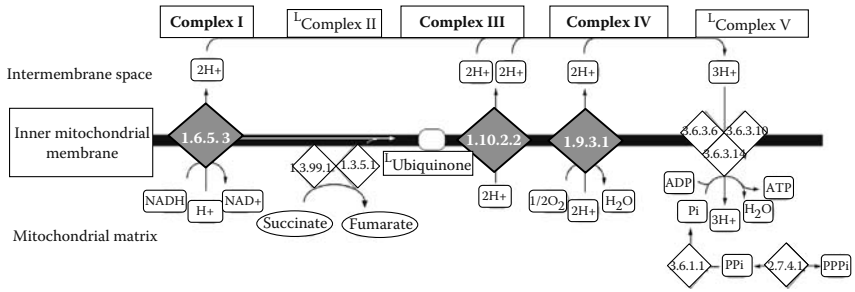
** Fold changes in average difference values were calculated using an Affymetrix software algolithm (GCOS ver. 1.0).

TABLE 14.3
Ingenuity Caronical Pathway Analysis for Upregulated Genes in Diabetic db/db Mice

Canonical Pathway	Significance	Genes
Oxidative phosphorylation	3.93×10^{-8}	COX15, COX5A*, COX5B*, COX6B, COX6C, DAP13, MTND6, NDUFA1, NDUFA2, NDUFA5, NDUFAB1, NDUFB9, NDUFC1, NDUFC2, NDUFS3, NDUFV2, UQCRB, UQCRC2
Citrate cycle	1.64×10^{-6}	ACLY, ATP5G3, CS, FH, IDH1*, IDH3B, MDH1*, MDH2, SUCLA2
Ubiquinone biosynthesis	4.07×10^{-6}	DAP13, MTND6, NDUFA1, NDUFA2, NDUFA5, NDUFAB1, NDUFB9, NDUFC1, NDUFC2, NDUFS3, NDUFV2
Pyruvate metabolism	2.33×10^{-4}	ACAA1, ACAS2L, ACAT1, ADH5, AKR1A1*, ALDH9A1, GLO1, HADHB, LDHB, MDH1*, MDH2
Fatty acid biosynthesis (path 2)	6.99×10^{-4}	ACAA1, ACAT1, EHHADH, HADHB, HADHSC*
Synthesis and degradation of ketone bodies	7.57×10^{-3}	ACAT1, HMGCS1*, OXCT*

Source: From Naito, Y. et al., *Int. J. Mol. Med.* 18, 685, 2006. With permission.

* Duplicates. Gene/protein IDs marked with an asterisk indicate that multiple identifiers from the input list are mapped to a single gene in the global molecular network.



Pathway Node Name	Ingenuity Node Name
1.6.5.3	**DAP13, MTND6, NDUFA1, NDUFA2, NDUFA5, NDUFAB1, NDUFB9, NDUFC1, NDUFC2, NDUFS3, NDUFV2**
1.10.2.2	**UQCRB, UQCRC2**
1.9.3.1	**COX15, COX5A*, COX5B*, COX6B, COX6C**

FIGURE 14.1 The oxidative phosphorylation pathway includes 20 sets of probes for genes located at the inner mitochondrial membrane. (From Naito, Y. et al., *Int. J. Mol. Med.* 18, 685, 2006. With permission.)

TABLE 14.4
The Effects of Astaxanthin Treatment on Oxidative Stress-Related Gene Expression

		Average Difference*			Diabetic/Normal		Astaxanthin/Diabetic	
Probe Set ID	**Description**	**Normal (db/m)**	**Diabetic (db/db)**	**Astaxanthin**	**Fold Change****	**Change*****	**Fold Change****	**Change*****
1426385_x_at	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, epsilon polypeptide	62.2	3762.1	1053.6	**90.51**	I	**0.25**	NC
1436691_x_at	Peroxiredoxin 1	367.1	39422.3	5075.8	**64.00**	I	**0.14**	D
1425993_a_at	Heat shock protein 105	34.5	1804.2	174.5	**34.30**	I	**0.09**	NC
1427442_a_at	Amyloid beta (A4) precursor protein	340.3	20915.3	4786.4	**34.30**	I	**0.25**	D
1439443_x_at	Transketolase	190.2	7265.6	1082.8	**32.00**	I	**0.14**	D
1416430_at	Catalase	53.5	1011.5	360.9	**25.99**	I	**0.33**	NC
1419821_s_at	Isocitrate dehydrogenase 1 (NADP+), soluble	118.3	2713.1	1141.9	**21.11**	I	**0.33**	NC
1416429_a_at	Catalase	666.3	12753.2	3273.1	**18.38**	I	**0.13**	D
1433866_x_at	Peroxiredoxin 1	899.7	8801.4	1255.8	**18.38**	I	**0.18**	D
1418180_at	*Trans*-acting transcription factor 1	73.0	1358.2	527.7	**18.38**	MI	**0.38**	NC
1451124_at	Superoxide dismutase 1, soluble	169.9	5222.6	1160.1	**17.15**	I	**0.23**	D

1418127_a_at	Programmed cell death 8	130.7	1978.9	1072.6	**16.00**	I	**0.27**	D
1434731_x_at	Peroxiredoxin 1	1701.0	19840.5	4196.7	**12.13**	I	**0.11**	D
1448808_a_at	Expressed in nonmetastatic cells 2, protein	1462.9	9564.2	1215.9	**11.31**	I	**0.13**	D
1422433_s_at	Isocitrate dehydrogenase 1 (NADP+), soluble	520.3	7709.1	271.7	**10.56**	I	**0.03**	D
1426384_a_at	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, epsilon polypeptide	63.7	1486.3	592.5	**9.85**	I	**0.25**	NC
1438839_a_at	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, epsilon polypeptide	495.2	3111.5	899.5	**8.00**	I	**0.31**	NC
1437014_x_at	Peroxiredoxin 1	507.4	2643.9	600.8	**8.00**	MI	**0.27**	D
1449254_at	Secreted phosphoprotein 1	1563.0	5812.8	593.1	**4.92**	I	**0.13**	D
1434831_a_at	Forkhead box O3a	514.0	1987.5	116.4	**4.29**	I	**0.05**	D
1449106_at	Glutathione peroxidase 3	2488.7	7390.7	1745.1	**4.29**	MI	**0.20**	D

Source: From Naito, Y. et al., *Int. J. Mol. Med.* 18, 685, 2006. With permission.

* Average difference indicates the level of expression of the gene.

** Fold changes in average difference values were calculated using an Affymetrix software algorithm (GCOS ver. 1.0).

*** I, MI, D, MD, and NC indicate increase, marginal increase, decrease, marginal decrease, and no change, respectively, in the average difference.

Peroxiredoxin 1 has been shown to play an important role in the defense against oxidative stress indicates the presence of such stress [27], and thus the enhanced expression of peroxiredoxin 1. Similar to peroxiredoxin 1 expression, the mRNA expression of catalase (1416430_at, 1416429_a_at), superoxide dismutase 1 (1451124_at), and glutathione peroxidase 3 (1449106_at) was also increased in diabetic mice, and these increases were significantly inhibited by treatment with astaxanthin. In addition, our previous study showed that astaxanthin accumulated in the kidneys of mice after 3 weeks of the administration of a diet containing 0.02% astaxanthin [20]. Therefore, the inhibition of the expression of these oxidative stress-responsive genes by an astaxanthin-containing diet, demonstrated in the present study, may indicate that astaxanthin absorbed from the intestine can act as an antioxidant in the glomerular lesion *in vivo*. The present data are also consistent with those of the our previous study, in which urinary and kidney 8-hydroxydeoxyguanosine levels, an index of oxidative DNA damage, exhibited marked reduction by treatment with astaxanthin, in spite of high blood glucose conditions.

14.5 CONCLUSION

In conclusion, changes in the gene expression profile of glomerular cells in the early phase of diabetic nephropathy in db/db mice were surveyed by laser capture microdissection/GeneChip analysis. In a comparison of db/m and db/db mice, we found that 779 probes showed a more than 1.5-fold difference with respect to the expression levels in each type of mouse; we identified the mitochondrial oxidative phosphorylation pathway as the caronical pathway that is most significantly affected by diabetic nephropathy in mice. Chronic treatment with astaxanthin significantly decreased the expression of upregulated probes, including those genes associated with oxidative phosphorylation and oxidative stress

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15 Insulin Resistance and Inflammatory Signaling Pathways Modulated by High-Fat Diet

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CONTENTS

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15.1 INTRODUCTION

Obesity and associated disorders, of which incidences have increased dramatically worldwide in the last decades, give a serious threat to the current and future health of mankind on Earth. The World Health Organization (WHO) estimates that more than 1 billion adults worldwide are overweight and 300 million are clinically obese, based on the body mass index (BMI) equal to or over 30 kg/m^2 [1]. Remarkably frightening is the similarly marked increase in obesity among children not only in Western countries, but also in the developing countries of the world [2,3]. Obesity is associated with many additional health problems, including increased risk of insulin resistance, type 2 diabetes, nonalcoholic fatty liver, atherosclerosis, degenerative disorders such as dementia, some immune-mediated disorders like asthma, and certain cancers [4,5]. This cluster of obesity-related pathologies has also started to emerge in children at young ages, a manifestation that was implausible only a few decades ago.

The worldwide consumption of dietary fat appears to be stable or declining in relative terms, although there is an epidemic of obesity [6], leading some to conclude that dietary fat is not related to obesity [7,8]. However, an analysis of the cross-cultural data and trends within populations suggests that dietary fat may play a direct role in obesity [9–11]. Although it is clear that genetic factors contribute to the propensity of an individual to become obese, the striking increase in overweight people occurs as previously underdeveloped countries modernize and the continued growth in the number of obese individuals in developed countries indicated an important role for environmental factors as well [12]. The consumption of a high-energy density diet is thought to be one of the main environmental factors. The consumption of a high-fat diet (HFD) increased free fatty acids (FFAs) or saturated fatty acid influx from the diet in rodents or humans, which may induce the adipogenesis and modulate the inflammatory responses.

Obesity is the most important well-known factor that leads to insulin resistance [13,14]. The physiological mechanisms linking obesity to insulin resistance include altered production of various adipocytokines (adiponectin, resistin, leptin, etc.), proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), retinol binding protein 4 (RBP4), and FFAs themselves [4,13–18]. Now, recent studies have focused the relationship between the inflammatory response and

metabolic diseases, including an HFD-induced insulin resistance. The general consensus is that HFD-induced obesity is characterized by chronic activation of inflammatory responses and that the inflammatory responses in obesity are closely linked to the insulin resistance. In this chapter, we focus on the molecular mechanisms of dietary fatty acid, especially saturated fatty acids, on the development of pathogenesis of obesity and related metabolic disorders. An HFD-induced modulation of signaling pathways implicated in the adipogenesis and in the glucose transporter 4 (GLUT4) translocation was observed in the visceral adipose tissue of rodent obesity models. Finally, we discuss the relationship between pathogen-sensing pathways and insulin resistance in HFD-induced obesity; the molecular and cellular links between HFD-induced metabolic disorders and alterations in inflammatory signaling pathways have been explored.

15.2 CHARACTERISTICS OF HIGH-FAT DIET–INDUCED VISCERAL OBESITY AND INSULIN RESISTANCE

Most cases of human obesity are polygenic and represent the interaction between multiple genes and the environment, of which diet is a major component [19,20]. High-fat intake has been recognized as a prime risk factor that leads to most cases of human obesity. Dietary fat is calorically dense, extremely palatable, and causes less satiety than carbohydrates and proteins [21]. Chronic exposure to an HFD can affect the generation and reception of meal-related signals that control energy metabolism, brain's generation, and reception of adiposity-indicating signals that regulate food intake and metabolism, thereby causing obesity [22]. Rodents with HFD-induced obesity have been proven to be useful experimental models for human obesity [23]. The extent to which obesity is induced by diet varies depending on the length of the feeding period, the types and levels of dietary fat, and the presence of other modifications in the dietary ingredients [22,24–28]. Substantial evidence suggests that not only the level but also the type of fat can influence body weight, body composition, and plasma comorbidity factors; a diet high in saturated fat induces a relatively low resting metabolic rate and reduces the diet-induced thermogenesis that would exacerbate weight gain [29,30].

Identifying the regulatory processes that mediate HFD-induced obesity is of fundamental importance and requires a well-established animal model. Authors have recently established a model for a rat with HFD-induced obesity, presenting a body weight gain and distinctive characteristics of metabolic syndrome that are more profound than those that had been previously reported in rats with diet-induced obesity [31,32]. The normal diet (ND) was formulated based on the AIN-76 rodent diet composition, and the HFD contained 200 g of fat/kg (170 g of lard plus 30 g of corn oil to provide essential fatty acids) and 1% cholesterol by weight (Table 15.1) [31,32]. The HFD was formulated to provide 40% of the total energy generated by the diet from fat, by replacing carbohydrate energy with lard and corn oil, and had the same amount of vitamins and minerals per kJ as the ND. The compositions of the experimental diets are shown in Table 15.1.

TABLE 15.1
Composition of Experimental Diets

Ingredient	ND (g/kg diet)	HFD
Casein	200	200
DL-methionine	3	3
Cornstarch	150	111
Sucrose	500	370
Cellulose	50	50
Corn oil	50	30
Lard	—	170
Vitamin mix[a]	10	12
Mineral mix[b]	35	42
Choline bitartrate	2	2
Cholesterol	—	10
tert-Butyhydeoquinone[c]	0.01	0.04
Total (g)	1,000	1,000
Fat (% calorie)	11.5	39.0
Total energy, kJ/kg diet	16,439	19,315

Source: Kim, Y.J. & Park, T., *Nutr. Res.*, 28: 414, 2008.

[a] AIN-76 Vitamin mixture (g/kg mix); thiamin HCl 0.6; riboflavin 0.6; nicotinamide 25; pyridoxine HCl 0.7; nicotinic acid 3; D-calcium pantothenate 1.6; folic acid 0.2; D-biotin 0.02; cyanocobalamin (Vitamin B_{12}) 0.001; retinyl palmitate (250,000 IU/gm) 1.6; DL-α-tocopherol acetate (250 IU/gm) 20; cholecalciferol (Vitamin D_3) 0.25; menaquinone (Vitamin K_2) 0.05; sucrose, finely powdered 972.9.

[b] AIN-76 Mineral mixture (g/kg of mix); $CaHPO_4$ 500; NaCl 74; $K_2H_6O_7H_2O$ 220; K_2SO_4 52; MgO 24; $MnCO_3$ 3.57; Fe ($C_6H_5O_7$) $6H_2O$ 6; $ZnCO_3$ 1.6; CuCO3 0.3; KIO3 0.01; $Na_2SeO_3 \cdot 5H_2O$ 0.01; CrK (SO4)2 0.55; sucrose, finely powdered 118.

[c] Antioxidant agent, 0.01 g/50 g lipids.

Sprague-Dawley (SD) rats given the HFD for 9 weeks weighed 55% more (exhibited almost a 100% greater body weight gain; Table 15.2) [31] and accumulated significantly greater visceral fats (85%–133% greater depending on the site) than the rats fed the ND (Figure 15.1) [31]. The rats with free access to the HFD were not only provided with more energy but also showed a higher energy efficiency ratio relative to the animals with free access to the ND in this study (Table 15.2) [31].

Interestingly, the HFD containing 17% lard plus 3% corn oil as a dietary lipid source induced a much greater increase in the final body weight compared to the animals fed a regular AIN-76 diet containing 5% corn oil, than those achieved by the diet containing 20% beef tallow [24]. A couple of speculations may explain this marked discrepancy in the body weight gain of the rats that were the HFD. First, the amount of essential fatty acids (EFA, linoleic acid + α-linolenic acid)

TABLE 15.2
Serum and Hepatic Biochemistries of Rats Fed Experimented Diets for 9 Weeks

	ND	HFD
Final body weight (g)	420 ± 5.75	650 ± 20.3
Body weight gain (g/10 wk)	233 ± 5.38	463 ± 23.3***
Food intake (g/day)	21.7 ± 0.42	27.6 ± 0.54***
Food efficiency ratio[a]	0.183 ± 0.004	0.226 ± 0.009***
Serum		
Total cholesterol (mol/L)	2.05 ± 0.15	4.22 ± 0.55*
LDL + VLDL cholesterol[b] (mmol/L)	0.50 ± 0.13	2.95 ± 0.50***
HDL cholesterol (mmol/L)	1.25 ± 0.05	1.26 ± 0.09
HTR[c] (%)	62.3 ± 3.35	31.8 ± 2.63**
Triglyceride (mmol/L)	0.43 ± 0.05	0.39 ± 0.03
Free fatty acid (mmol/L)	0.64 ± 0.08	0.58 ± 0.04
Aspartate transaminase (Unit/L)	70.7 ± 4.15	183 ± 29.0*
Alanine transaminase (Unit/L)	22.9 ± 0.86	51.2 ± 11.4*
Glucose (mmol/L)	8.83 ± 0.29	10.8 ± 0.61**
Insulin (pmol/L)	162 ± 30.6	508 ± 132*
C-peptide (pmol/L)	706 ± 59.4	1,862 ± 262**
Leptin (ng/mL)	3.04 ± 0.31	11.0 ± 1.57***
Insulin resistance index[d]	1.47 ± 0.35	5.32 ± 1.51*
Liver		
Weight (g/100 g body weight)	2.67 ± 0.13	5.01 ± 0.18***
Total lipid (mg/g liver)	369 ± 5.84	581 ± 13.3***
Cholesterol (μmol/g liver)	16.2 ± 1.32	41.1 ± 0.75***
Triglyceride (μmol/g liver)	3.67 ± 0.27	7.07 ± 0.19***
Free fatty acid (μmol/g liver)	3.46 ± 0.32	6.66 ± 0.81*
G6PDH (nmole · min^{-1} · mg $protein^{-1}$)	40.0 ± 4.45	25.7 ± 4.67*
Malic enzyme (nmole · min^{-1} · mg $protein^{-1}$)	25.0 ± 2.30	15.2 ± 3.01*

Source: Kim, Y.J. & Park, T., *Nutr. Res.*, 28: 414, 2008.

Note: Values are mean ± SEM for 10 rats.

[a] Food efficiency ratio = body weight gain/food intake

[b] LDL + VLDL cholesterol = total cholesterol – HDL cholesterol

[c] HTR (%) = [HDL cholesterol/total cholesterol] × 100

[d] Insulin resistance index = 10^{-3} pmol insulin × mmol glucose × L^{-2}

*, **, *** Significantly different from the value for rats fed ND by Student's t-test at $P < 0.05$, < 0.01, and < 0.001, respectively.

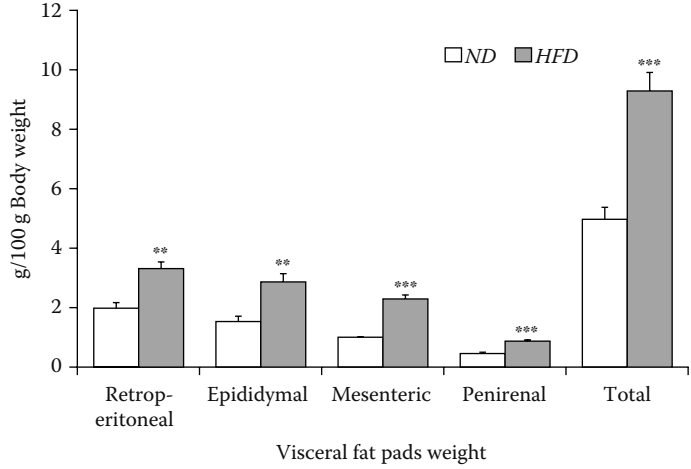

FIGURE 15.1 Visceral fat pads weights of rats fed the ND or the HFD diet. Values are means ± SEM, $n = 10$. **, *** Different from the ND group, $^{**}p < 0.001$ and $^{***}p < 0.0001$ [31].

was limited in the 20% beef tallow diet (5.6 g EFA/kg), compared to the regular AIN-76 diet containing 5% corn oil (29.0 g EFA/kg) [33] and the HFD containing 17% lard and 3% corn oil (29.6 g EFA/kg). Therefore, based on the assumption that 12 g linoleic acid and 2 g α-linolenic acid/kg were the minimal EFA requirements for rats [34,35], it was speculated that the HFD composed of 17% lard plus 3% corn oil, which was used in the current study, could attain a much greater weight gain by relieving the deficiency of essential fatty acids that was manifested in the rats that had been fed 20% beef tallow as a single source of fat. Second, the amounts of vitamin and mineral mixtures that were provided in the experimental diet were equalized per kJ for the high-fat and normal diets in the present study, while in the previous report [24], identical amounts of vitamin and mineral mixtures were used regardless of the caloric density of the diet. The percentage of increase in the body weight gain induced by the HFD formulated as shown in Table 15.1 (as compared with that induced by the ND) was approximately three to five times that observed in rats that had been fed a AIN-93G-based 20% (19% butter + 1% soybean oil) or 30% fat diet (23% shortening + 7% corn oil) [27]. These results indicate that diet-induced obesity can be achieved more successfully in rats by feeding them an HFD based on the AIN-76 composition rather than on the AIN-93 composition, and also by providing them with EFAs, vitamins, and minerals adjusted to the caloric density of the diet, for a minimum of 8 to 9 weeks.

Obesity is associated with a spectrum of nonalcoholic fatty liver disease. Losing 10% or more body weight can correct abnormal liver chemistries, decrease the liver's fat content, and improve the features of steatohepatitis [36]. The direct exposure of liver cells, through portal circulation, to high concentrations of FFAs and/or other metabolites derived from intraperitoneal adipose tissues has been

postulated to be responsible for the increased frequency of dyslipidemia, fatty liver, hyperinsulinemia, and other metabolic complications associated with abdominal obesity [37]. Feeding rats the HFD led to a significant increase in the relative weight of the liver compared with that in the ND rats. The hepatic levels of the total lipids, triglyceride, cholesterol, and free fatty acid were significantly higher in the HFD rats than in the ND rats. The HFD rats exhibited significantly elevated activities of serum aspartate transaminase and alanine transaminase compared to those for the ND rats. The hepatic activities of G6PDH (36% lower, $P < .05$) and malic enzyme (39% lower, $P < .05$) were significantly downregulated in rats given the HFD than in the ND rats (Table 15.2) [31]. The HFD-induced downregulation of hepatic malic enzymes and G6PDH activities observed in this study coincides with previous observations [38] and appears to represent a feedback regulation of lipogenesis.

Many studies have provided evidence that insulin resistance characterizes the diet-induced obesity in animal models [39–41], and this is comparable with the metabolic condition in the majority of obese humans. Rats fed the HFD acquired distinctive dyslipidemia, hyperinsulinemia, and hyperleptinemia (Table 15.2) [31], which are typically associated with human obesity. The fact that slight hyperglycemia (22% higher) evolved in spite of the marked hyperinsulinemia (213% higher) indicates a successful induction of insulin resistance in the current model of a rat with diet-induced obesity (Table 15.2) [31]. Endogenous insulin secretion is assessed best by measuring C-peptide, a 31-amino-acid peptide cleaved from the processing of proinsulin to insulin [42], which is cosecreted with insulin in a one-to-one molar ratio, but, unlike insulin, experiences little first-pass clearance by the liver. The serum C-peptide level is increased in patients with insulin resistance, whereas it is decreased in patients with insulin deficiency [42]. Leptin is a protein produced primarily in adipocytes and regulates food intake and energy balance [43–45]. The vast majority of obese humans have a high plasma leptin concentration, relatively proportionate to their fat mass [46]. Leptin enters the brain through a saturable transport system, of which activity is lower in obese individuals than in age- and sex-matched, normal-weight subjects [47]. The plasma insulin and leptin levels are generally correlated since insulin stimulates leptin synthesis and release through the regulation of glucose metabolism in the adipocytes [48,49].

15.3 HIGH-FAT DIET–INDUCED MODULATION OF SIGNALING CASCADES IN ADIPOGENESIS

15.3.1 Adipogenic Transcriptional Cascades

Adipocyte growth and differentiation are complex processes characterized by many changes in cell morphology and hormone sensitivity, and eventually lead to the phenotype of the mature adipocyte, which expresses the genes that control lipogenesis and lipolysis [50]. Knowledge of the molecular events that regulate the differentiation of preadipocytes to adipocytes is needed to better understand

the mechanisms of HFD-induced obesity and to develop strategies for controlling diet-induced obesity. Adipocyte differentiation involves a regulated set of gene-expression events, and understanding the underlying transcriptional networks is of fundamental importance. Several transcription factors act cooperatively and sequentially to trigger the terminal adipocyte differentiation program. These include members of the peroxisome proliferator-activated receptor-γ2 (PPARγ2), the CCAAT/enhancer binding proteins (C/EBPs), and sterol regulatory element-binding protein-1c (SREBP1c) family [51–53]. The C/EBP family and PPARγ are the two main groups of transcription factors involved in the hormonally induced regulation of adipogenesis [20,21]. PPARγ, a member of the nuclear-receptor superfamily, is the master regulator of adipogenesis and is also required for the maintenance of the differentiated state [54]. Forced expression of PPARγ is sufficient to induce adipocyte differentiation in fibroblasts, and no factor that promotes adipogenesis in the absence of PPARγ has been discovered so far [55]. The PPARγ2, a splicing isoform of PPARγ, is expressed selectively in the adipose tissues and promotes the differentiation and proliferation of adipocytes from fibroblasts, thus causing an increase in fat [52].

The C/EBP family, expressed at high levels in adipose tissues, is also induced during adipogenesis. The C/EBPβ is involved in the early events of cell differentiation and enhances the expression of PPARγ2, whereas the C/EBPα, in powerful synergy with the PPARγ2, promotes the terminal differentiation of preadipocytes [56]. Several C/EBP family members expressed in adipocytes, including the C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and CHOP (transcription factor homologous to CCAAT/enhancer binding protein), work as a cascade, whereby early induction of C/EBPβ and C/EBPδ leads to the induction of C/EBPα. Despite the importance of C/EBPs in adipogenesis, these transcription factors cannot function efficiently in the absence of PPARγ [57,58]. SREBP1c was identified as a proadipogenic basic helix-loop-helix (bHLH) transcription factor that induces the PPARγ expression and possibly the generation of PPARγ ligands, which are yet to be explored [59,60].

15.3.2 Extracellular Signaling in Adipogenesis

The program of adipogenesis is modeled by the sequential activation of transcription factors, which function downstream of signaling pathways that transduce the information about the appropriateness of intracellular and extracellular conditions for differentiation. Almost every important cellular signaling pathway has a positive or negative effect on adipocyte development, and some pathways exert both pro- and antiadipogenic effects depending on factors that are still poorly understood. Although many pathways influence the adipocyte differentiation *in vitro*, the importance of these pathways for adipocyte development *in vivo* has not been tested as persistently. The Wnt signaling inhibits adipocyte differentiation *in vitro* by blocking the expression of PPARγ and C/EBPα, whereas the Wnt proteins produced by preadipocytes function as a "brake" during the differentiation [61–64]. The transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), and myostatin are also known to regulate the differentiation of adipocytes [65].

Several studies have examined the effects of mitogen-activated protein kinase (MAPK) family members on the adipogenesis with conflicting results [66]. The consensus favors a role for the extracellular signal-regulated kinase 1 (ERK1) and p38 in adipocyte differentiation, with confusion stemming from the divergent effects in different phases of differentiation. ERK1, for example, is required in the proliferative phase of differentiation, whereas in the terminal differentiation phase, it leads to the phosphorylation of PPARγ, which inhibits the adipocyte differentiation [67,68].

Insulin has distinct effects on adipogenesis. In the early stages of adipogenesis, insulin functions predominantly through the insulin growth factor-1 (IGF1) receptor signaling, as preadipocytes express many more receptors for IGF1 than for insulin, although this ratio shifts as the differentiation proceeds [69]. Downstream components of the insulin/IGF1 signaling cascade are also remarkably important for adipogenesis. The loss of individual insulin receptor substrate (IRS) proteins inhibits adipogenesis, with an order of importance of IRS1 > IRS2 > IRS3 > IRS4 [70]. Moving down the insulin signaling cascade, the inhibition of the phosphatidylinositol 3-kinase (PI3K), as well as the loss of the v-akt murine thymoma viral oncogene homolog 1 (Akt1)/protein kinase B (PKB) or Akt2/PKB, represses adipogenesis [71]. Other downstream effectors of insulin action, such as the mammalian target of rapamycin (mTOR), have also been shown to be involved in adipogenesis [72].

Early studies indicated that fibroblast growth factors (FGFs) inhibit adipogenesis, but in these experiments, high concentrations of FGFs were delivered during or after the adipocyte differentiation had begun [73]. More recent studies indicate a positive role for FGFs in adipogenesis [74]. The FGF1 was identified as a substance released from the microvascular endothelial cells that had proadipogenic activity on human preadipocytes [75]. The 3T3-L1 cells secrete large amounts of FGF1, which might account for the ability of these cells to differentiate more readily than human preadipocytes, which do not express FGF1. Progression of cells through the cell cycle depends on the coordinated expression of a variety of cyclins, which are essential for the activation of cyclin-dependent kinases (CDKs) [76]. Activated CDKs phosphorylate a number of key substrates including the retinoblastoma tumor suppressor gene product (Rb) which are negative regulators of cell cycle progression. The CDKs regulate the activity of the transcription factors of the E2F family, and thereby the expression of a variety of genes necessary for the S-phase entry, including the gene for thymidine kinase [77]. The cyclin D/CDK4 and CDK6 complexes appear to be important for the progression through the early G1-phase, whereas the cyclin E/CDK2 and cyclin A/CDK2 complexes are essential for the late G1- and S-phase entry.

15.3.3 High-Fat Diet–Induced Modulation of Adipogenic Transcriptional Cascades

Many investigators agreed that the expression levels of PPARγ2 and C/EBPα genes were increased during the growth and differentiation of adipocytes in the *in vitro* system. However, animal and human studies investigating the role of HFD

on the expression of these adipogenic transcription factors in the visceral adipose tissue have led to conflicting results. Increased levels of PPARγ2 and C/EBPα gene expression were reported in the visceral adipose tissues of high-fat fed rodents compared to their normal-weight counterparts [19,31,78–81], as well as in the visceral fat tissue of adolescent girls with increased BMI [82]. By contrast, other studies showed that the expression levels of these adipogenic transcription factors were decreased in the rodent models of HFD-induced obesity [32,83,84], as well as in obese subjects [85]. Further studies need to be conducted in well-characterized animal models with HFD-induced obesity to draw a conclusion for the acting mechanism of an HFD on the visceral adipogensis. The authors observed that feeding an HFD to mice significantly upregulated the expressions of FGF1, E2F1, and C/EBPα in the epididymal adipose tissue. The immunoblot results of the whole-tissue extract showed that the HFD led to a 30% increase ($p < 0.05$) in the level of PPARγ protein in the epididymal adipose tissue compared to that in the ND mice. Furthermore, the HFD enhanced the phosphorylations of the key FGF1-signaling molecules, such as FGFR-1 and Rb, compared with those for the ND group (unpublished data).

15.4 HIGH-FAT DIET–INDUCED MODULATION OF GLUT4 TRANSLOCATION SIGNALING PATHWAYS

15.4.1 Insulin-Mediated GLUT4 Translocation Signaling Pathways: Overview

The stimulation of glucose transport by insulin is a two-step process, with the first step being GLUT4 translocation and the second being GLUT4 activation. It is widely recognized that insulin stimulates glucose uptake via the rapid recruitment of GLUT4 to the plasma membrane of skeletal muscle cells and adipocytes. Three major signaling cascades, which are involved in the insulin-stimulated GLUT4 translocation, have been recognized (Figure 15.2): the PI3K/Akt pathway; the Cbl-associated protein (CAP)/casitas b-lineage lymphoma (Cbl)/TC10 pathway; and the RAS/RAF/mitogen-activated protein kinase (MAPK) pathway [86–90]. The PI3K/Akt pathway [88,89] and the CAP/Cbl/TC10 pathway [90] have gained wide acceptance as critical components in the insulin-stimulated glucose uptake by promoting the translocation of the GLUT4 to the plasma membrane, whereas the RAS/RAF/MAPK pathway does not have an established role in insulin-stimulated glucose uptake.

Binding of insulin to its receptor leads to the autophosphorylation of tyrosine residues of the receptor and then the phosphorylation of IRS. Phosphorylated IRS activates the PI3K/Akt pathway in which the IRS1 binds and activates the enzyme PI3K after tyrosine phosphorylation. The activation of PI3K increases the serine phosphorylation of Akt, which in turn stimulates the glucose transport in the muscle and adipose tissue (Figure 15.2). The protein phosphatase 2A (PP2A) is a multimeric serine/threonine phosphatase that has been highly conserved during the evolution of eukaryotes [91]. Recent evidence indicated that the PP2A

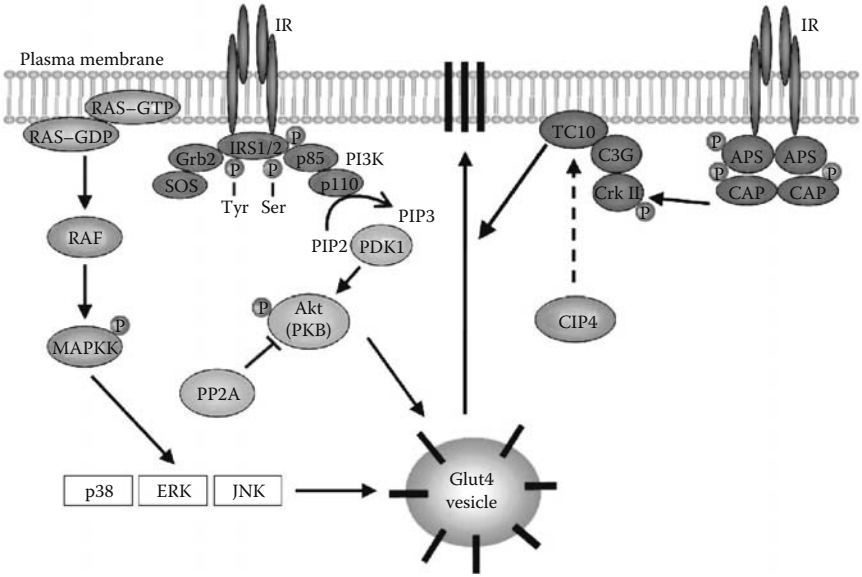

FIGURE 15.2 Major signaling pathways involved in the translocation of the GLUT4 from the cytosol to the plasma membrane in the adipose tissue.

dephosphorylates a diversity of kinases *in vitro*, including the Akt, protein kinase C (PKC), mitogen-activated protein/extracellular-regulated kinase kinase (MEKK), and MAPK [91]. The PP2A is one of the most abundant phosphatases regulating the activities of signal transduction proteins [91], and it has been known to negatively regulate the insulin signaling pathway by inhibiting the Akt activity in 3T3-L1 adipocytes [92]. A couple of recent reports cited the possible role of PP2A in the metabolic actions of insulin: okadaic acid, an inhibitor of PP2A, activated glucose transport and GLUT4 translocation *in vitro* [93], whereas PP2A expression was increased in the biopsy samples of skeletal muscle obtained from the patients with type 2 diabetes [94].

The activation of TC10, a Rho family GTPase that is highly expressed in the muscle and adipose tissues, is essential for insulin-stimulated GLUT4 translocation via the alternative insulin signaling pathway, the CAP/Cbl/TC10 pathway. TC10 is involved in the remodeling of the actin cytoskeleton, which is necessary for GLUT4 translocation [90]. The cell division cycle 42 (Cdc42)-interacting protein 4 (CIP4) appears to interact with TC10, and may have a role in the regulation of actin dynamics, and eventually, in modulating the GLUT4 translocation (Figure 15.2) [95]. CIP4 is known to be involved in the regulation of the actin cytoskeleton and membrane trafficking through the interaction with the GTP-bound Cdc42 [96]. A possible link between the CIP4 protein and insulin resistance has been raised from the *in vitro* study (3T3-L1 adipocytes), suggesting that the CIP4 protein is required for the insulin-stimulated GLUT4 translocation via its interaction with TC10 [97].

In the insulin-stimulated RAS/RAF/MAPK pathway, the phosphorylated IRS via the activated IR subsequently recruits the growth factor receptor-bound protein (Grb)-son of sevenless (SOS) proteins, then it causes the GTP–GDP exchange of RAS, which recruits the protein RAF to the membrane, where it is activated (Figure 15.2). RAF is also known as a MAP kinase kinase kinase (MAPKKK) because it phosphorylates tyrosine as well as serine and threonine residues of MAPKK, which phosphorylate MAPK on both the threonine and tyrosine residues of a tripeptide sequence (Thr-X-Tyr) near their catalytic site, thereby activating the enzyme. The p38 MAPK is an important stress kinase that is involved in inflammation, cell growth and differentiation, cell cycle, and cell death [98,99]. Available evidence from the skeletal muscle and cultured cells indicates that the p38 MAPK might also be involved in the regulation of glucose transport [100,101]. Muscle contraction, which increases GLUT4-mediated glucose transport, activates the p38 MAPK [101,102]. Glucose uptake induced by ischemic preconditioning may also be mediated by the p38 MAPK in the rat heart [103]. In addition, the p38 MAPK activates plasma membrane glucose uptake in 3T3-L1 adipocytes and L6 myotubes [86,104].

15.4.2 High-Fat Diet–Induced Modulation of GLUT4 Translocation

Although the relationship between the HFD-induced visceral adiposity and insulin resistance is compelling, its precise mechanism has not yet been fully elucidated. In most animal and cellular models of insulin resistance, insulin-stimulated GLUT4 translocation to the plasma membrane is reduced [105]. Until recently, few studies have demonstrated the relationship between an HFD and impaired insulin-stimulated GLUT4 translocation in the skeletal muscle of rodent models. The activation of several components involved in the CAP/Cbl and IRS/PI3K signaling cascades has garnered some preliminary evaluations in skeletal and cardiac muscles obtained from genetic, pharmacologically induced, or HFD-induced models of insulin resistance [106–109]. Recently, authors observed, from the cDNA microarray analysis of the epididymal adipose tissue gene expression, that PP2A and CIP4 were up- and downregulated, respectively, in rats fed the HFD [31]. It was hypothesized that the alterations of PP2A and CIP4, which are potential downstream components of the IRS/PI3K/Akt and CAP/Cbl/TC10 pathways, respectively, might be linked to impaired GLUT4 translocation in the visceral adipose tissue of rats with HFD-induced insulin resistance. The exposure of rats to the HFD for 8 weeks resulted in a significant increase in the expression of PP2A at both the transcriptional and translational levels, along with marked reductions in the levels of phosphorylated Akt (at Ser 473) and IRS1 (at Tyr 941) in the visceral adipocytes, compared to those for the ND rats (Figure 15.3) [95].

Tyrosine dephosphorylation and/or the serine phosphorylation of IRS1 has been recognized as one of the main mechanisms that lead to insulin resistance [110]. IRS proteins contain many potential serine phosphorylation sites, in addition to tyrosine phosphorylation sites [111], and the serine phosphorylation of IRS1 is

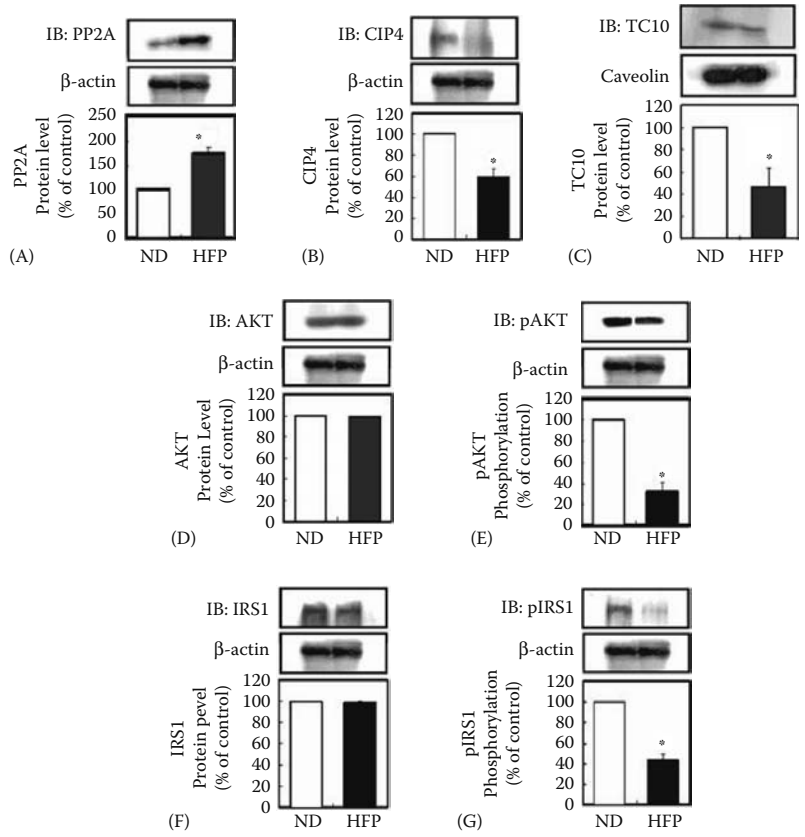

FIGURE 15.3 The HFD led to an induction of the PP2A protein in parallel with suppressed phosphorylation of IRS1 and Akt in the PI3K/Akt pathway, and led to the suppression of CIP4 and TC10 protein expression in the CAP/Cbl/TC10 pathway. Protein from whole-tissue extract (80 μg/lane) or plasma membrane proteins (80 μg/lane) were probed using the following antibodies: (A) anti-PP2A; (B) anti-CIP4; (C) anti-TC10; (D) anti-Akt; (E) anti-pAkt (Ser 473); (F) anti-IRS-1; (G) anti-pIRS-1 (Tyr 941). The blot is a representative experiment of a total of three independent experiments. Each bar represents the mean ± SEM, $*p < 0.05$. (From Jun, H. S. et al, *Obesity* (forthcoming). With permission.)

capable of regulating insulin signal transduction both positively [112] and negatively [113]. The serine 307 phosphorylation of IRS1 is particularly important in modulating the interaction between IRS1 and the insulin receptor (IR) [114], and stimulated phosphorylation of IRS1 at serine 307 was found in insulin resistance induced by a variety of agents [115]. The increased serine phosphorylation of IRS1 protein has been elucidated as a major mechanism of insulin resistance in a rodent model with HFD-induced obesity by suppressing the phosphorylation activity of IRS1 at tyrosine residue that contributes to insulin signaling [99]. Serine phosphorylation of IRS proteins by c-Jun N-terminal kinase (JNK) contributes to the

dysregulation of insulin signaling pathway in *in vivo* models of HFD-induced obesity, as interfering with the tyrosine phosphorylation of IRS1 protein, which is the starting point of PI3K/Akt pathway. Although several serine/threonine kinases that phosphorylate IRS1 have been reported [112–114], the phosphatases that act on these sites have not yet been identified. Based on recent studies using 3T3L1 adipocytes [92,116], the PP2A appears not to be involved in the dephosphorylation of the serine 307 residue of IRS1. Further work is needed to determine whether serine phosphorylated IRS1 serves as a substrate for PP2A.

Although several publications have described the relationship between Cbl activation and insulin resistance in muscles [106,109], the regulation of downstream components such as TC10 and CIP4 during the progress of HFD-induced obesity and insulin resistance has not been clearly established. Significant decreases in the mRNA and protein levels of CIP4 and TC10 were observed in the adipose tissue of rats rendered obese by the HFD (Figure 15.3) [95]. These results contradict the recent findings by Bernard *et al*. that the TC10 protein level was not altered in the skeletal muscle of rats fed the HFD [106], but correspond to the reports of Gupte and Mora that the TC10 protein level was suppressed in the adipose tissue of *ob/ob* mice [109].

15.5 HIGH-FAT DIET–INDUCED MODULATION OF INFLAMMATORY SIGNALING PATHWAYS

15.5.1 Evolutional Perspectives on the Lineage of Body Fat, Inflammation, and Insulin Resistance

During the past decade, it became clear that inflammation is a key phenomenon of obesity and type 2 diabetes [4]. In the classic literature, inflammation is described as the short-term adaptive response of the body invoked to deal with injuries, the hallmarks of which include swelling, redness, pain, and fever [117]. Although this is a crucial component of tissue repair that involves the integration of many complex signals in distinct cells and organs, the long-term consequences of prolonged inflammation are not often beneficial. This certainly seems to be the case in the metabolic diseases. A distinct form of injury response or low-grade and chronic inflammation has recently been described with a new term: *metaflammation* (metabolically triggered inflammation) [118]. This condition is principally triggered by nutrients and metabolic surplus, and engages a similar set of molecules and signaling pathways to those involved in classical inflammation.

There is a close relationship between the immune and metabolic systems that has evolutionary evidence. The functional units that regulate metabolic and immune functions in higher organisms have evolved from common ancestral structures. One such structure is the *Drosophila* fat body, which is organized as the equivalent of mammalian adipose tissue, liver, haematopoietic system, and immune systems in one efficient unit [119,120]. The adipose tissue, liver, and haematopoietic system in higher organisms have maintained their developmental heritage, which was shared in earlier organisms. Therefore, it is possible to imagine

a situation in which common or overlapping pathways regulate both metabolic and immune functions through the common key regulatory molecules and signaling systems [118]. Further evidence indicates that the adipose tissue and the liver have an architectural organization in which metabolic cells (adipocytes or hepatocytes) are in close proximity to immune cells (Kupffer cells or macrophages), and both have access to a vast network of blood vessels for soluble mediators. In this configuration, both tissues shape a suitable atmosphere for continuous and dynamic interactions between immune and metabolic responses and also establish communications with other peripheral organs such as the pancreas and skeletal muscle [118]. Ample evidence has suggested that metabolic diseases, including obesity and type 2 diabetes, are linked to the inflammatory response in our body.

15.5.2 Adipocytokines: Mediators of Insulin Resistance

Adipose tissues exist as two forms in the body: the white adipose tissue and the brown adipose tissue. Most adipose tissues in mammals are white adipose tissue and its major function is the site of energy storage. However, brown adipose tissue is found in human neonates and regulates body temperature through the nonshivering thermogenesis. In addition to adipocytes, which are the most abundant cell type in white adipose tissue, adipose tissues also contain preadipocytes, endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages, which are bone-marrow derived, and the number of these cells present in the white adipose tissues correlates directly with obesity of an individual [5].

Therefore, the adipose tissue is no longer thought to play an inert role as energy storage, but is emerging as an important organ regulating many pathological processes. Various products of adipose tissues have been characterized, and some of the soluble factors produced by this tissue are known as *adipocytokines*, the term used to describe certain cytokines that are mainly produced by adipose tissue, although they are not exclusively derived from this organ. Adiopnectin, leptin, resistin, and visfatin are adipocytokines and are considered to provide an important link between obesity, insulin resistance, and inflammatory disorders [4,121–125].

Adiponectin circulates at high concentrations (5–10 mg/ml) in human serum, compared to leptin, which circulates at a concentration of a few nanograms per milliliter. Adiponectin has a wide range of biological activities [126]. Serum levels of adiponectin are markedly decreased in individuals with obesity, insulin resistance, nonalcoholic fatty liver disease, atherosclerosis, and type 2 diabetes mellitus [127]. A major role for adiponectin in regulating insulin sensitivity has been demonstrated [128]. Adiponectin also stimulates β-oxidation in hepatocytes and downregulates the expression of SREBP1c, which is the main transcription factor regulating the expression of genes encoding mediators of lipid synthesis and GLUT4 translocation through the AMP-activated protein kinase (AMPK) activation [5]. In contrast, the TNF suppresses the transcription of adiponectin in an adipocyte cell line, which might explain the lower levels of serum adiponectin in individuals who are obese [129]. Expression of adiponectin is also regulated by

other proinflammatory mediators such interleukin 6 (IL-6), which suppresses the adiponectin transcription and translation in an adipocyte cell line [130].

Besides its well-known function for the regulation of appetite, the leptin, which is generated mostly by adipose tissue, is an important mediator of inflammtory processes [123]. Serum levels of leptin reflect the amount of energy stored in the adipose tissue and are proportional to overall adipose mass in both mice and humans [123,131]. However, unlike adiponectin, the leptin promotes the proinflammatory cytokines, such as TNF-α, IL-6, and IL-12. Mice with a mutation in the gene encoding the leptin (*ob/ob* mice) or the gene encoding the leptin receptor (*db/db* mice) have obese phenotypes and, at the same time, have various defects in cell-mediated and humoral immunity [132–134]. Resistin has also been reported in the pathogenesis of obesity-associated insulin resistance and type 2 diabetes in a mouse model [16], but such a role in human is still argued [135–137]. Visfatin has recently been identified as an adipocytokine that is secreted by adipocytes and that decreases insulin resistance [138]. This molecule binds to and activates the IR but dose not compete with insulin, since the two proteins bind at different sites on the IR [138].

15.5.3 Molecular Signaling That Links Inflammation and Insulin Resistance

Obesity-triggered IκB kinase (IKK)/nuclear factor κB (NFκB) and the JNK pathways in adipose tissues encompass adipocytes, hepatocytes, and associated macrophages [139–146]. Stimuli that have been shown to activate these IKK/NFκB and JNK pathways during the metabolic dysregulation include ligands for TNF-α [147,148]; IL-1; Toll [149,150]; advanced glycation end-product receptors (RAGE) [151,152]; intracellular stresses, including the ROS [153–155] and endoplasmic reticulum (ER) stress [156]; and various PKC isoforms (e.g., PKC-θ, PKC-βII, and PKC-δ) [157,158]. Obesity-induced IKK activation leads to NFκB tanslocation and the increased expression of numerous markers and potential mediators of inflammation, such as IL-6 and TNF-α, that can induce insulin resistance. The IR signaling is induced normally through a tyrosine kinase cascade. However, obesity-induced JNK activation promotes the phosphorylation of IRS-1 at serine sites (serine 302 and 307) that negatively regulate the normal signaling through the IR/IRS-1 axis [114,145,156,159] (Figure 15.4).

In the adipose tissue, dietary excess and obesity cause lipid accumulation in adipocytes [160,161], initiating a state of cellular stress and activation of JNK and NFκB [4,5,118]. These inflammatory signaling pathways regulate protein phosphorylation and cellular transcriptional events, thereby leading to increased adipocyte production of proinflammatory cytokines, including TNF-α, IL-6, adiponectin, leptin, resistin, chemokines (e.g., monocyte chemoattractant protein-1), and other proatherogenic mediators (e.g., plasminogen activator inhibitor-1 ([PAI1]) [4,5,118]. Endothelial adhesion molecules—such as the intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)—and the chemoattractant molecules bind integrins and chemokine receptors, respectively, on the monocyte surface to recruit them to the adipose tissues.

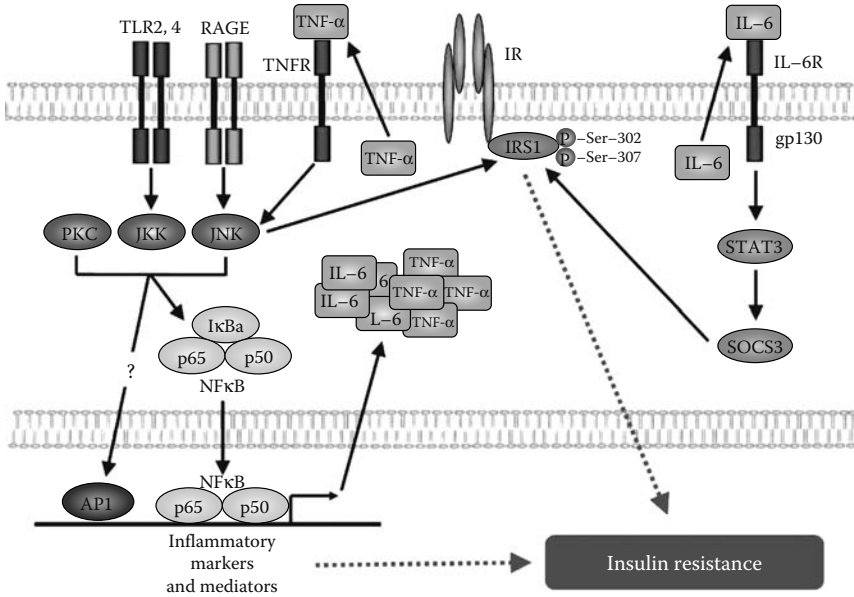

FIGURE 15.4 Molecular signaling that links inflammation and insulin resistance.

Hepatic steatosis observed in obesity is accompanied by the activation of inflammatory signaling pathways in the liver. Healthy liver contains a broad repertoire of cells that participate in the inflammatory and immune responses, including the resident hepatic macrophages (Kupffer cells), B and T cells, natural killer (NK) and natural killer T (NKT) cells, dendric cells (DCs), liver sinusoidal endothelial cells, hepatic stellate cells, and hepatocytes [146,162–165]. Proinflammatory cytokines and FFAs, produced either by hepatocytes in response to steatosis or by abdominal fat tissue, may activate Kupffer cells [146]. Numbers of regulatory NKT cells decrease in parallel with Kupffer cell activation [146,163–165]. Moreover, increasing adiposity activates the inflammatory responses in the fat tissue and liver, with associated increases in the production of cytokines and chemokines. Immune cells, including monocytes and macrophages, are recruited and/or activated, and together these cause local insulin resistance. Portal delivery of abdominal fat-derived cytokines and lipids contributes to hepatic inflammation and insulin resistance.

15.5.4 Toll-Like Receptor Signaling Pathways

Vertebrates are frequently exposed to the invasion of microbial pathogens and have evolved systems of immune defense to remove infective pathogens in the body. The mammalian immune system can be divided into the innate and acquired immunity, with the innate immunity being the first line of host defense against

pathogens. The acquired immune responses are slower processes, which are characterized by specificity and are involved in the elimination of pathogens in the late infection by T and B cells. Contrary to the large repertoire of rearranged receptors utilized by the acquired system, the innate immune system senses infectious microorganisms through a limited number of germline-encoded pattern-recognition receptors (PRRs) [149,166]. The innate immune response is not completely nonspecific, and recent studies have shown that the innate immunity is able to discriminate between host and a variety of pathogens [167].

Toll-like receptors (TLRs) are a family of PRRs that play a critical role in the innate immune system by activating proinflammatory signaling pathways in response to microorganisms [168]. The finding of the TLR family began with the identification of Toll, a receptor that is expressed by insects, including *Drosophila*, and was found to be critical for establishing dorsoventral polarity during embryogenesis [169]. Subsequent studies indicated that the Toll also has an important function in the insect innate immune response against fungal infection [170]. TLRs are the mammalian homolog of Toll and the type I integral membrane glycorprotein with extracellular leucine-rich repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R (TIR) homology domain [171,172]. Until recently, 12 members of the TLR family have been identified in mammals [149]. TLRs recognize the conserved pathogen-associated molecular patterns (PAMPs) of invading microbial pathogens, of which structures include lipids, carbohydrates, nucleic acid, and various proteins [173–175]. Stimulation of different TLRs caused distinct patterns of gene expression, which not only leads to the activation of innate immunity but also stimulates the progress of antigen-specific acquired immunity [166].

TLRs occur as dimers by the PAMPs [176]. For example, TLR1 and TLR2 heterodimerize, and the resulting dimer recognize bacterial triacylated lipopeptides, whereas TLR2, heterodimerizing with the TLR6, senses bacterial diacylated lipopeptides. Homodimerized TLRs include TLR4, a receptor for the Gram-negative bacterial product lipopolysaccharide (LPS); TLR9, a receptor for the unmethylated CpG-containing DNA motifs occurring in bacterial and viral DNA; TLR3, which senses synthetic and viral double-stranded RNA (dsRNA); and TLR5, which binds flagellin from bacteria [149,166,173,174,177]. TLR8, which binds viral single-stranded RNA (ssRNA) and synthetic imidazoquinolene compounds, heterodimerizes with TLR7 or TLR9. Ligands for TLR8 antagonize signaling by TLR7 or TLR9, while TLR9 antagonizes signaling through TLR7 [177]. These interactions, therefore, indicate that there is added complexity in the subsets of TLRs [178].

Recent evidence suggests that nonmicrobial molecules can also activate TLRs. For example, TLR4 can be activated by fibronectin, fibrinogen, heparin sulfate, and taxol [179,180]. The heat shock proteins also induced the activation of TLR2 and TLR4 [181–183], although it is still controversial if the activation of TLR4 by the heat shock protein 60 (Hsp60) may be due to the contamination with LPS [184]. Therefore, the activation of TLRs by microbial pathogens, tissue injury, and stress leads to the expression of mediators for both immune responses and

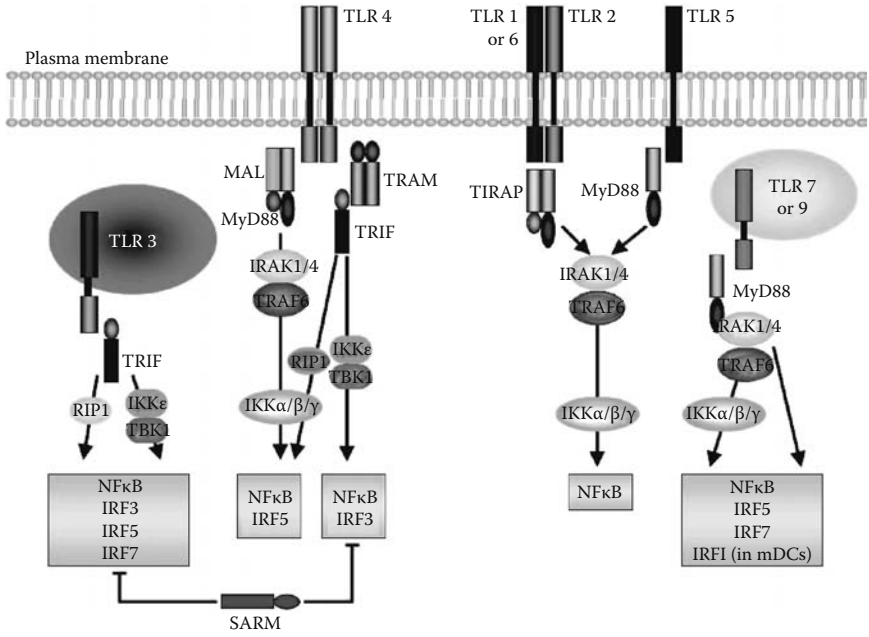

FIGURE 15.5 Overview of Toll-like receptor signaling pathways.

inflammation processes. Although the original purpose of TLR activation is to initiate and to amplify the immune responses as a host defense system against pathogens, the accompanying inflammation responses are unavoidable.

The overall TLR signaling pathways are summarized in Figure 15.5 [149,166,177,185]. TLR signaling involves a family of five adaptor molecules, which couples to downstream protein kinases that ultimately lead to the activation of transcription factors, such as NFκB, and members of the interferon (IFN)-regulatory factor (IRF) family (IRF 1, 3, and 7). The key signaling domain, which is unique to the TLR system, is the TIR domain, located in the cytosolic part of each TLR and in the adaptors [186]. The adaptors are MyD88, MyD88-adaptor-like (MAL/TIRAP), TIR-domain-containing adaptor protein inducing IFNβ (TRIF/TICAM1), TRIF-related adaptor molecules (TRAM/TICAM2), and sterile α- and armadillo-motif-containing protein (SARM) [149,166,177,185]. Different TLRs trigger signals via different combinations of adaptors. TLR4 activation recruits four major adaptors: MyD88, MAL, TRIF, and TRAM. TLR3 signaling is mostly dependent on the TRIF, whereas TLR2 requires MyD88 and MAL. The activation of downstream signaling pathways of TLR5, TLR7, and TLR9 is also dependent on MyD88. The MyD88-dependent pathway includes the association and phosphorylation of IL-1 receptor-associated kinase 4 (IRAK4) and IRAK1. The tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is recruited to the

receptor complex, resulting in the activation of IKK complex and the phosphorylation of IκB. The latter leads to the degradation of IκB and the translocation of NFκB into the nucleus, which induces the expression of target genes. The TRIF-dependent pathway is essential for the MyD88-independent pathway. The IKKε and TRAF family member-associated NFκB activator (TANK)-binding kinase 1 (TBK1) are the downstream kinases of TRIF leading to the activation of IRF3 and the expression of type I interferons (IFNs). The receptor-interacting protein 1 (RIP1) is activated through the TRIF pathway resulting in the activation of NFκB. TLR7 and TLR9 induce the expression of type I IFNs through the MyD88-IRF7 pathway [149,166,177,185].

15.5.5 High-Fat Diet–Induced Modulation of Toll-Like Receptor 4–Mediated Insulin Resistance

The two significant observations have generated a critical interest in the molecular networks for the relationship among obesity, inflammation, and insulin resistance. First, in addition to energy-regulating hormones, adipose tissue releases proinflammatory cytokines, including TNF and IL-6. TNF-α causes insulin resistance in the peripheral organs [187] via the serine phosphorylation of IRS1 [147], while IL-6 changes glucose metabolism in the peripheral organs, such as the liver and skeletal muscle [188]. Second, studies of the complex network of insulin signal transduction and observations of impaired insulin signaling in a diabetic organ proposed the mechanism for the FFA-induced insulin resistance. FFAs, whose levels are increased in obese subjects, have been considered as proximate causes of insulin resistance in several models [139,145,189–195] and have been shown to induce inflammatory signaling in fat tissues and skeletal muscle [158,190,196–201]. Shulman [202] reported that intracellular accumulation of lipid metabolites, such as fatty-acyl-CoAs, diacylglycerol (DAG), and ceramadies, activates PKC-θ, which causes serine phosphorylation of IRS-1 and insulin resistance in skeletal muscle. FFAs have been shown to induce JNK and activate TNF-α expression in 3T3-L1 adipocytes, while blockage of JNK or TNF-α can prevent FFA-induced insulin resistance, indicating that fatty acid-induced insulin resistance may be mediated in part by these proinflammatory signaling pathways [196]. Moreover, FFAs also stimulate the proinflammatory IKK/NFκB signaling in the 3T3-L1 adipocytes, leading to the expression of the cytokines, TNF-α, and IL-6 [197,198].

Recently, Shi *et al*. [203] suggested that TLR4 may be one gateway by which fatty acids impact inflammation and metabolism. In this article, the authors demonstrated that nutritional fatty acids, such as palmitate and oleate, initiated the IKK/NF-κB pathway and induced the production of TNF-α and IL-6 by macrophages, dependent on the TLR4 signaling. Cytokine induction was dependent on the dose of nutritional fatty acid. The effect was more potent with saturated fatty acids than with unsaturated fatty acids, which omega-3 polyunsaturated fatty acids did not activate the TLR4 signaling pathway. The authors also found that the expression levels of TLR4 were elevated in adipocytes from obese rodent models (*ob/ob*, *db/db*, and HFD-induced obese mice). FFAs-induced stimulation of TLR4

and IKK/NFκB also increased the local secretion of TNF-α and IL-6 that indirectly develops insulin resistance in other organs. Activated IKK, JNK, and PKC directly increase the serine phosphorylation of IRS that reduces the insulin signaling associated with IRS tyrosine phosphorylation, PI3K, pyruvate dehydrogenase kinase (PDK), and Akt, resulting in reduced glucose transport into cells (insulin resistance). The FFA-induced stimulation of the TLR4/IKK/NFκB signaling and the IL-6 and TNF-α secretions further downregulate insulin signaling via the suppressor of cytokine singling 3 (SOSC3) protein and JNK, and indirectly induce insulin resistance in the peripheral organs (Figure 15.4).

The two different groups reported the attenuation of inflammation and insulin resistance in high-fat fed TLR deficient mice. Poggi et al. [204] reported that the TLR4 deficient mice (*C3H/HeJ* mice) ate less, but exhibited a higher epididymal adipose tissue weight compared to their wild-type counterparts fed an HFD. Increased food efficiency ratio in the TLR4 deficient mice was correlated with lower expression of uncoupling protein 1 (UCP1) gene in the brown adipose tissue. Another group [205] showed that the *C3H/HeJ* mice (TLR4 deficient strain) were protected against the development of diet-induced obesity. The *C3H/HeJ* mice exhibited decreased adiposity, increased oxygen consumption, decreased respiratory exchange ratio, improved insulin sensitivity, and enhanced insulin-signaling capacity in their adipose tissue, muscle, and liver, compared with those for the control mice during the high-fat feeding. Moreover, the control mice fed an HFD exhibited an increase in IKK complex and JNK activity in these tissues, whereas the activities of these signaling molecules were prevented in the tissues of *C3H/HeJ* mice.

15.5.6 Dietary Fatty Acid and Toll-Like Receptor 2–Mediated Insulin Resistance

The TLR2 and TLR4 not only recognize LPS, peptidoglycan, and lipopeptide from microorganisms, but also interact with a large number of other lipid-containing molecules (e.g., oxidized low density lipopeptide [LDL]), as well as endogenous proteins (e.g., Hsp60) [206]. The critical component of LPS involved in the activation of TLRs is the lipid A subunit, which is composed almost entirely of long-chain fatty acid. The specific fatty acid composition, such as triacyl or diacyl, appears to determine which TLR (TLR2 or TLR4) is activated [207]. Recently, three different groups have demonstrated that the activation of the TLR2 signaling pathway is related to the development of insulin resistance in adipocytes or myotubes [208–210].

Senn [208] examined the role of TLR2 in the palmitate-induced insulin resistance in the C2C12 myotubes. Treatment with palmitate rapidly stimulated the association of MyD88 with the TLR2, and activated the stress-related kinases, such as p38, JNK, and PKC, in the C2C12 cells. The activation of kinases induced the degradation of IκBα, and increased the nuclear translocation of NFκB and its DNA binding. The activation of these signaling pathways by palmitate was sensitive and temporally controlled and occurred within the upper physiological concentration

range of saturated fatty acid, suggesting a receptor-mediated event. Palmitate inhibited the insulin signaling in the C2C12 cells beginning 1–2 h after the exposure and reached a maximum at 12–16 h. An antagonist TLR2 antibody led to a 50%–60% decrease in the palmitate-induced IL-6 production and partially restored the insulin signal transduction, whereas an isotype-matched control antibody had no effect. Murakami et al. reported that high-fat intake increased the expression of TLR2, in addition to the TNF-α in the visceral adipose tissue [209]. Flow cytometry analysis showed that the presence of adipocytes coexpressing TLR2 and TNFα (TLR2/TNFα-adipocytes), and the number of TLR2/TNFα-adipocytes in the visceral fat tissues were increased by high-fat intake compared to that in subcutaneous fat tissues. FFAs induced TNFα expression in the 3T3-L1 adipocytes through the TLR2 signals. Taken together, the authors suggested that TLR2/TNFα possibly caused the induction of TNFα expression in the visceral fat tissues, being associated with the development of high-fat-induced insulin resistance [209]. Similarly, Creely et al. [210] observed that the expressions of TLR2, MyD88, TRAF6, and NFκB protein were increased in the human abdominal subcutaneous adipocytes obtained from the type 2 diabetes patients. The circulating LPS level was 76% higher in the type 2 diabetes subjects compared with matched controls.

Most of researchers indicate that the TLR is an attractive solution to the century-old question concerning the link between obesity, inflammation, and insulin resistance, and their discoveries will lead to the uncovering of new therapeutic targets to fight this old disorder. Recent studies have shown that the polymorphisms of the TLR2 gene are correlated to populations at a higher risk of the development of insulin resistance and type 2 diabetes [211,212]. A different frequency of polymorphisms in the TLR2 gene and the proinflammatory signaling studies of TLR2 in insulin resistance [208–210] strongly suggest that TLR2 is associated with the development of insulin resistance and type 2 diabetes, and that TLR2 provides an excellent therapeutic target for the treatment of obesity and insulin resistance.

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16 Molecular Effect of Complex Food Matrices on Endothelial Functions—A Complex Issue to Study: The Case of Wine

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CONTENTS

16.1 INTRODUCTION

Diet is one of the most important lifestyle factors affecting the incidence and severity of several diseases, including cardiovascular disease [1]. Multiple risk factors for atherosclerosis and cardiovascular disease include disordered lipid profiles, homocysteine, smoking, elevated blood sugar, C-reactive protein, hypertension, and genetic predisposition [2–4]. Many of them act in a coordinated way,

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through multiple inflammatory pathways [5], and can act synergistically to increase relative risk.

Risk factors act on three cell types that coordinate their action to influence cardiovascular dynamics function and structure. These cells are endothelial cells (EC), smooth muscle cells (SMC), and immune cells, including monocytes/macrophages and T lymphocytes.

EC line the vascular lumen and act as selective filters regulating the intra- and transcellular flow of nutrients and hormones. In the course of the inflammatory response, EC express chemotactic molecules for the recruiting inflammatory cells and receptors that modulate the interaction with immune cells. EC also contribute to the control of blood clotting and the vascular tone [6]. SMC maintain vascular tone and structure, and are therefore one of the major determinants in blood flow and in vascular dysfunction leading to occlusion and infarction [7]. Their activity and thickness is determined by an entangled interaction with EC-released factors acting on endothelium and SMC as a physiological response to different stimuli, including chemical and biological insult. The disruption of the coordinated activities of these cells generates a "proinflammatory" environment predisposing to the formation of atheroma. In fact, inflammation is one of the most common condition disrupting the activities of these cells.

Diet mostly affects atherogenesis by modulating, at cellular level, proinflammatory processes that initiate and maintain endothelial dysfunction, plaque formation, and, eventually, plaque rupture.

16.2 ENDOTHELIAL CELL FUNCTION

The endothelium is a continuous monolayer formed by cells linked to one another by different types of adhesive structures or cell-to-cell junctions. Adhesion junctions are formed by vascular endothelial (VE)-cadherin present on the surface of endothelial cells and anchored to catenins. Catenins are intracellular cytoplasmic proteins, connected to the actin-based microfilament system. Endothelial cells are the main regulator of vascular homeostasis interacting with circulating cells and smooth muscle cells present in the vascular wall. They make up a selective barrier for the transport of small and large molecules between blood and tissue [6].

Healthy endothelium has both anticoagulant and antithrombotic activities regulating blood coagulation and platelet function. Under basal conditions, endothelial cells produce a variety of vasoactive substances, such as prostacyclin and nitric oxide (NO) that inhibit platelet aggregation and promote vasodilation. In the quiescent state, cells maintain blood fluidity by promoting the activity of different anticoagulant factors. Among them, of particular importance is the protein C. This factor inactivates factors VIIIa and Va, two downstream cofactors essential for blood coagulation. Moreover, endothelium synthesizes inhibitors of tissue factor (TF) pathways [8]. TF, the receptor for factor VII and activated factor VII (FVIIa), is the major initiator of blood coagulation and induces thrombin generation leading to fibrin formation and platelet activation. TF also plays a major role in cell migration and angiogenesis. TF activity is downregulated by tissue factor pathway inhibitor

(TFPI), a multivalent Kunitz-type serine protease inhibitor mainly expressed by endothelial cells, which forms a neutralizing complex with TF, FVIIa, and activated factor X. In physiological conditions, TF is absent from vascular cells that come into contact with flowing blood and is present as an inactive pool in fibroblasts and SMC. Endothelium also participates to fibrinolysis by releasing tissue-type plasminogen activator (t-PA) that converts plasminogen into plasmin, in turn acting on thrombus by digesting fibrin network. The inhibitor of t-PA, plasminogen activator inhibitor-1 (PAI-1), is also secreted by endothelial cells that take control of proteolytic activity within the endothelial lumen [9].

16.3 ENDOTHELIAL CELL DYSFUNCTION

Endothelial dysfunction is a generic term referring to a state of activation of endothelial cells characterized by the activation of proinflammatory, proliferative, and procoagulatory cellular responses that in turn generate a specific environment leading to an impairment of endothelium-dependent vasodilation and eventually to atherosclerosis.

On the basis of this relationship, endothelial function may reflect the predisposition of an individual to develop atherosclerotic disease. Many of the risk factors related to atherosclerosis and cardiovascular disease were also associated with endothelial dysfunction. NO is believed to be the main component responsible for endothelium-dependent vasorelaxation and therefore for endothelial function integrity. Hypercolesterolemia, hypertension, diabetes, and smoking are associated with the overproduction of reactive oxygen species or increased oxidative stress. One of the hypothesis that correlates oxidative stress to endothelial dysfunction considers that the reaction of NO with reactive oxygen species reduces vascular NO bioavailability promoting an impairment of endothelium-dependent vasorelaxation [10,11]. Reduced NO bioavailability due to oxidative stress seems to be the common molecular feature comprising stable atherosclerotic narrowing lesions [12]. Free radicals generation is one of the typical characteristics of the inflammatory environment and is likely to play a part in the mechanisms finally leading to plaque generation and activation. Hence, increased oxidative stress has been considered pivotal in the pathogenesis of endothelial dysfunction and progression of vascular diseases.

Endothelial cells are metabolically very active and release chemical mediators acting in a manner similar to that of paracrine or endocrine gland cells, and generate a number of active signaling molecules as a response to injury or toxic chemicals or pro-oxidant stimuli [6]. Inflammation is a localized fundamental protective response aimed to life preserving that needs to be tightly regulated to avoid inappropriate, excessive, or chronic inflammation possibly leading to a pathological situation. Adhesion of circulating leukocytes to the endothelium is one of the earliest steps in atherogenesis. The entry of inflammatory cells into the arterial wall depends on the interaction between adhesion molecules on the surface of endothelial cells and their counterligands on leukocytes. Several lines of evidence have implicated oxidized low density lipoprotein (Ox-LDL) in the development and

progression of atherosclerosis [13]. Ox-LDL is undoubtedly present in atherosclerotic lesions [14], where it exhibits a variety of biological features: including participation in foam cell formation [15], induction of the expression and release of different cytokines, growth factors and chemotactic protein from endothelial cells, promoting in turn further inflammatory response [16]. Macrophages respond by attempting to remove oxidized LDL by binding and engulfing modified LDL. This activity results in a foam cell. Foam cells are the major components forming the fatty streak, the first identifiable characteristic lesion of advanced atherosclerosis. At the same time, increased endothelial wall adhesiveness also alters its permeability, making easier leukocyte, macrophage, and LDL passage through the wall, and transfer to the intima space [17]. The inflammatory response triggers the multiplication and migration of smooth muscle cells, which accumulate in the plaque, and inflammatory cells eventually produce a degenerative lesion inside the artery wall [18]. Once this lesion is covered by a fibrous cap that protects the area, the artery dilates to accommodate this lesion making the arterial lumen narrower. The loss of a finely tuned control of endothelial barrier function can lead to extracellular oedema. When exposed to an inflammatory stimulus, endothelial cells also express and release tissue factor, which is not expressed in homeostatic conditions, activating coagulation pathways through the activity of thrombin [19]. This protease converts circulating fibrinogen to fibrin monomer, which polymerizes to form fibrin, the fibrous matrix of blood clot. Once coagulation has been initiated, endothelial cells promote platelets and thrombi activation [18]. A simplified sketch describing the endothelial environment in the early stages of atheroma generation is provided in Figure 16.1.

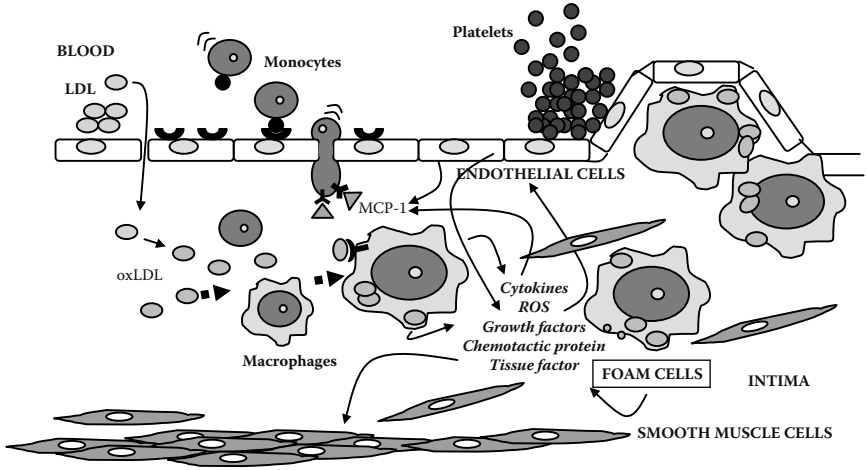

FIGURE 16.1 A simplified sketch of the early and later stages of atheroma formation illustrating the components of pathogenic process considered in the text. MCP-1, monocytes chemoattractant protein-1; ROS, reactive oxygen species.

The increased risk of chronic disease development, such as cardiovascular disease, is the result of the interplay between human genetic variation, environmental factors, and lifestyle, particularly diet and physical activity.

Diet is indeed one of the major risk determinants in vascular disease and also an important component of disease prevention and treatment by maintaining a "healthy endothelium" and by countering the process of atherosclerosis acting through specialized mechanisms in which inflammation is directly targeted [20].

16.4 DIETARY FACTORS PLAY AN IMPORTANT ROLE IN THE RISK OF DEGENERATIVE DISEASES

Three dietary traits have been reported to be significantly able to beneficially affect vascular and heart function. Namely:

- Replacing saturated fats, and *trans* fat with more monounsaturated (MUFA) and polyunsaturated fats (PUFA), such as those found in olive, canola, and soybean oils, as well as in fatty fish, avocados, nuts, and seeds [21]
- Eating more omega-3 fatty acids in order to reach the ideal ratio of omega-6:omega-3 between 3:1 and 5:1 [22]
- Regularly eating more fruits, vegetables, nuts, and whole grains [23]

There are several reasons why these specific actions may improve heart health. Replacing saturated fat with PUFA and MUFA has been shown to improve blood levels of total and LDL cholesterol [24]. In addition, evidence is accumulating that omega-3 fatty acids improve artery function and blood flow favorably altering the eicosanoid profile and modulating inducile nitric oxide synthase activity [25,26]. A number of hypotheses addressing the mechanisms underlying the role of specific constituents of fruits and vegetables have been proposed, including the antioxidant hypothesis as a result of vitamin C and E content [27] and the homocysteine hypothesis; the latter suggesting that folate and vitamin B6 largely present in these food items can modify homocysteine blood levels acting in a protective fashion [28].

The interest in the role of dietary fats in the development of heart disease has led to a considerable amount of research in the past three decades. The Mediterranean diet, rich in olive oil, is associated with significantly lower mortality from cardiovascular disease than in countries characterized by different dietary patterns. Available data suggested that MUFA fatty acid of olive oil plays an important role in the prevention of cardiovascular disease through the reduction of LDL oxidation, changes in lipid ratios, and reduction of macrophage uptake of LDL cholesterol [29,30]. However, it is possible that the natural predisposition of researchers to attempt to simplify the problem in order to build up and pursue hypotheses to be easily tested leads to the underestimation of the exposure to complex food matrices in favor of single diet components. Diet–health interaction is obviously very complex, food items most probably act through multiple pathways, and the isolation of the specific role of a single component is essentially impossible. A study on the effect of feeding with different dietary oils can

provide a paradigm of this complexity. Patients with mild-to-moderate hypertension were supplemented with extra virgin olive oil and the effect of this diet on blood pressure was compared with a diet enriched with sunflower oil, which provides a higher proportion of PUFA. All diets were controlled and only differed in the type of oil supplemented. At the end of the trial, patients receiving olive oil had a lower blood pressure in comparison to those supplemented with sunflower oil. The authors could not explain the mechanism of the observed effect of diet on blood pressure on the basis of a simple modification of serum lipid profile, since this parameter was similar in the two dietary groups. The authors therefore concluded that minor components, such as polyphenols, found in olive oil could be responsible for this effect [29]. This example suggests that the isolation of a single "active component" within a complex dietary profile could be misleading. Polyphenols contained in high concentration in fruits and vegetables have been initially considered antinutrients because of the adverse effect of tannins, one type of polyphenol. Tannins can bind proteins, carbohydrates, fats, and minerals, making them unavailable to absorption. More recently, flavonoids and, in general, polyphenols received an unprecedented interest in the field of human nutrition due to their antioxidant capacity and to a very large spectrum of other biological activities.

16.5 IMPROVEMENT OF ENDOTHELIAL FUNCTION BY POLYPHENOLS: THE CASE OF WINE

Polyphenols are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolates, and beverages (e.g., tea, coffee, or wine), and therefore are the most abundant "minor components" in the diet. Experimental studies on animals or cultured human cell lines support a role of polyphenols in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, or osteoporosis. The two main classes of polyphenols are flavonoids and phenolic acids. Flavonoids are further classified into several classes: flavones, flavonols, flavanones, isoflavones, proanthocyanidins, and anthocyanins [31]. Owing to a strong antioxidant capacity, they can increase the availability of NO and protect cell constituents against oxidative damage, therefore limiting the risk of a number of degenerative diseases associated with oxidative stress, preventing endothelial dysfunction associated with NO reduced availability [12]. In fact, the phenolic groups in polyphenols can accept an electron to form relatively stable phenoxyl radicals, blocking oxidation reaction chains in cellular components [32].

Red wine is considered one of the major sources of polyphenols and is therefore suggested as a "healthy food" by a large number of medical associations, at least in specific groups of populations at risk of cardiovascular disease [33]. Several reports indicated that light-to-moderate wine consumption reduces the risk of mortality by cardiovascular disease [34]. The protective effects associated with polyphenols might be attributed to their ability to retard the progression of early atherosclerotic lesions to advanced plaque. This hypothesis is corroborated by experiments on animal models addressing the efficacy of polyphenols in preventing the progression

of atherosclerosis. Several studies have demonstrated that the consumption of polyphenols reduces the development of atheromatous lesions. Supplementation of dealcoholized wine, catechins, or quercetin reduced the size of these lesions in apoE-deficient mice. These effects are associated with reduced LDL uptake by macrophages, lower oxidation of isolated LDL, and decreased susceptibility of LDL to aggregation [35,36]. Isolated polyphenols administered to rats produced a progressive decrease of blood pressure in normal and hypertensive rats [37–39]. Moreover, red wine polyphenols have been shown to inhibit platelet aggregation *in vivo* and *in vitro* [40] thereby preventing thrombosis. Short-term administration of red wine polyphenolic compounds has been reported to induce a decrease of blood pressure through the NO-dependent pathway [41]. The antioxidant capacity of polyphenols constitutes the most popular hypothesis on their beneficial effects; however, polyphenols have been shown, in experiments *in vitro*, to possess multiple biological activities independently from their antioxidant activity, such as inhibition of platelet aggregation and vasorelaxation [42,43], and the ability to modulate the adhesion process [44,45] and to promote fibrinolysis [46]. Diverse mechanisms have been proposed to explain the biological activity of polyphenols, including the capacity to bind protein and eventually affect enzyme activity by either competitive or allosteric interactions [47,48], regulation of signal transduction, modulation of redox-sensitive transcription factors (including Nrf2, NF-κB and AP-1) [49,50], glutathione biosynthesis [51], and, in general, gene expression [52–54].

The majority of available *in vitro* studies addressing the understanding of polyphenol mechanisms of action [44,45] have considered concentrations in the 10–100 μM range, which is largely too high to be achieved in circulation in physiological conditions. Indeed, at least in theory, these concentrations could be observed in specific tissues such as skin, where high amounts of polyphenols can be topically applied, or in the gastrointestinal tract after a meal [55]. Moreover, chemical and structural modifications due to gastrointestinal absorption and metabolization have usually not been taken into account. The comprehensive understanding of the protective mechanisms exerted by polyphenols is hindered by the lack of complete knowledge of their bioavailability. Information about absorption, distribution, metabolism, and excretion of individual flavonoids is scarce. It is known that polyphenols are extensively metabolized during first-pass metabolism and their structure significantly modified, making the molecular forms reaching the peripheral circulation and tissues quite different, in general, from those originally present in foods. An essential initial step is the hydrolysis catalized by bacterial β-glucosidases in the small intestine [56]. Once absorbed, the aglycone undergoes the phase II metabolization by the drug metabolizing enzymes. With very few exceptions, the overall result of this extensive metabolism is that the predominant forms in plasma are sulfates and glucuronide or methyl conjugates [31,57]. Conjugates differ in size, polarity, and ionic form from their parent molecule. Consequently, their physiological effect is likely to be different from that of native compounds. In addition, there are different sites of possible conjugation and not all the possible existing metabolites have been identified. For example, plasma samples from volunteers receiving quercetin orally contained 12 distinct

conjugated forms of quercetin which were not present in the original food [58]. According to these considerations, a really major issue still open for the understanding of the molecular mechanism underlying the effect of red wine on human health is in the effects of metabolism on the biological activities of polyphenols. Different studies address the effect of biotransformed polyphenols on endothelial response to proatherogenic stimuli [59]. However, it is important to note that no studies are available at present, addressing the effects of wine metabolites in the form circulating into the body, once ingested, absorbed, and distributed to target tissues and organs. Even though a few studies have addressed the effect of biotransformed polyphenols on endothelial response to proatherogenic stimuli [59], the majority of available *in vitro* studies have been designed and performed by adding wine (or other food items) "as they are in the food" to experimental cultured cell models. Alternatively, single, purified phenolic compounds, either as glycone or in their aglycone form, have been added to the cultured cells. These approaches are obviously somehow "naïve" and unable to take into account both the extensive metabolism of polyphenols during gastrointestinal absorption and the possible interaction of different molecules, therefore excluding the assessment of possible synergic/cooperative activity within the same food item. These experimental weaknesses can only provide a very nebulous picture. As an obvious consequence of their complex metabolism and poor bioavailability, the direct transfer of *in vitro* observations to *in vivo* conclusion must be cautious. Molecular effects of polyphenols in the form found in food, detected *in vitro*, could not necessarily be relevant, *in vivo* as suggested by our laboratory [60] and by others [59,61,62].

Overall, these considerations strongly suggest the need of a model able to mimic the complex metabolism as it occurs in humans that can be applied to cellular models for the study of the molecular mechanisms of complex food matrices undergoing significant modification during metabolism. In our lab, we have recently set up a novel model to study the effect of red wine (and possibly also other complex food matrices) on molecular aspects of cell functions according to a more physiological approach, utilizing healthy human subjects as "bioreactors." According to this experimental model, subjects were fed with 5 ml/kg body weight of red wine (RW) and, at appropriate time points after drinking (40 minutes), blood was withdrawn and serum (RWS) utilized to enrich the culture medium of human primary endothelial cells (HUVEC) [60]. Figure 16.2 illustrates the experimental design of the bioreactor approach coupled to cultured endothelial cells.

Two groups of genes were arbitrarily selected on the basis of data coming from *in vitro* experiments that deal with the beneficial effects of red wine and the regulation of the expression of genes involved in the early and progressive stages of atherosclerosis. To assess the effect of RWS on endothelial cell function, the expression of the selected genes was measured at the level of transcription. Real-time polymerase chain reaction (PCR) was used to address whether RWS was able to modulate VCAM, ICAM, and MCP-1, or t-PA, PAI-1, and PAI-2 gene expression in endothelial cells as representative genes involved in cell adhesion and fibrinolysis, respectively. Gene expression was assessed in HUVEC after 12 h of incubation with RWS. In order to provide a comparison of RWS effect on HUVEC with a

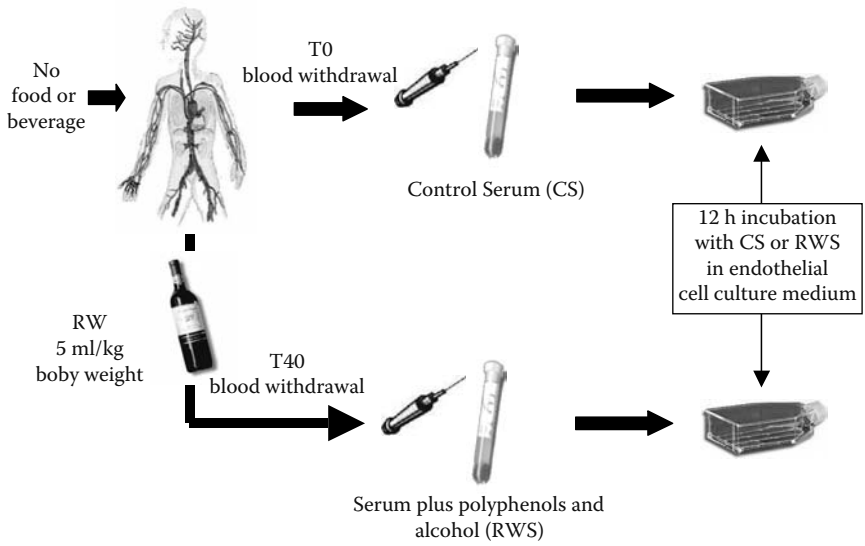

FIGURE 16.2 Schematic representation of a novel *in vivo/in vitro* model to study molecular mechanism of the components of complex food matrices.

"classical" experimental approach, the effect of a direct addition of wine (RW) to the culture medium of endothelial cells for 12 h was also considered. The volume of wine to be provided to cultured cells was calculated to reach an amount of alcohol (0.015% w/v) in the same range of that utilized in experiments conducted with RWS. RW induced a differential gene expression profile in endothelial cells that could not be simply attributed to alcohol because it was totally different to that induced by RWS, as shown in Figures 16.3 and 16.4. In fact, feeding cells with RWS was associated with a downregulation of the expression of all the genes considered, with the only exception of MCP-1, in comparison to control cells.

Data obtained by this model suggest that the profile of gene expression induced by RWS results from a combined effect of wine components and alcohol. On the other hand, the addition of red wine "as it is" to the culture medium induced a very strong and, evidently, not physiological, inflammatory and procoagulant pattern of expression in endothelial cells.

These results underscore the significant difference in biological activity of red wine components "as they are in food" in comparison to red wine ingested and metabolized during gastrointestinal absorption. Experiments *in vitro* should consider the "food–organism interaction" in its whole complexity in order to understand the mechanisms underlying the effect of diet in human health. Biotransformation, and possibly synergism and cross-activity between different components, must be taken into account. However, it appears more and more evident that it is not a single dietary factor that acts as a "magic bullet," but the overall dietary pattern has a pivotal role on cardiovascular outcome by interplaying with other risk factors, including lifestyle and individual genetic profile.

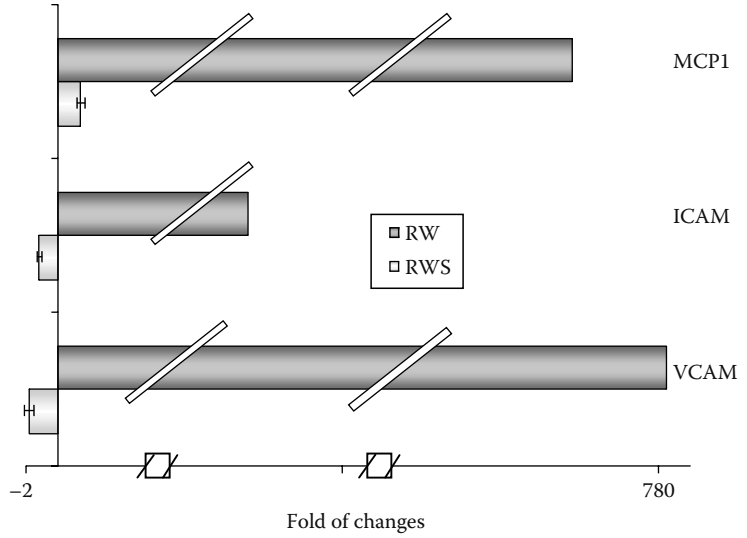

FIGURE 16.3 Effect of RWS and direct wine addition (RW) on mRNA expression in HUVEC. Culture medium was supplemented with RWS (20% final concentration) and with RW (0.02% ethanol final concentration). Cells were incubated for 12 h. At the end of the incubation time, RNA was isolated and the expression of adhesion molecule genes assessed by real-time PCR. Values are presented as mean values ± S.D. of the fold of changes of the gene expression compared to control.

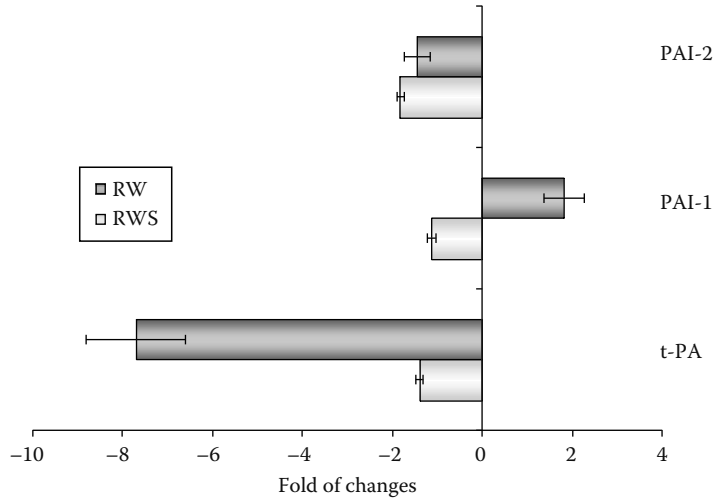

FIGURE 16.4 Effect of RWS and direct wine addition (RW) on mRNA expression in HUVEC. Culture medium was supplemented with RWS (20% final concentration) and with RW (0.02% ethanol final concentration). Cells were incubated for 12 h. At the end of the incubation time, RNA was isolated and the expression of genes involved in fibrinolytic pathway was assessed by real-time PCR. Values are presented as mean values ± S.D. of the fold of changes of the gene expression compared to control.

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17 Role of Oxidative Stress in β-Thalassemia and the Antioxidative Effect of Fermented Papaya Preparation

Eitan Fibach, Ada Goldfarb, and Johnny Amer*

CONTENTS

17.1 β-THALASSEMIA AND OXIDATIVE STRESS

β-Hemoglobinopathies (sickle cell anemia and thalassemia) are hereditary chronic hemolytic anemias caused by mutations in the β-globin gene [1]. In

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β-thalassemia various mutations cause absence (β^o) or reduced (β^+) synthesis of β-globin leading to absence or reduced levels of hemoglobin (Hb) A ($\alpha_2\beta_2$). β-Thalassemia is associated with a relative access of the α-globin chains, which form unstable tetramers that precipitate and cause cellular damage [2]. Although the basic lesion is in the globin genes, the pathology of β-thalassemia involves oxidative stress-mediated cell damage in the bone marrow (defective red cell production, ineffective erythropoiesis) due to apoptosis of early erythroid precursors and in the peripheral blood (short survival of mature red blood cells [RBC]) [3,4]. In addition to chronic anemia, these patients experience high incidence of thromboembolic complications [5] and recurrent bacterial infections [6], the etiology of which is thought to be caused, at least in part, by oxidative stress in platelets and polymorphonuclear neutrophils (PMN), respectively.

Oxidative stress involves increased generation of free radicals, especially reactive oxygen species (ROS), and a concomitant decrease in cellular antioxidants, the major one being reduced glutathione (GSH) [7]. Factors contributing to oxidative stress in β-thalassemia are Hb-instability and iron overload [4]. The latter is caused by increased dietary iron absorption and by a failure to dispose off excess iron acquired by frequent therapeutic blood transfusions [4]. Iron participates in cellular biochemical reactions leading to generation of ROS (Fenton reaction) [8,9]. Despite extensive research on the role of oxidative stress in β-thalassemia, yielding promising results, there is a need for additional basic knowledge and for the results of preclinical and clinical trials with new antioxidants.

17.2 USE OF FLOW CYTOMETRY FOR THE STUDY OF OXIDATIVE STRESS IN BLOOD CELLS

To study the role of oxidative stress in the pathology of thalassemia, we developed flow cytometric techniques to measure its various aspects and effects in blood cells [10–12].

ROS generation was measured by staining with nonpolar compound 2′,7′-dichlorofluorescein diacetate (DCF-DA), which readily diffuses across the membrane, deacetylated by intracellular esterases into a nonfluorescent polar derivative that is trapped inside the cells. When oxidized by ROS, a fluorescent product dichlorofluorescin (DCF) is emitted [13,14]; the intensity of its fluorescence is proportional to the cellular concentration of ROS. The applicability of the method was validated by the increased fluorescence following treatment with the ROS-generating agents hydrogen peroxide and butyl-hydroxyperoxide (BHP), and with the catalase inhibitor sodium azide, and the decreased fluorescence observed after treatment with the ROS scavenger N-acetylcysteine (NAC). In some experiments ROS was measured also by dihydrorhodamine 123. This dye enters the cells freely, and after oxidation by ROS to rhodamine 123, emits a bright fluorescence [15]. Comparable results were obtained using both dyes.

GSH was measured using mercury orange [10], which forms fluorescent adducts with GSH via the sulfydryl group, producing a S-glutathionyl derivative

that emits red-orange fluorescence [16]. The molecule reacts more rapidly with nonprotein thiols, such as GSH, than with thiol proteins, allowing specificity under controlled staining conditions [17]. Confirming the validity of this method, N-ethylmaleimide, which blocks total thiol groups, decreased the fluorescence of mercury orange in a dose-dependent manner [18]. To ascertain that nonprotein thiols were being stained under the staining conditions, we incubated cells with diethylmaleate, a specific nonprotein thiol-depleting agent. This is a weak electrophil of the α,β-unsaturated carbonyl group, which reacts with GSH only in the presence of glutathione transferase. Diethylmaleate markedly suppressed the mercury orange fluorescence, suggesting that GSH was the principle thiol being stained by the dye [18]. Although we have not confirmed that mercury orange is specific for GSH, the assay measures predominantly GSH, since it is the main nonprotein thiol constituent of the thiol pool [19].

Other parameters of oxidative stress measured by flow cytometry were membrane lipid peroxidation, by staining with fluor-DHPE [10]; and externalization of phosphatidyl serine (PS) moieties, a marker of membrane damage, by fluorochrome-conjugated Annexin-V.

Following staining with the specific dyes, cells were analyzed and specific subpopulations were gated based on their size and granularity (forward and side light scatter). The gated cells were identified by staining them with antibodies to lineage-specific surface antigens: glycophorin A (GPA) for RBC, CD61 for platelets, CD15 for neutrophils, CD19 for B lymphocytes, and CD3 for T lymphocytes. Thus, various oxidative stress parameters could be assigned to each cell type (Figure 17.1).

Flow cytometry offers several advantages, mostly the ability to measure simultaneously various populations of blood cells based on the results of their individual cells rather than the mean values. The latter may suffer from inaccuracy if the

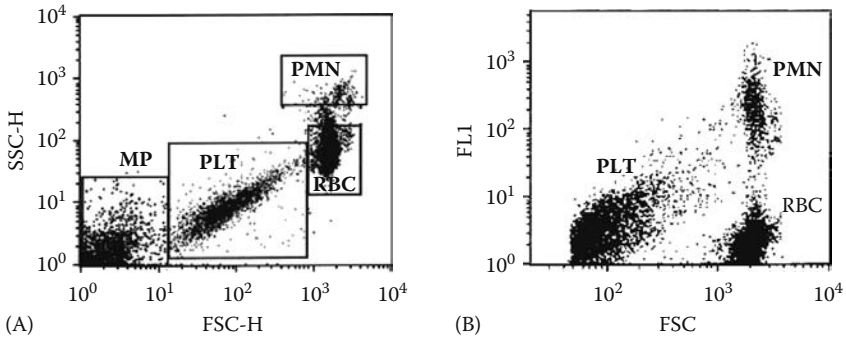

FIGURE 17.1 Flow cytometry measurement of the oxidative status in thalassemic RBC, platelets, and PMN. A dot plot of the size and granularity (FSC X SSC) of blood cell populations. Microparticles (MP), platelets (PLT), RBC, and PMN are shown in part (A). The DCF fluorescence of each cell population is depicted in part (B).

population studied is contaminated with other types of cells (e.g., RBC contaminated with PMN). Although the data are expressed in arbitrary fluorescence units rather than weight or molar concentrations, it is useful for comparative purposes.

17.3 OXIDATIVE STRESS IN THALASSEMIC ERYTHROID CELLS

RBC derived from the peripheral blood of patients with β-thalassemia showed higher DCF fluorescence at basal level and following stimulation with oxidants, such as hydrogen peroxide, compared to normal RBC [20] (Figure 17.2). These results

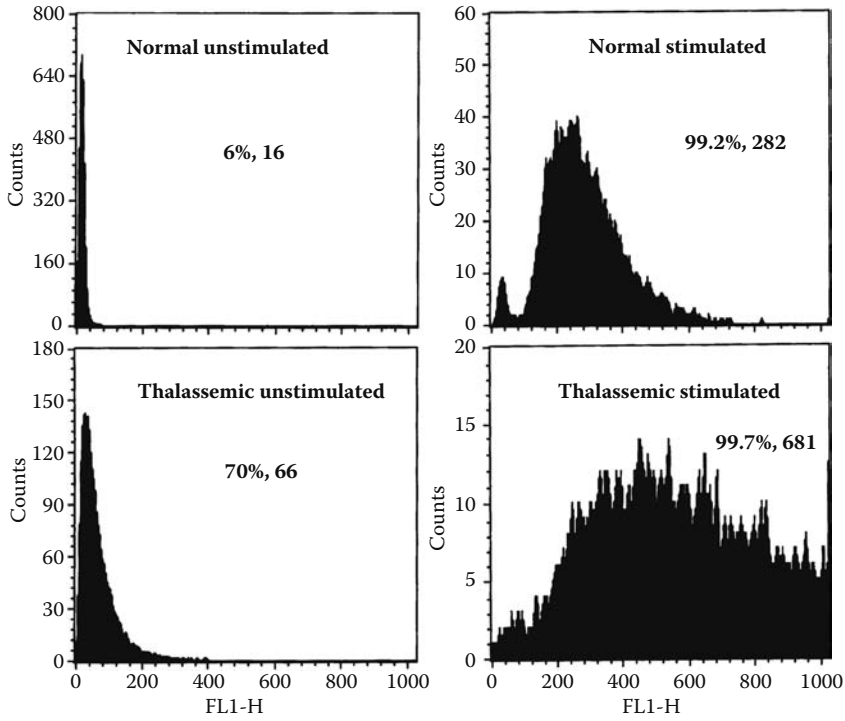

FIGURE 17.2 ROS production by normal and thalassemic RBC. Normal and thalassemic RBC were incubated with 0.4 mM DCFH-DA for 15 min, then washed and either stimulated or not stimulated by 2 mM H_2O_2 for 30 min. The histograms show that in unstimulated normal RBC, 6% were fluorescent (as compared to RBC that had not been incubated with DCFH-DA). The fluorescence intensity (mean fluorescence channel, MFC) of the entire RBC population was 16.45 (as compared to 2.7 for control RBC). In normal H_2O_2-stimulated RBC, 99.2% were fluorescence positive, with MFC of 282. Comparing unstimulated and H_2O_2-stimulated RBC obtained from different normal donors showed that 2 mM H_2O_2 increased cellular fluorescence by 10–30-fold. Unstimulated thalassemic RBC were 70% positive, with MFC of 66, while 99.7% of the stimulated RBC were positive, with MFC of 681.

indicate an increased generation of ROS in thalassemic RBC at baseline level and following oxidant stimulation. The latter is due to a decreased intracellular antioxidative capacity as a result of continued oxidative insult. In both cases, the results demonstrate that thalassemic RBC are under oxidative stress. Analysis of other parameters supported this conclusion: thalassemic RBC had decreased GSH, and increased lipid peroxidation and PS externalization compared to their normal counterparts.

To investigate whether iron overload and Hb instability induced oxidative stress, normal RBC were treated with compounds that simulate conditions that exist in β-thalassemia. These treatments included incubation with oxidants such as hydrogen peroxide and BHP; phenylhydrazine, a known Hb-denaturizing agent; and iron-containing compounds, such as ferric salts and hemin. All these treatments induced oxidative stress in normal cells, suggesting that intra- and extracellular conditions are responsible for oxidative stress in thalassemia.

17.4 OXIDATIVE STRESS IN RBC IS ASSOCIATED WITH VARIOUS CELLULAR ABNORMALITIES

17.4.1 Oxidative Stress: Effect on RBC Resistance to Valinomycin

Exposure of normal RBC to the K^+ ionophore valinomycin (val) causes loss of KCl and water, resulting in cell dehydration and increased density [21]. We recently demonstrated [22] that in β-thalassemia a substantial portion of the RBC fail to dehydrate and maintain a light density. Val-resistance (*val-res*) of RBC is related to their oxidative status:

- Treatment of normal RBC with oxidants (hydrogen peroxide, BHP, hemin, ferric ammonium citrate, or phenylhydrazine) increased the frequency of *val-res* RBC, while treatment of β-thalassemia RBC with antioxidants (e.g., NAC or vitamin C) reduced their frequency (Figure 17.3).
- *Val-res* RBC have higher oxidative statuses compared with other RBC of the same sample.
- Thalassemic *val-res* RBC are at elevated oxidative statuses compared with that of normal *val-res* RBC.

Taken together, these findings strongly suggest that val-resistance represents damage to RBC by oxidative stress, probably by affecting their ion flux across the membrane. The exact mechanism involved in this phenomenon and its physiological or pathological significance are a subject of further research.

17.4.2 Oxidative Stress: Effect on Hemolysis and Phagocytosis

In vivo oxidative stress in RBC results in intra- and extravascular hemolysis. We demonstrated the increased tendency of thalassemic RBC to undergo hemolysis *in vitro* by incubating thalassemic and normal heparinized blood overnight at 37°C. Oxidants enhanced hemolysis, while antioxidants, such as NAC and vitamin C,

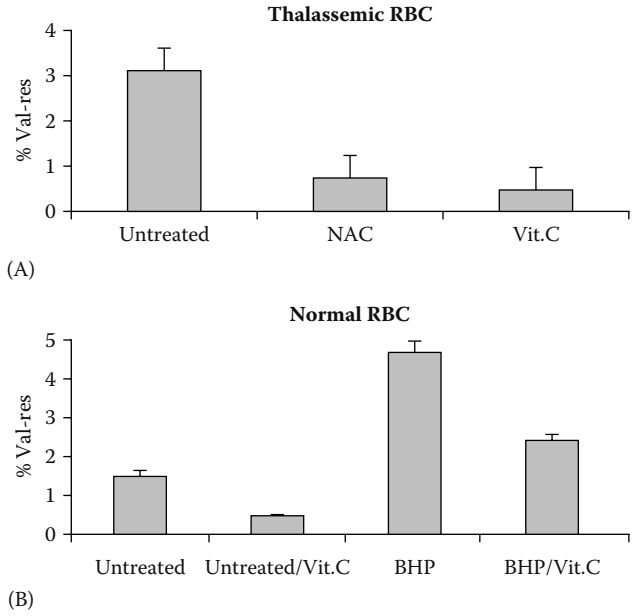

FIGURE 17.3 The effect of oxidants/antioxidants on the frequency of valinomycin-resistant (*val-res*) RBC. (A) RBC obtained from thalassemic patients were washed with PBS, diluted to 5% hematocrit, and treated for 45 min at 37°C with the antioxidants NAC and vitamin C, both at 1 mM. (B) Normal RBC were washed and diluted as in (A) and pretreated with BHP (1.5 mM) for 45 min and then treated with vitamin C (1 mM) for another 45 min. Following treatment, RBC were washed and suspended in 15K buffer, treated with valinomycin and separated on Larex as previously described [22]. RBC in the intermediate layer (≤1.091 g/ml) were collected, counted, and the percentage of *val-res* RBC out of the total (unfractionated) RBC calculated. The results are expressed as the mean ± SD of four experiments, each with cells derived from a different donor. The results in (A) indicated that treatment of thalassemic RBC with an oxidant decreased the percent of *val-res*. The results in (B) indicated that treatment of normal RBC with an oxidant increased the percent of *val-res* RBC to levels present in thalassemia. Subsequent treatment with an antioxidant decreased *val-res* RBC to almost normal levels.

reduced it. In thalassemia, hemolysis occurs mainly extravascularly, for example, in the spleen and liver, and is executed by macrophages of the reticuloendothelial system that harbor a specific PS receptor by which they phagocyte RBC. To study this process, normal RBC were treated with BHP. Figure 17.4 shows that significant fractions of these RBC carry external PS. When cocultured with macrophages derived from peripheral blood monocytes, RBC were observed first to adhere to macrophages, then to be engulfed by them and 24 h later the majority of the RBC were phagocytosed, and showed signs of intracellular lysis (Figure 17.5). These results suggest that a similar sequence of events occurs in thalassemia and that extracellular hemolysis due to phagocytosis could be mediated in part by oxidative stress.

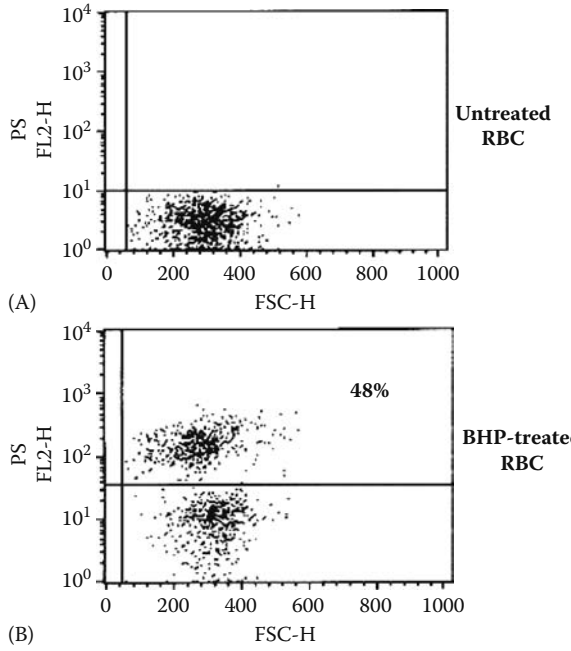

FIGURE 17.4 The effect of oxidants on phosphatidylserine (PS) externalization by RBC. Normal RBC were (A) untreated or (B) treated for 30 min with 1 mM butylhydroperoxide (BHP) and then analyzed for PS. The results show a flow cytometry dot plot, indicating that 48% of the RBC were induced by the treatment to externalized PS.

17.4.3 Oxidative Stress and Ineffective Erythropoiesis

To study the effect of oxidative stress on developing erythroid precursors we applied a two-phase liquid culture protocol for growing normal and pathological erythroid cells [23]. Peripheral blood mononuclear cells are first cultured for 7 days

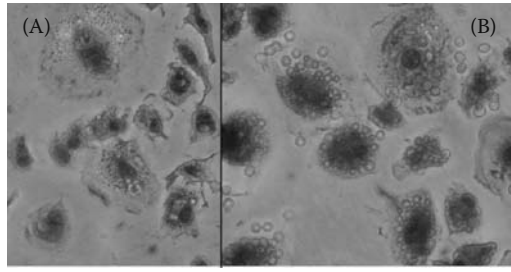

FIGURE 17.5 RBC phagocytosis by macrophages. Normal RBC were (A) untreated or (B) treated for 30 min with 1 mM butylhydroperoxide (BHP), washed, and incubated overnight with autologous macrophages. After 24 h, free RBC were washed away. BHP treated, but not untreated, RBC are readily seen within macrophages.

in the presence of various cytokines, not including erythropoietin. During this phase, early erythroid committed progenitors (BFUe) proliferate and differentiate to mature erythroid progenitors (CFUe). Nonadherent cells are then harvested, washed, and recultured in the presence of erythropoietin. The latter cells continue to proliferate and mature into erythroid precursors, first to proerythroblasts, and, after 12 days, to Hb-containing orthochromatic normoblasts.

Analysis of the development of erythroid precursors in the second phase of the cultures showed a time-dependent decrease in ROS generation. Since cultures of cells obtained from different donors developed at different rates, in order to compare ROS generation at various stages of maturation, the cells were double stained for ROS and GPA. The intensity of GPA increased with maturation. When values of ROS were plotted versus GPA intensity (i.e., cell maturity), a reverse relationship was found, indicating that ROS decrease as cells mature. ROS could be modulated by iron levels in both normal and thalassemic erythroid cultures; adding iron-saturated transferrin increased ROS, whereas adding iron chelator (L1) decreased it. Hemin (added as heme chloride or heme arginate) also increased ROS. Comparing β-thalassemic to normal erythroid precursors under culture conditions of equal iron concentrations indicated higher ROS in thalassemic cells, especially at late stages of maturation (Figure 17.6). These results suggest that increased ROS generation by thalassemic cells can be due to environmental factors, such as iron overload, as well as internal factors, such as Hb instability and release of heme. Oxidative stress in thalassemic developing erythroid precursors was associated with an increased apoptosis as manifested externalization of PS, suggesting that oxidative stress is responsible for the ineffective erythropoiesis.

17.4.4 Oxidative Stress in Thalassemic Platelets

Thromboembolic complications are an important cause of morbidity and mortality in β-thalassemia [5]. As platelets play a key role in homeostasis and thrombosis, many studies examined whether platelet function is altered in this disease. Chronic platelet activation in thalassemia was demonstrated by the presence of an increased fraction of platelets carrying the activation markers CD62P (P-selectin) and CD63 [24,25] and PS externalization [26]. In addition, morphological changes in platelets, increased spontaneous whole-blood platelet aggregation and decreased platelet life span, as well as elevated plasma platelet factor 3 and augmented urinary excretion of T_xA_2 metabolites were all reported in thalassemia [5,27].

ROS profoundly affect platelet function and promote platelet activation [28,29]. Furthermore, many studies have shown that the platelets produce low levels of ROS, which may be increased by various platelet activators such as thrombin [30,31].

We showed [11] that platelets from β-thalassemic patients contain higher ROS and lower GSH levels than do platelets from normal donors, indicating a state of oxidative stress. In the absence of any known inherent abnormality in thalassemia platelets, this may be attributed to oxidative insults from extraplatelet sources. Thus, exposure of platelets to oxidants (such as hydrogen peroxide and BHP) or to the platelet activators thrombin, calcium ionophore, or phorbol myristate

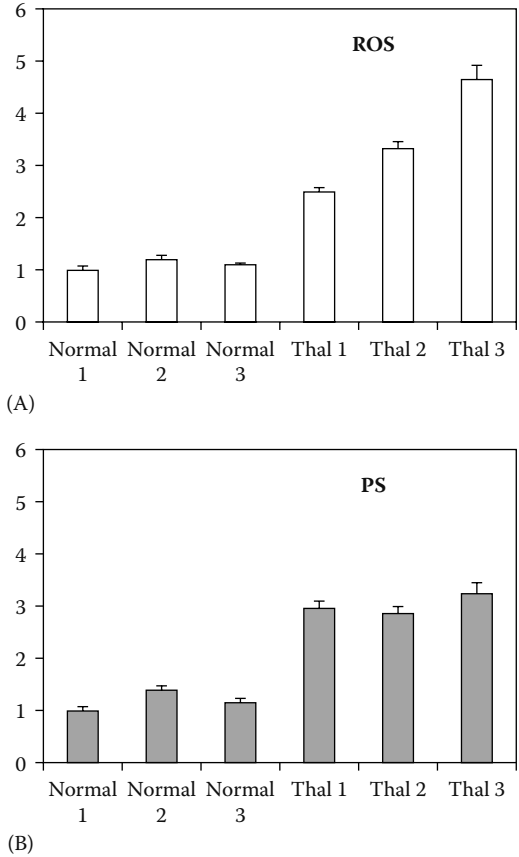

FIGURE 17.6 ROS and phosphatidylserine (PS) generation by cultured normal and thalassemic erythroid precursors. Normal and thalassemic cells were cultured according to the two-phase liquid culture protocol [23]. On day 10 of phase II, non-adherent cells were harvested, stained for (A) glycophorin A and ROS or (B) glycophorin A and PS. The results show the ratio ROS/GPA and PS/GPA. The results indicate higher ROS and PS in thalassemic cells versus normal cells.

acetate (PMA) stimulated oxidative stress. This was also increased by plasma of thalassemia patients, and decreased following treatment of the plasma with the iron-chelator Desferoxamin. Iron and hemin, the levels of which are augmented in plasma of thalassemia patients [29], stimulated the platelets' oxidative stress. The oxidative status of the platelets was also affected by RBC; it was higher in normal platelets when incubated with thalassemic RBC than when incubated with normal RBC. Normal RBC stimulated with hydrogen peroxide had a greater effect on platelets than did unstimulated RBC. The platelets' oxidative stress was ameliorated by antioxidants such as NAC and vitamin C. Our findings indicate that in thalassemia, platelets are in a state of oxidative stress, leading to their activation and potentially to thromboembolic consequences.

17.4.5 Oxidative Stress in Thalassemic PMN

The antibacterial effect of polymorphonuclear neutrophils (PMN) involves production of a burst of ROS (respiratory burst). Their response to PMA measures the potency of their antibacterial activity. We reported [12] that at basal level, ROS generation, measured as the mean fluorescence channel, of thalassemic PMN was much higher than that of normal PMN, 95.6 ± 19.8 versus 12.7 ± 4.5. Upon PMA stimulation, it changed to 39.5 ± 14.3 and 283.4 ± 72.5, respectively, indicating that thalassemic PMN have a reduced response to PMA. Treatment of normal PMN with the oxidants hydrogen peroxide and BHP, as well as iron and hemin increased their basal ROS by 5–22-fold, but their PMA response was abolished. Treating thalassaemic PMN with antioxidants (NAC or vitamins C and E) reduced their basal ROS but enhanced their PMA response. These findings indicate that in thalassaemia, chronically stressed PMN have reduced capacity to elicit a respiratory burst, which may compromise their antibacterial capacity and result in recurrent infections.

In conclusion (Figure 17.7), in β-thalassemia, both developing erythroid precursors and mature RBC are under oxidative stress, which may cause ineffective erythropoiesis in the bone marrow (and extramedullar sites) as well as short survival of RBC due to extravascular hemolysis by phagocytosing cells. In addition, platelets and PMN, as well as other cells (such as vascular endothelial cells),

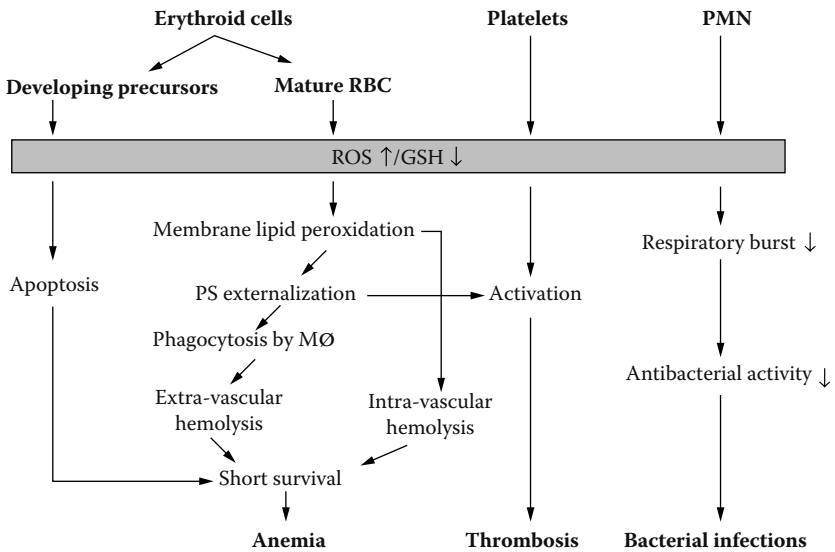

FIGURE 17.7 The outcome of oxidative stress in thalassemia. In thalassemia, various cells in the bone marrow (developing erythroid precursors) and in blood (RBC, platelets, and PMN) are under oxidative stress. The scheme illustrates the outcome of this situation as manifested by clinical symptoms: anemia, thrombosis, and recurrent bacterial infections. Abbreviation: M∅, macrophages.

might also suffer from oxidative stress leading to thromboembolic complications and recurrent infections. All these symptoms might be potentially ameliorated by treatment with antioxidants.

17.4.6 Antioxidant Effect of Fermented Papaya Preparation on Blood Cells of Patients with β-Thalassaemia

Fermented papaya preparation (FPP) is a natural Japanese health food obtained by biofermentation of *carica papaya*. FPP was reported to be endowed with a potent free radical-scavenging property, as ascertained by recent experimental and clinical studies [32–36]. We tested FPP for its antioxidative effects *in vitro* and *in vivo* on RBC, platelets, and PMN of patients with β-thalassemia.

The patients that participated in the study had different mutations and different clinical severity, and they underwent different treatment regimes. Nevertheless, most of the patients were β-thalassemia major, polytransfused, and on iron-chelation therapy. In the case of transfused patients, blood was obtained prior to blood transfusion. Informed consent was obtained in all cases. The blood was diluted with saline and mixed with gelatin and left to stand for 30 min at room temperature, as previously described [12]. The upper fraction, containing RBC, leukocytes, and platelets, was collected, washed, and used within 2 h of blood withdrawal. For *in vitro* studies, FPP (Osato Research Foundation, Gifu, Japan) was dissolved in water and added (10 mg/ml) to a suspension of blood cells derived from thalassemia patients, and after 1 h their ROS, GSH, content, and PS externaliztion were determined. Table 17.1 summarizes the results of cells derived from four patients. The results show a marked decrease in ROS and PS exposure, which was accompanied by an increase in GSH.

TABLE 17.1
Effect of *In vitro* Treatment with FPP of Blood Cells Obtained from Thalassemic Patients on Their Oxidative Stress

	ROS (MFC)		GSH (MFC)		PS (%)	
	−FPP	**+FPP**	**−FPP**	**+FPP**	**−FPP**	**+FPP**
RBC	221	130	124	266	4	0.77
Platelets	109	44	51	91	N/A	N/A
PMN	1990	700	531	540	N/A	N/A

Note: Results represent the average of four thalassemic patients and the difference between FPP-treated and -untreated cells is significant ($p < 0.05$), MFC = mean fluorescence channel, N/A = not assayed.

TABLE 17.2
Effect of Treating Thalassemic Patients with FPP on the Oxidative Status of Their Blood Cells

	RBC			
Patient Number	ROS		GSH	
	Before	After*	Before	After*
1	422	364	250	669
2	614	464	591	663
3	379	114	284	500
4	398	127	200	270
5	326	220	N/A	N/A
6	259	147	135	545
	Platelets			
Patient Number	ROS		GSH	
	Before	After*	Before	After*
1	414	331	290	422
2	598	421	550	598
3	340	180	195	476
4	422	111	239	312
5	330	220	N/A	N/A
6	299	127	202	405

* Samples were taken about 2 months following initiation of treatment.

Other parameters of oxidative stress were improved as well. These included lipid peroxidation, decreased valinomycin resistance, phagocytosis by macrophages, hemolysis, and osmotic resistance (data not shown). The *in vivo* effect of FPP was studied in a group of six patients. Patients were given 3 g per os 3 times a day. ROS and GSH were measured in their RBC and platelets at several time points prior and during treatment. Table 17.2 shows the results of each patient before and 2 months after beginning the treatment. In all cases, a marked decrease in ROS and an increase in GSH were observed in both RBC and platelets.

FPP may be more potent than specific antioxidants used individually. Its antioxidant potential may be amplified by its various components working in harmony, by exerting additive/synergistic action, and/or by each affecting specifically various cellular components such as the DNA, proteins, and lipids. We are currently studying the mechanism underlying the antioxidant effect of FPP and the *in vivo* hematological response to treatment in a mouse model of β-thalassemia and in larger scale clinical trials.

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18 *Ginkgo Biloba* Leaf Extract (EGb 761) Modulates Cell Signaling Associated with Amyloid Beta Oligomers in Alzheimer's Disease

*Yuan Luo**

CONTENTS

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18.1 INTRODUCTION

With the World Health Organization's shift in focus from the treatment of manifest diseases to disease prevention, the use of herbal medicines continues to grow in the United States, as well as many in other countries. A consumer survey in the United States estimated that 49% of all adult Americans had used at least one herbal medicine during the previous year, with 24% acknowledging regular use of herbal medicines for their well-being and for prevention or treatment of chronic diseases [1,2]. *Ginkgo* leaf extracts (such as EGb 761) is one of the best-selling herbal medicine products in the United States [3]. It has been used for primary neurodegenerative dementias associated with aging, Alzheimer's disease (AD), peripheral vascular diseases, and neurosensory problems (e.g., tinnitus) [4]. The disease prevention theory associated with herbal medicine has the potential to both increase quality of life and reduce health care costs in our society. Substantial experimental evidence supports neuroprotective properties of EGb 761, but the actual mechanisms remain unknown. Application of contemporary neuroscience theories and methodologies may provide a better understanding of the efficacy of the herbal extract, which may, in turn, facilitate an explanation of the mechanism by which the disease progresses.

18.2 ALZHEIMER'S DISEASE AND THE ANIMAL MODELS

AD, a multifactorial brain dysfunction, is recognized as a serious public health problem [5]. Currently, more than 12 million individuals worldwide and more than 5 million Americans are affected. Half of the people over 80 years old are suffering from this disorder. Memory impairment progressing to dementia is the main clinical symptom of AD, which is thought to be the consequence of the selective degeneration of nerve cells in the brain regions critical for memory, cognitive performance, and personality [6]. A common feature of AD shared with other neurodegenerative diseases is the characteristic senile plaques observed in the brain tissues of the cortex, hippocampus, and amygdale. The primary constituents of the plaques are aggregates of amyloid β-peptide (Aβ), a 4 kD peptide cleaved by β and γ secretases from the amyloid precursor protein (APP) [7]. The Aβ monomers form oligomers and polymers, which assemble into protofilaments and then fibrils [8]. Recent studies indicate that accumulation of Aβ oligomers seems to be one of the earliest events in the transgenic mice of AD [9], which impairs long-term potentiation and memory [10,11] and correlates better with severity of dementia in AD patients than the density of amyloid plaques [12].

Current management for mild and moderate AD includes acetylcholinesterase inhibitors, Tacrine (Cognex), Donepezil (Aricept), Rivastigmine (Exelon), and so on; and an NMDA (N-methyl-D-aspartate) antagonist, Memantine, to improve cognitive function [13]. These drugs provide only symptomatic modification of the disease and, thus, are of limited benefit to most patients [14]. Thus, new therapeutic strategies are focused on disease-modifying or preventive therapies [15].

Many attempts have been made to generate transgenic mouse models of AD for a mechanistic approach and pharmacological evaluation *in vivo* [6,16]. The double transgenic mice coexpressing the Swedish mutation APPswe and PS1 [17] exhibit accelerated earlier deposition of Aβ in hippocampus and cortex regions than the transgenic mice of single APP mutation [17,18]. Subsequently, the triple transgenic mice model of AD was developed, which provided a better link to the tau-related pathology, and a temporal profile of Aβ toxicity [9,19]. Although these mice do not model the full phenotype of AD [20], they represent useful tools for the investigation of Aβ toxicity and of cognitive impairment [21].

Use of simple microscopic invertebrate organisms to model AD is relatively new [22]. The round worm, *C. elegans*, was genetically engineered to carry the human gene for $A\beta_{42}$. The "sick" worms show amyloid aggregates of different sizes, just as seen in the Alzheimer's brain. Instead of cognitive impairment observed in the transgenic mice, the worms become paralyzed. In addition, the worms only lived for 20 days, allowing my colleagues and I to evaluate the time sequence of events in these worms during their entire life. Thus, the transgenic *C. elegans* expressing $A\beta_{42}$ has been used extensively for the mechanistic study of Aβ toxicity because of its ability to express muscle-specific human Aβ peptide, which forms intracellular Aβ deposits [23,24] and exhibits increased levels of protein carbonyls [25] similar to those observed in the AD brain [26]. The transgenic strain also develops concomitant progressive paralysis phenotype (CL4176) [27]. DNA microarray assay of the transgenic strain indicates that several stress-related genes were upregulated, particularly two genes homologous to human αB-crystalline and tumor necrosis factor-induced protein, which were also found upregulated in the postmortem AD brain [28]. Even though this is an invertebrate system and thus may not have relevance to AD pathology, it is a well-suited model for correlating Aβ expression and toxicity in an *in vivo* model organism.

To understand the early events in development of AD and to develop therapeutic strategies, it is fundamentally important to determine the temporal sequence of events leading to neurodegeneration. *C. elegans* is suitable for mechanistic examination of the transgene products as well as for pharmacological analysis of time course and kinetics of drug effect [29]. For example, a relationship between Aβ amino-acid sequence, amyloid formation, and oxidative damage was established using this model. Yatin et al. [25] showed both *in vitro* and in the *C. elegans* model that methionine (Met^{35}) is critical for free radical-production by $A\beta_{1-42}$, and it is also critical for β-sheet formation in the transgenic *C. elegans* lines [30]. A correlation between a progressed paralysis phenotype with increased levels of protein carbonyls in CL4176 [27] supports the advanced "amyloid hypothesis," which states that Aβ-induced oxidative stress leads to neuronal cell death seen in AD [31]. It is likely that the temporal sequence of events manifested in the transgenic worms is the same as the one demonstrated in a *Drosophila* model of AD [32], that is, accumulation of $A\beta_{42}$ in the brain is sufficient to cause cognitive impairment and neurodegeneration.

18.3 AMYLOID HYPOTHESIS FOR ALZHEIMER'S DISEASE

An amyloid cascade hypothesis has been well accepted. It states that accumulation of Aβ deposition initiates a series of downstream neurotoxic events, which results in neuronal dysfunction and death seen in AD [33]. The strongest evidence supporting this hypothesis comes from molecular genetic studies. Patients with Down's syndrome, with an extra copy of chromosome 21 containing the APP gene, always develop AD and the formation of Aβ deposits is their early sign of brain lesion [34]. All familiar forms of AD (FAD)-linked mutations, in the APP gene or two presenilin genes (PS1 and PS2), result in increased production of $A\beta_{42}$, which is the more amyloidogenic form [35]. Furthermore, transgenic mice overexpressing the mutant APP developed Aβ-containing amyloid plaques similar to those found in AD. In addition, other structure lesions, including neurofibrillary tangles and aproE, might contribute to an imbalance between Aβ production and clearance [33]. Therefore, modulation of Aβ production and clearance in the brain is becoming one of the rationale approaches for treatment of AD [14].

Despite a large body of experimental evidence supporting the hypothesis that Aβ deposition is critical in the pathogenesis of AD, the theory remains controversial. An intriguing question is whether the Aβ fibrils or the oligomers are the cause of neuronal death in AD, which is critical to determining the mechanism of Aβ toxicity and the specific therapeutic strategies. The controversial theories are: (1) neurotoxicity of Aβ is directly linked to its state of aggregation in that only fibrillar Aβ is toxic [36–38]; (2) fibrils are not necessary for neurotoxicity, rather the intracellular [19,39,40], small aggregates or oligomers of the soluble Aβ are the neurotoxic species [41–44]; and (3) generation of oxidative stress by Aβ is a possible cause for neurodegenerative diseases in AD [45–47]. The evidence for or against these hypotheses is critical for determining the mechanism of Aβ toxicity and the specific therapeutic strategies.

18.4 Aβ AND CELL SIGNALING

Aβ has many targets that activate different second-messenger cascades. Soluble Aβ-induced cell death was found to be mediated by activating a redox-sensitive cytosolic phospholipase A_2-arachidonic acid pathway [48], by activating the NF-κB pathway (the nuclear translocation of p65 and p50 subunits) [49], and may be dependent upon IL-1beta-triggered activation of c-Jun-N-terminal kinase [50]. Acute application of Aβ or APP overexpression inhibited activity-dependent regulation of several protein kinase pathways that require Ca^{2+} influx via NMDA receptors for activation, including Ca^{2+}/calmodulin-dependent protein kinase II, protein kinase A, and extracellular regulated kinases (ERK), implicating multiple Ca^{2+}-regulated signaling pathways involved in the synaptic action of Aβ, and malfunction of these pathways may underlie the synaptic dysfunction in early AD [51].

Aβ has been found to markedly impair hippocampal long-term potentiation [52,53]. A recent report demonstrated that the use of the nitricoxide (NO) donor and the soluble Guanylyl cyclase stimulator (sGC), or the cGMP-analogs

reversed the Aβ-induced impairment of CA1-LTP through cGK activation and reestablished the enhancement of CREB phosphorylation occurring during LTP in hippocampus slices, suggesting that Aβ downregulates the NO/cGMP/cGK/CREB pathway [54]. Thus, enhancement of NO/cGMP signaling may provide a novel approach to the treatment of AD and other neurodegenerative diseases with elevated production of Aβ. Along the same line, Aβ treatment of cultured hippocampal neurons led to the inactivation of protein kinase A (PKA) and a decreased CREB phosphorylation in response to glutamate, which is reversed by rolipram, a phosphodiesterase inhibitor that raises cAMP and leads to the dissociation of the PKA catalytic and regulatory subunits [55]. One of the downstream effectors of CREB is brain-derived neurotrophic factor (BDNF). Synaptic plasticity induced by BDNF (25 ng/ml) was impaired by sublethal Aβ (5 μM), which underlies the deficits of synaptic plasticity occurred at the early stage of AD before significant neuronal loss [56]. Glabe and coworkers proposed that the amyloid oligomers share a common primary mechanism of pathogenesis, including intracellular calcium dyshomeostasis, production of reactive oxygen species, altered signaling pathways, and mitochondrial dysfunction that represent key effectors of cellular dysfunction and cell death in amyloid-associated degenerative diseases [57].

Within the paradigm of the amyloid hypothesis, many attempts have been made to use amyloid-binding ligands as therapeutic tools in AD. Congo red and the fluorescent dye thioflavin S, both preferentially binding to stacked β-sheets, were the first agents known to reduce Aβ toxicity in mice [58]. Since these molecules do not cross the blood–brain barrier, derivatives were generated that were shown to inhibit Aβ fibrillization *in vitro* and in cultured cells [59]. Ingram and coworkers have employed high-throughput screening of small molecules of known activity in relation to Aβ. They reported that 6 compounds out of more than 3000 candidate molecules more or less reduced the β-sheet content and eliminated neuronal toxicity in cultured cells [38].

Extensive efforts have been made to develop Aβ vaccine and inhibitors of amyloidogenic secretases. Both active and passive immunization have shown promising results in mice [60,61] and in small human trials [62,63]. There is an ongoing debate in the scientific community on the pros and cons of immunotherapy for AD [64]. Although it currently seems premature for clinical applications in humans, immunotherapy is valuable for research on pathological mechanisms of AD in animals [65].

Recent technological advances have led to new interest in natural products as a source of drug discovery [66] because the natural products' structures possess high chemical diversity, biochemical specificity, and other molecular properties. These intrinsic basic characteristics of natural products set the basic premise for drug discovery for treatment of AD. Revealing molecular mechanisms of drug action is referred to by Christen as an example of "reverse pharmacology." The approach is being successfully applied to the *Ginkgo biloba* extract EGb 761. The extract was put on the market in France by IPSEN many years before cellular and molecular tools became available [67].

18.5 *GINKGO BILOBA* LEAF EXTRACT (EGb 761) AND ALZHEIMER'S DISEASE

The standard *Gingko biloba* leaf extract (EGb 761) has been routinely given as a prescription drug in many countries and as a dietary supplement in United States for Alzheimer's dementia [67]. Several clinical trials have provided evidence of efficacy as a symptomatic treatment for AD [68–72] and suggestive for AD prevention [73]. Currently, an NIH-supported Ginkgo Evaluation of Memory (GEM) study in the United States and a GuidAge study in Europe, both of which include more than 3000 individuals older than 70 years old, are underway to evaluate EGb 761 as a preventive drug [74].

Ginkgo biloba tree leaves and fruit have been recorded in ancient and modern Chinese herbal pharmacopoeia as treatment for dysfunctions of the heart and lung, and as promoters of longevity [75]. The G*inkgo* tree has a life span of more than 4000 years because its leaves are resistant to infection and diseases [4]. The standardized *Ginkgo biloba* leaf extract (EGb 761) was developed and put on market in the early 1970s by IPSEN in France and Dr. Willmar Schwabe Pharmaceuticals in Germany. EGb 761 contains 24% flavonol glycosides (the flavonoid fraction) and 6% terpene lactones (terpenoid fraction). The flavonoid fraction is primarily composed of quercetin, kaempferol, and isorhamnetin. The terpenoid fraction primarily contains ginkgolides A, B, C, J, and M, as well as bilobalide. The chemical structure of flavonoids preferentially reacts with hydroxyl radicals [76] and chealate pro-oxidant transition heavy metal ions [77], which consequently inhibits the formation of new hydroxyl radicals. The ginkgolides are known to be platelet activating factor (PAF) antagonists, able to improve blood circulation [78].

During the past decade, *in vivo* and *in vitro* experiments in mammalian systems and clinical studies in humans demonstrated that EGb 761 exhibits a range of biochemical and pharmacological effects [4]. Major biochemical and pharmacological activities of EGb 761 include free-radical scavenger activities; inhibition of membrane lipid peroxidation [75]; cognition enhancement, particularly in aging rats, and alleviating stress in the experimental animals [79–81]; anti-PAF activity contributing to improvements in cerebral insufficiency [78]; enhancing neuronal plasticity [77]; anti-inflammatory effects [82]; and antiapoptotic activities in neuronal cells [83–86]. As summarized by Christen at a recent conference ("*Ginkgo biloba* Extract: From Traditional Medicine to a Medicine of the Future," Berlin, 2002), EGb 761 seems to act at all levels of life: from molecules, cells, and tissues, to the entire organism [67].

In human studies, more than a dozen clinical trials have supported the clinical efficacy of EGb 761 in primary degenerative dementia of Alzheimer's type [68]. A meta-analysis study reviewed over 50 clinical studies using EGb 761 for treatment of dementia and cognitive functions associated with AD and concluded that the administration of 120 to 240 mg/day of EGb 761 for 3 to 6 months had a small but significant effect [68,69,87]. The effect of EGb 761 was comparable to the current drug Donepezil (Aricept) [70,72]. The evidence supporting EGb 761 enhancement of learning in healthy humans is inconclusive [88]. Other clinical effects of

EGb 761 include improvements in peripheral arterial insufficiency in cerebral disorders, including cognitive decline, short-term memory, tinnitus, acute cochlear deafness, and disturbance in equilibrium [89], and cognitive deficits that follow stress or traumatic brain injury [75]. Upon considering all studies conducted to date, it appears that EGb 761 has a beneficial effect on brain functions [90].

18.5.1 EGb 761 and Aβ

Studies concerning whether EGb 761 protects against Aβ toxicity were first conducted in cultured cells treated with Aβ [85,91,92]. To identify the specific neurotoxic species of Aβ and the nature of its effects, an Aβ-expressing neuronal cell line was used and demonstrated that EGb 761 appears to inhibit formation of an extracellular, SDS-stable Aβ species at molecular weight around 7–10 kD, which corresponds to an Aβ dimer [84]. The SDS-stable Aβ oligomers (Mr ~ 8–12 kD) have been detected by Western blotting in the soluble fraction of Alzheimer's diseased cortex [93], in certain cultured cells [94,95], and have been shown to inhibit hippocampal long-term potentiation [10,95].

To further verify this inhibitory effect of EGb 761 *in vivo*, we analyzed tissue samples from an intracellular Aβ-expressing *C. elegans* strain CL2006 (23) fed with or without EGb 761 by Western blotting with an anti-Aβ antibody for Aβ species. Our data show (Figure 18.1A) that multiple Aβ immunoreactive bands were detected in the Aβ-expressing *C. elegans*, and an Aβ species with molecular weight at around 14 kD (oligomers) were decreased in EGb 761-fed *C. elegans* [96]. Interestingly, Congo red also decreased this Aβ species. The oligomers species inhibited by EGb 761 is similar to and/or identical to, in terms of its size, the neurotoxic small diffusible Aβ oligomers referred to as ADDLs (Aβ-derived diffusible ligands), which were found to kill mature neurons in cultured hippocampal slices at nanomolar concentrations [52] and can be inhibited *in vitro* by EGb 761 in a dose-dependent manner [97].

Whether EGb 761-induced inhibition of Aβ oligomerization in the *C. elegans* is associated with cellular function was subsequently determined by using temperature-inducible paralysis in CL4176 strain. We observed a convincing delay of paralysis in the worms fed with EGb 761. Congo red did not generate a significant decrease in Aβ-induced paralysis in CL 4176 (Figure 18.1B). We reasoned that EGb 761 offers more protective activities than anti-Aβ aggregation alone. Using the "wonder" worms, we correlated Aβ aggregates with their toxicity. Our findings strongly support the current theory that the smaller amyloid beta aggregates are more risky than the larger aggregates, which were originally thought to be the main cause of Alzheimer's disease. Interestingly, a known antioxidant vitamin C alone was not sufficient to ease the toxicity in the paralyzed worms. We assume that it is the combined properties (antioxidative and antiamyloidogenic) of the *Ginkgo biloba* extract that protect the brain against cognitive dysfunction.

In addition to clearance of Aβ oligomers toxicity, an independent study demonstrated that EGb 761 induced the metabolism of APP toward the

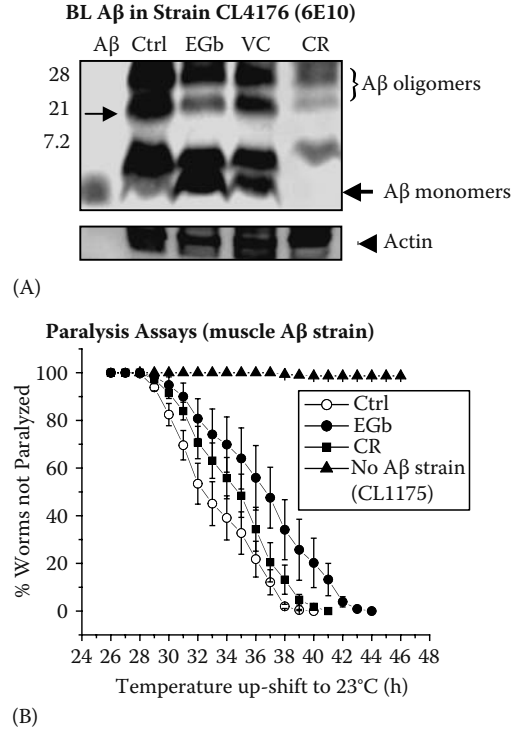

FIGURE 18.1 (A) Representative Western blot (BL) of Aβ species in the transgenic *C. elegans* CL4176 fed with or without EGb 761 (EGb), L-ascorbate acid (VC), and Congo red (CR). The CL4176 worms maintained at 16°C were fed with a vehicle (Ctrl), EGb 761 (100 μg/ml), L-ascorbate acid (50 μg/ml), or Congo red (139 μg/ml) at day 1 of age for 72 h. The worms were collected and equal amounts of protein were loaded on each gel lane and immunoblotted with an anti-Aβ antibody (6E10). (B) Time course of paralysis assays in CL4176 fed with different drugs. Synchronized eggs of CL4176 *C. elegans* were maintained at 16°C, on the 35 × 10 mm culture plates (~100 eggs/plate) containing vehicle (Ctrl), EGb 761 (100 μg/ml), or Congo red (139 μg/ml). The hatched worms were grown for 38 h at 16°C followed by upshifting the temperature to 23°C to induce the transgene expression. The paralysis was scored at 60 min intervals. Paralysis in the transgenic strain CL4176 is due to Aβ expression as compared with the control strain CL1175, which does not express Aβ transgene (filled triangles).

α-secretase in hippocampal slices and *in vivo* [98]. EGb 761 was also reported to influence the production of brain APP and Aβ by lowering the levels of circulating free cholesterol [99].

18.5.2 EGb 761 Modulating Cell Signaling

Accumulating evidence suggests that many of the actions of EGb 761 are so-called *polyvalent* actions, that is, the therapeutic activity of EGb 761 is the net

effect of interactions between various biological activities of the individual substances in the extract. Presumably, this is one of the advantages of using chemicals obtained from natural products for the prevention and treatment of infirmity, as well as the maintenance of health. As opposed to pharmacologically manufactured or synthetic drugs, which provide a single target for a single receptor as its mechanism of action, EGb 761 is able to up- or downregulate signaling pathways, gene transcription, and cellular metabolism, that is, the general physiological states of the cell and organism in response to both normal and stressed conditions [100]. At the same time, it appears that it is the multiplicity of effects by EGb 761, or the polyvalent action, that complicates the mechanistic studies. Genomic and proteomic microarray methods provide researchers with the tools to decode the diverse effects of complex natural substances on biological systems. Gene microarray assays yielded molecular evidence for the neuromodulatory action of EGb 761 in separate brain regions of mice fed with EGb 761 for 4 weeks [101]. In these mice, transcription of transthyretin and several other molecules with neuroprotective roles was all significantly upregulated. Thus, the therapeutic effects of EGb 761 on cognitive impairment (dementia) probably involve modification of the expression of many genes by actions involving several of its active constituents [90]. Combining functional genomic and behavioral analysis to yield an objective assessment of the *in vivo* effects of EGb 761 is certainly merit for future studies [77] and for therapeutic development.

To determine the polyvalent activities of EGb 761 reflected in global gene expression changes, we first compared the transcriptional profiles in a neuronal cell line (PC12) treated with or without EGb 761 using the DNA microarray technique. We discovered that multiple gene transcripts (more than 70 out of 816 aging-related genes) are altered more than twofold in EGb 761-treated PC12 cells (Table 18.1). The transcript level for an antiapoptotic Bcl-2-like protein was elevated, whereas the transcript level for proapoptotic caspase12 was decreased in EGb 761-treated cells. We confirmed it by the biochemical assays [84,86,102] and indicated that the protective action of EGb 761 may be carried out, at least in part, by modulating cellular apoptotic machinery. Similarly, expression of genes encoding transcriptional factors, antioxidant defenses, and stress response is strongly modified by EGb 761 treatment of human hNT neurons [103]. In mice fed with EGb 761, the hippocampus and cerebral cortex regions displayed upregulation of more than 10 neuromodulatory genes, especially that for transthyretin, which could be involved in neuroprotection [101]. However, interpretation of microarray results from the postmortem AD brain tissue is often complicated by the genetic and environmental heterogeneity of the sample, the time lag between the onset of pathology and tissue recovered, and the DNA chips selected for hybridization.

For these reasons, Link et al. [28] used DNA microarray analysis to look at changes in gene expression resulting from the induction of human Aβ expression in a transgenic strain CL4176 and identified 67 upregulated and 240 downregulated genes, of which 40% of these genes have recognizable human homology. Among them, the small heat-shock protein gene *hsp-16*, which is closely colocalized with intracellular Aβ [104], is particularly interesting. *Hsp-16* was reported

TABLE 18.1
Representative Transcriptional Effects of EGb 761 on NGF Differentiated PC12 Cells

Gene/Function	Gene ID	Clone Description	Fold Change
Apoptosis			
Bcl-2 interacting protein	H3103B07	Mus musculus Bcl2/adenovirus E1B 19 kDa-interacting protein 3-like	1.95
Tumor necrosis factor	H3091D11	Mus musculus tumor necrosis factor superfamily member 19	1.86
Caspase 12	H3131G02	Mus musculus caspase 12	−1.74
Apoptosis regulator	H3038E03	PRKC, apoptosis, WT1, regulator	−1.84
Other MitoChip Clones			
ATPase-like proton channel	H3027A10	Mus musculus ATPase-like vacuolar proton channel (Atpl)	1.89
Choline transporter	H3102C06	Choline transporter (CHOT1)	1.76
Brain cDNA clone MNCb-0663	H3122H03	Mus musculus brain cDNA, clone MNCb-0063, liver regeneration-like	1.71
Voltage-gated sodium channel	H3149E11	Homo sapiens sodium channel, voltage-gated type II	−1.71
Glutathione-S-transferase	H3111F09	Rat Y-b3 glutathione–S-transferase mRNA	−1.81
Glycogen phosphorylase	H3117G06	Rat glycogen phosphorylas brain isozyme mRNA	−1.91
Serine protease	H3045E05	Mus musculus serine protease OMI	−2.02
Glycerol-3-phosphate dehydrogenase	H3005G01	Mouse mRNA for glycerol-3-phosphate dehydrogenase	−2.09
Mitotic arrest deficient	H3124D11	Mus musculus mitotic arrest deficient 1-like	−3.16

Note: Total mRNA was extracted from the PC12 cells treated with or without EGb 761 (100 μg/ml for 48 h). cDNAs for array printing were amplified by PCR. Mouse Mitochip array consists of 816 cDNA clones originated from the NIA 15 k mouse cDNA library. Positive numbers and negative numbers indicate upregulation or downregulation of the transcription by EGb 761 treatment [86]. Multiple gene transcripts (more than 70 out of 816 aging-related genes) are altered in EGb 761-treated PC12 cells. The transcript level for an antiapoptotic Bcl-2-like protein was elevated, whereas the transcript level for proapoptotic caspase 12 was decreased in EGb 761-treated cells.

to be upregulated in Aβ-expressing *C. elegans* [28], which exhibited a protective function [105]. However, using the GFP-reporter transgenic *C. elegans* (*hsp-16/GFP*) to visualize the expression of *hsp-16 in vivo*, we found that in these worms fed with EGb 761 the expression of the *hsp:GFP* gene in response to oxidative stresses was significantly suppressed [106]. We speculate that the presence of EGb 761 reduces the cellular flux of free radicals, leading to a concomitant

decrease in damaged proteins, and a reduced requirement for the stress-response gene produced, such as *hsp-16*. Consistent with this notion, we demonstrated that treatment of the nematodes with EGb 761 increased their resistance to an acute oxidative stress by 33%, and their thermo-tolerance by 25% [107], suggesting that EGb 761 can successfully counteract oxidative and thermal stress. As a consequence, the wild-type *C. elegans* fed with EGb 761 lived longer than their untreated controls [107].

In order to demonstrate a possible link between oxidative stress and Aβ-expression, we established an assay for the measurement of intracellular levels of H_2O_2-related reactive oxygen species (ROS), using 2′,7′-dichlorofluorescein (DCF) methods. Our data indicate that EGb 761 significantly attenuates both Aβ and Juglone-induced ROS production in the transgenic Aβ-expressing neuroblastoma cells [108]. We then modified the assay to be used in the *C. elegans* and showed that the intracellular level of ROS is significantly higher in the mutant *C. elegans* in an AD-associated strain CL2006 than in the wild-type [108] supporting the free-radical hypothesis of Aβ toxicity [31]. We further demonstrated that EGb 761 attenuates elevated levels of ROS in the transgenic *C. elegans*. The flavonoid components kaempferol and quercetin provided the most significant effect. As a comparison, the known antioxidant ascorbic acid (vitamin C) also attenuated elevated ROS in *C. elegans*, but to a lesser extent than the falvonoids [108].

Contemporary neuroscience utilizes the best molecular, cellular, physiological, behavioral, and imaging techniques to address a range of concerns related to the function, and dysfunction, of the brain. For example, neuroplasticity and learning can be measured *in vitro* at the synaptic level electrophysiologically as long-term potentiation, at the cellular level as image of waves of molecular changes, *in vivo* as gene expression profile, and at a behavioral level in a transgenic animal model of neurodegenerative diseases or in the human brain via visualization by functional imaging of brain activity in real time. Apparently, modern neuroscience has at its disposal the best tools for approaching the most challenging questions, such as the mechanism complexity of herbal medicine, which is a mixture of molecules synthesized not by a chemist but by the natural selection.

EGb761 was found to attenuate the apoptosis, a programmed cell death, in the culture, which in effect increases the number of cells [84,85]. On the other hand, the same extract also inhibits cell proliferation, inhibiting growth of cultured cancer cells [109]. This dual effect is in line with the bidirectional modulator theory of herbal medicine. Thus, the regulatory and adaptive effects of EGb761 can vary neurotransmitter or neuromodulator activities according to the circumstances [67].

18.6 CONCLUSION

Our research demonstrates that EGb 761 exerts an anti-Aβ aggregation effect in a neuroblastoma cell line expressing Aβ [84]. The antiapoptotic properties of EGb 761 are supported by the DNA microarray method in PC 12 cells (Table 18.1) [86].

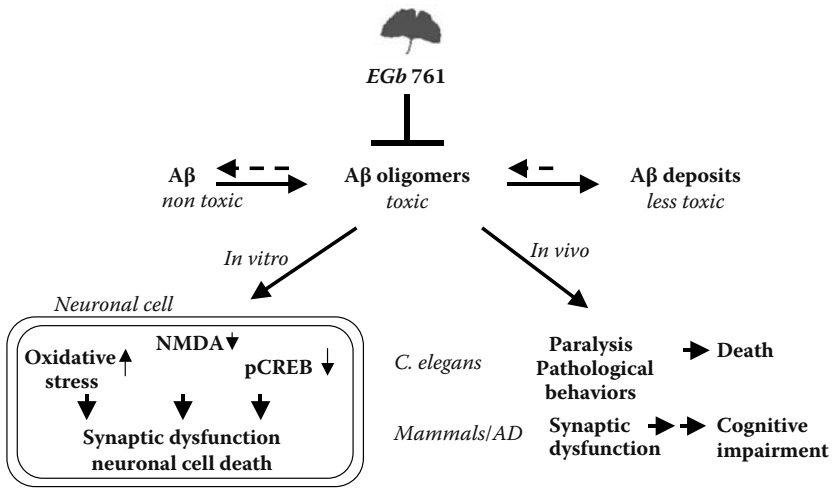

FIGURE 18.2 A proposed mechanism by which EGb 761 suppresses Aβ-induced pathological behaviors. The equilibrium between monomers and oligomers is reached more rapidly than between oligomers and larger deposits. The process of oligomerization may generate multiple forms of oligomers. We speculate that the unique structures in EGb 761 bind to certain confirmations of Aβ oligomers and protect Aβ-induced behaviors by shifting toxic oligomers to nontoxic monomers.

Furthermore, EGb 761 exhibits antistress effects in wild-type *C. elegans* [107], reduces intracellular free-radical production in the transgenic *C. elegans* [108], and significantly attenuates expression of stress-response protein hsp16-2 in *C. elegans* [106]. These observations suggest a functional linkage between antioxidative and stress-response pathways in EGb 761 neuroprotection. We have recently reported that EGb 761 decreases Aβ oligomerization and Aβ-induced pathological behaviors in transgenic *C. elegans* [96]. These findings suggest that the beneficial effects of EGb 761 in AD patients and in transgenic mice are mediated by a combination of antioxidative, antiproteotoxicity, and antiapoptotic cell signaling pathways [Figure 18.2].

Egb 761 research serves as an example of how the combined power of contemporary neuroscience and theory of traditional medicine can provide new clues about the complexity of EGb 761 neuroprotection. Given the increasing use of herbal medicine by Western health consumers, it is imperative that we achieve a better understanding of the mechanisms of action of herbal remedies. Modern neuroscientists are ideally equipped to address both of these critically important needs and should take a proactive leadership role in these endeavors.

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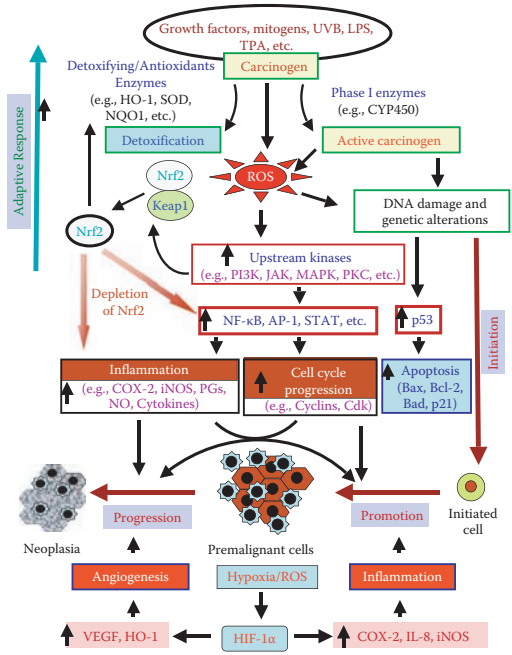

FIGURE 1.1 Major molecular events in multistage carcinogenesis.

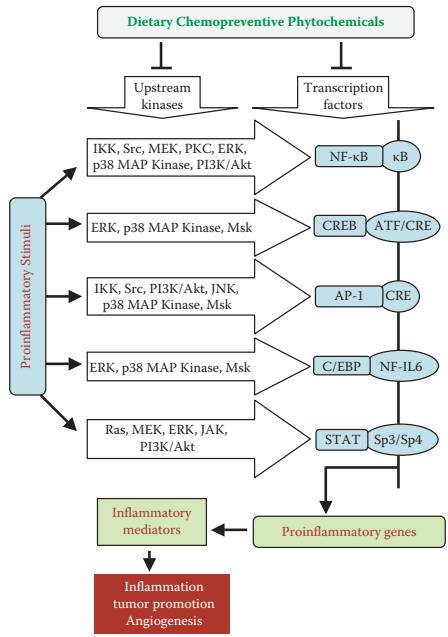

FIGURE 1.2 Some representative upstream kinases and transcription factors as potential targets of dietary chemopreventive phytochemicals.

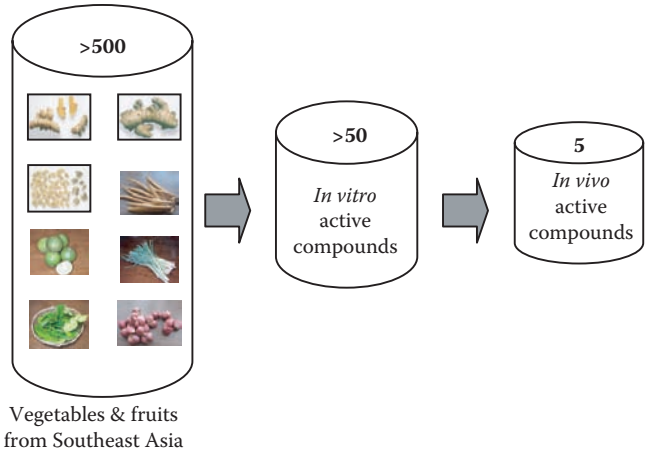

FIGURE 3.2 Screening for cancer preventive compounds from vegetables and fruits. Most of the *in vitro* active compounds could not be subjected to animal experiments because of sample limitations and their *in vivo* cancer preventive efficacy remains unknown.

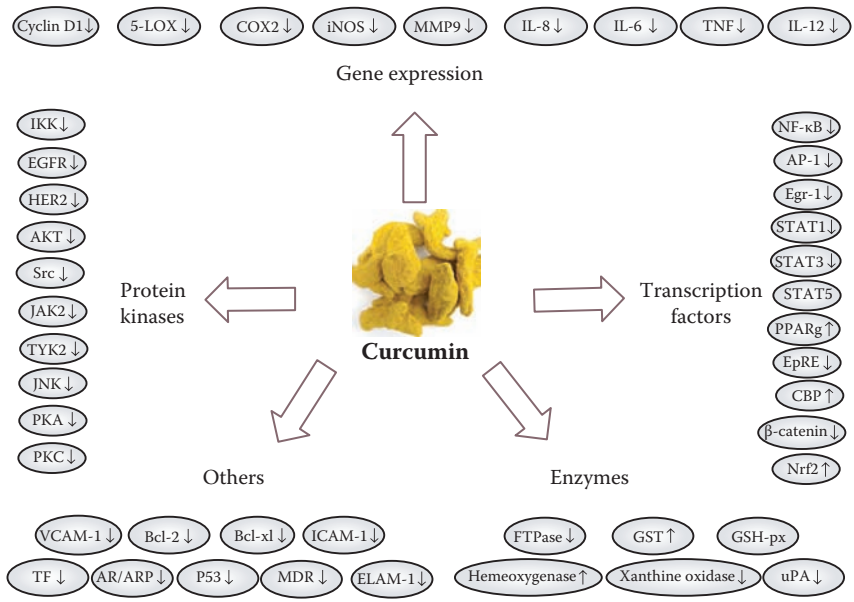

FIGURE 4.2 Molecular targets of curcumin.

Biological Sciences

Dietary Modulation of Cell Signaling Pathways

A consequence of rapid progress in the science of nutrigenomics and nutrigenetics is the substantial accumulation of data covering nutrienal modulation of gene expression at the cellular and subcellular levels. Current research is increasingly focused on the role of nutrition and diet in modifying oxidative damage in the progression of disease. **Dietary Modulation of Cell Signaling Pathways** reviews some of these findings, focusing on nutrient-gene interactions with particular emphasis on the intracellular signaling network.

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Features

- Provides a comprehensive introduction to dietary modulation of cell signaling
- Discusses chemopreventive agents, food phytochemicals, flavonols, and polyphenols
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