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REVIEWS: CURRENT TOPICS

A review of the interaction among dietary antioxidants and reactive oxygen species

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Abstract

During normal cellular activities, various processes inside of cells produce reactive oxygen species (ROS). Some of the most common ROS are hydrogen peroxide (H_2O_2), superoxide ion (O_2^-), and hydroxide radical (OH^-). These compounds, when present in a high enough concentration, can damage cellular proteins and lipids or form DNA adducts that may promote carcinogenic activity. The purpose of antioxidants in a physiological setting is to prevent ROS concentrations from reaching a high-enough level within a cell that damage may occur. Cellular antioxidants may be enzymatic (catalase, glutathione peroxidase, superoxide dismutase) or nonenzymatic (glutathione, thiols, some vitamins and metals, or phytochemicals such as isoflavones, polyphenols, and flavanoids).

Reactive oxygen species are a potential double-edged sword in disease prevention and promotion. Whereas generation of ROS once was viewed as detrimental to the overall health of the organism, advances in research have shown that ROS play crucial roles in normal physiological processes including response to growth factors, the immune response, and apoptotic elimination of damaged cells. Notwithstanding these beneficial functions, aberrant production or regulation of ROS activity has been demonstrated to contribute to the development of some prevalent diseases and conditions, including cancer and cardiovascular disease (CVD). The topic of antioxidant usage and ROS is currently receiving much attention because of studies linking the use of some antioxidants with increased mortality in primarily higher-risk populations and the lack of strong efficacy data for protection against cancer and heart disease, at least in populations with adequate baseline dietary consumption.

In normal physiological processes, antioxidants effect signal transduction and regulation of proliferation and the immune response. Reactive oxygen species have been linked to cancer and CVD, and antioxidants have been considered promising therapy for prevention and treatment of these diseases, especially given the tantalizing links observed between diets high in fruits and vegetables (and presumably antioxidants) and decreased risks for cancer.
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1. Introduction

Since the late 19th and early 20th century, chemists have studied antioxidants, a loosely defined group of compounds characterized by their ability to be oxidized in place of other compounds present. Their uses ranged from food storage to the vulcanization of rubber, but it was only later that biologists realized the importance of antioxidants in health with the 1960s publications of vitamins and flavanoids, followed by later research in the 1970s on ascorbic acid (vitamin C), cancer, and the common cold [1]. With many well-known scientists actively researching and/or discussing antioxidants as protecting agents [2–6], explanations for the effects of antioxidants on cancer susceptibility and overall health expanded rapidly in subsequent decades with research into mechanisms, molecular targets, and molecular interactions [7]. It is no surprise then that the use of antioxidants has become widespread in US adults: as the National Health and Nutrition Examination Survey found, over half the population uses dietary supplements, a third take multivitamins, and over an eighth use vitamin E and/or C supplements [8].

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Along with the evidence of positive benefits, however, a substantive compendium of negative effects of antioxidant use, especially concerning dietary supplementation with vitamins C and E, β-carotene and selenium, has developed [9,10]. Of primary concern are the potentially deleterious effects of antioxidant supplements on reactive oxygen species (ROS) levels, especially when precise modulation of ROS levels are needed to allow normal cell function or to promote apoptotic cell death of precancerous or transformed cells [11]. The conflicting findings have led to a recent evidence report from the Agency for Healthcare Research and Quality on vitamins C and E and coenzyme Q10 [12]. This review will discuss the roles of antioxidants and ROS in normal cellular function with particular attention to signal transduction, apoptosis, and regulation and function of the immune response [13,14].

A number of epidemiological studies initially indicated utility of antioxidants in disease prevention, particularly for cardiovascular disease (CVD) and cancer. However, recent conflicting results from intervention trials have identified negative consequences associated with antioxidant supplement use and a presumed reduction in ROS. This apparent conundrum of antioxidant effects on ROS has recently been examined in light of molecular evidence for the role(s) of ROS in development and progression of cancer and CVD, especially since there has been a flurry of recent studies potentially linking some antioxidants with increased mortality and CVD. This aspect of antioxidants, ROS and their relationship to cancer and CVD, with an evaluation of related clinical findings, has been reviewed by us in a recent book chapter [15] and is referred to here in a more cursory manner. The focus of this report is on the more molecular aspects of the delicate balance between ROS and antioxidants in normal physiological systems, as well as external factors and other habits that may overwhelm that balance.

There are a number of areas where additional research is needed, e.g., to determine how specific antioxidants interact with ROS as well as cancer medications and radiation treatment. Because of the variety in the mechanisms of action for each antioxidant compound, the myriad differences within and between tumor types as well as some fulfilling more than one function in the cell cycle, it is imperative that they be evaluated separately to determine their effect, whether detrimental or beneficial, on conventional cancer therapies. This is a concern that repeatedly comes up in meetings, the literature and queries from the lay public. Additionally, antioxidants have shown some effects in a number of studies, but the data obtained seem contradictory when looked at in totality. Despite the uncertainties present, it appears that at least populations with inadequate baseline nutritional consumption may benefit from an increased intake of dietary antioxidants or supplements. A summary listing of overarching issues in antioxidant research is presented in Table 1.

Table 1
Overarching issues of antioxidant research

Smoker vs. nonsmoker risk/benefit assessment
Interaction with chemotherapeutic drugs
Protection against or exacerbation of radiotherapy
Validation of cardiovascular sequelae
Anticarcinogenic potential
Achievability of physiologically active doses
Issues of bioavailability and impact of formulation
Absorption, distribution, metabolism and excretion
Efficacy of combinations of antioxidants and/or drugs
Impact of polymorphic differences on diet and efficacy
Effectiveness of early versus late stage intervention

2. The role of ROS in normal physiology

Reactive oxygen species present a paradox in their biological function: on one hand, they prevent disease by assisting the immune system, mediating cell signaling and playing an essential role in apoptosis. On the other hand, they can damage important macromolecules in cells and may have a role in carcinogenesis and CVD.

Historically, the generation of ROS was viewed as indiscriminate and random, and their targets as primary determinants of disease and aging [16,17]. Research also demonstrates that ROS generation is a normal physiological process, particularly for proper immunocompetence and in coordination and activation of numerous signal transduction pathways [17].

The formation of ROS is a natural consequence of aerobic metabolism and is integral for maintaining tissue oxygen homeostasis [14]. Oxygen homeostasis — the balance between constitutive oxidants and antioxidants — is maintained through a natural series of reduction–oxidation (redox) reactions involving the transfer of electrons between two chemical species: compounds that lose electrons (oxidized) and those that gain electrons (reduced). When oxygen homeostasis is not maintained, the cellular environment becomes oxidatively stressed. Approximately 1–3% of oxygen consumed by the body is converted into ROS [18]. Three of the major ROS — superoxide radical, hydrogen peroxide and hydroxyl radical — are normal metabolic byproducts that are generated continuously by the mitochondria in growing cells [14,19,20]. Other significant intracellular sources of ROS include microsomal cytochrome P450 enzymes, flavoprotein oxidases and peroxisomal enzymes involved in fatty acid metabolism [14]. Potentially damaging oxidative stress can be generated by excess ROS, which are kept in check by endogenous cellular antioxidant mechanisms. Oxidative stress-related enzymes include superoxide dismutases for eliminating the superoxide radical as well as catalase and glutathione peroxidases for removing hydrogen peroxide and organic peroxides [14,19] (see Fig. 1).

Transient fluctuations of ROS levels influence activity of signal transduction pathways leading to cell proliferation, or

to apoptosis or necrosis, depending on the dosage and duration of ROS and also on cell type. Typically, low doses of ROS can be mitogenic, whereas medium doses lead to temporary or permanent growth arrest (replicative senescence), and high doses usually result in cell death either by apoptosis or necrosis [17]. Although necrosis and apoptosis may be viewed as negative events in terms of cell loss, these processes also have positive roles in the down-regulation of immune responses (discussed below) and elimination of transformed cells (tumor suppression).

2.1. Signal transduction and regulation of proliferation

Controlled production of ROS is essential for the activity of signal transduction pathways, and one broad class of signal transduction molecules on which ROS influence function is the mitogen-activated protein kinases (MAPKs). MAPKs are composed of three subfamilies: the extracellular signal regulated kinases (ERKs) ERK1 and ERK2; the c-*Jun* N-terminal kinases (JNKs) JNK1, JNK2 and JNK3; and the p38 kinases p38α, p38β, p38γ and p38δ [11,21]. Although their functions can overlap, ERKs traditionally regulate cell proliferation; JNKs and p38 kinases are more strongly linked to stress responses ultimately leading to apoptosis or necrosis [17]. ERKs coordinate signaling through growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor, and epidermal growth factor (EGF) [22,23]. Epidermal growth factor receptor (EGFR) has been demonstrated to be activated by ROS, similar to its activation by binding of its ligand, EGF [17,24]. Activation of EGFR causes receptor autophosphorylation, recruitment of accessory proteins, and activation of membrane-localized Ras, which initiates phosphorylation and activation of Raf and MAPK kinases (MAPKK), ultimately activating ERK [25]. Activation of ERKs by this route also triggers nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, producing superoxide anions and hydrogen peroxide [13,21]. This transient increase in intracellular ROS can mimic or augment ligand–receptor binding by facilitating autophosphorylation of the receptors and induction of signaling cascades; in fact, elimination of hydrogen peroxide by catalase has been demonstrated to inhibit EGF and nerve growth factor receptor autophosphorylation, as well as phosphorylation of downstream effector proteins. A summary of key signaling factors is in Table 2.

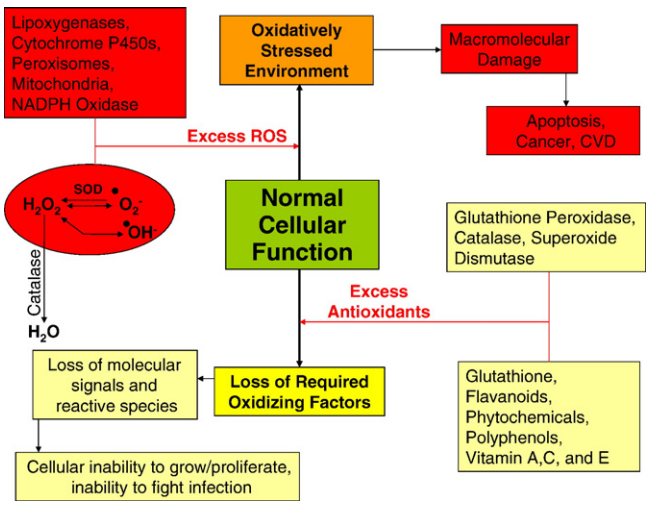

Fig. 1. Cellular oxidative interactions.

Another means by which ROS potentiates and coordinates growth factor action through MAPKs is by the action of hydrogen peroxide on glutathione-sensitive, membrane-bound phosphatases that are necessary for dephosphorylation and, thus, down-regulating active growth factor receptors [21]. These phosphatases contain redox-sensitive cysteine residues; increased levels of ROS can react with these residues, transiently inactivating the phosphatases [16,26,27]. With kinase activity proceeding unopposed by phosphatase activity, the net result is an increase in phosphorylation levels and pathway activation.

2.1.1. Signaling pathways

Increasing awareness of the role of ROS as second messengers in diverse signaling pathways has lead researchers to speculate that this may allow a cooperativity or synergism of response from different extracellular stimuli [11]. Ultimately, cellular response to oxidative stress and growth factors requires a balance between signals promoting cell proliferation or growth inhibition and/or cell death (see Fig. 2). ERK activation generally promotes survival of cells in response to ROS [16] but also can promote apoptosis under certain conditions and in certain cell types. For example, ERK facilitates hypoxia-induced apoptosis of macrophages and cisplatin-induced apoptosis of HeLa cells [11]. Whether ERK promotes cell survival or cell death may be determined by the characteristics of ERK activation. Survival appears to be enhanced by rapid, transient activation of ERK, whereas apoptosis results from a slower, more sustained activation. This may reflect differences in cellular response to acute vs. chronic oxidative stress [17].

The other two MAPK families, the JNK and p38 families, sometimes are known collectively as the stress-activated protein kinases (SAPKs) for their role in cellular response to different stresses, including cytokines, radiation, osmotic shock, mechanical injury, heat shock and oxidative damage [11]. Activation of these kinases involves multiple MAPKK and interacting regulatory proteins. As JNK and p38 kinases are activated by a wide array of stresses, the question arises of how a specific response to a given stressor can be achieved. For example, the redox regulated protein thioredoxin (Trx) binds to and inhibits the activity of the apoptosis signal-regulating kinase 1 (ASK1) under normal redox conditions. Oxidative stress causes dissociation of the Trx–ASK1 complex, leading to activation of JNK and p38 by ASK1 [11]. Similarly, binding of glutathione-s-transferase to JNK under nonstressed conditions can be disrupted by oxidative stress, resulting in activated JNK [11,17].

Table 2
Molecular targets affecting ROS

Target	Name	Function	Effect of ROS or oxidation
ASK1	Apoptosis signal-regulating kinase 1	ASK1 activates of JNK and P38 and is inactive when complexed with Trx	Causes dissociation of the Trx–ASK1 complex leading to ASK1 activation of JNK and P38
Caspase 8		A family of cytosolic aspartate-specific cysteine proteases involved in the initiation and execution of apoptosis; they are expressed as latent zymogens and are activated by an autoproteolytic mechanism or by processing by other proteases (frequently other caspases)	Activated by ROS
EGFR	Epidermal growth factor receptor	A member of the EGFR family of receptor tyrosine kinases, which activates Raf and MAPKK leading to activated ERK	Activated by ROS
ERK	Extracellular signal-regulated kinases	Members of the MAPK family; regulates cell proliferation and triggers NADH oxidase; ERK1 and ERK2 (also known as MAPK3 and MAPK1) are part of the Ras-Raf-ERK signal transduction cascade often found downstream of growth factor receptor activation.	Activated by ROS
HIF-1	Hypoxia-inducible factor	Induces erythropoietin, VEGF and TH; the hypoxia-inducible transcription factor 1 alpha (HIF-1 alpha) is the regulated member of the transcription factor heterodimer HIF-1 alpha; HIF-1 alpha binds to hypoxia-response elements in the promoters of many genes involved in adapting to an environment of insufficient oxygen or hypoxia.	Stimulated by low oxygen
IL1β	Interleukin 1-beta	Produced by a wide variety of cells in response to stimuli such as those produced by inflammatory agents, infections or microbial endotoxins	ROS increases activity
IL6	Interleukin 6, also known as interferon-beta	A multifunctional protein that plays important roles in host defense, acute phase reactions, immune responses and hematopoiesis.	ROS decreases activity
JNK	c-*Jun* N-terminal kinase	A member of the MAPK family; promotes phosphorylation and activation of AP-1 (c-*Jun* component); leads to apoptosis or necrosis activated by TNF-α; members of the MAPK family, the JNKs, are activated by environmental stresses and inflammatory cytokines.	ROS leads to activation following dissociation of the Trx-apoptosis signal-regulating kinase 1 or disruption of glutathione-s-transferase binding
MAPK	Mitogen-activated protein kinase	Activates ERK upon phosphorylation	
NADH oxidase	Nicotinamide adenine dinucleotide oxidase	A potential source for O_2^- in the proliferation-inducing activity of oxLDL	
NFκB	Nuclear factor of kappa light chain gene enhancer in B cells		Activated by ROS as IκB (inhibitor) and is degraded by oxidation: NκKB DNA-binding complex is inhibited by IκB proteins, which inactivate NFκB by trapping it in the cytoplasm is decreased
oxLDL	Oxidized low-density lipoprotein	Stimulates atherosclerotic plaques; NADPH oxidase is a potential source for O_2^- in the proliferation-inducing activity of oxLDL	
P38		A member of the MAPK family: substrates include transcription regulators activating transcription factor 2 (ATF2), myocyte enhancing factor 2c (MEF2C) and MAX (a Myc heterodimerization partner), cell cycle regulator Cdc25B, and tumor suppressor p53, which suggest the roles of this kinase in stress related transcription and cell cycle regulation, as well as in genotoxic stress response	Activated by ROS
P53		Tumor suppressor protein is a multifunctional transcription factor that regulates cellular decisions regarding proliferation, cell cycle checkpoints, and apoptosis	ROS Increases accumulation of protein but decreases binding to DNA
STAT1 and 2	Signal transducer and activator of transcription	Inflammatory cytokine STAT proteins are a family of latent cytoplasmic transcription factors involved in cytokine, hormone, and growth factor; signal transduction regulates numerous aspects of hematopoiesis and the immune response via activation of the JAK/STAT pathway; the activated Stat proteins, in turn, dimerize, translocate into the nucleus and activate a specific set of genes; in this way, each cytokine elicits its specific response from the target cell	Activated by ROS
VEGF	Vascular endothelial growth factor	on its ability to increase the permeability of capillary blood vessels	Decreased by ROS
SOD	Superoxide dismutase	SOD catalyzes the conversion of the O_2^- into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2^-), and, as such, is considered an important antioxidant in aerobic cells.	Accumulation of ROS, including the superoxide free radical (O_2^-), can damage membrane lipids, proteins and DNA.

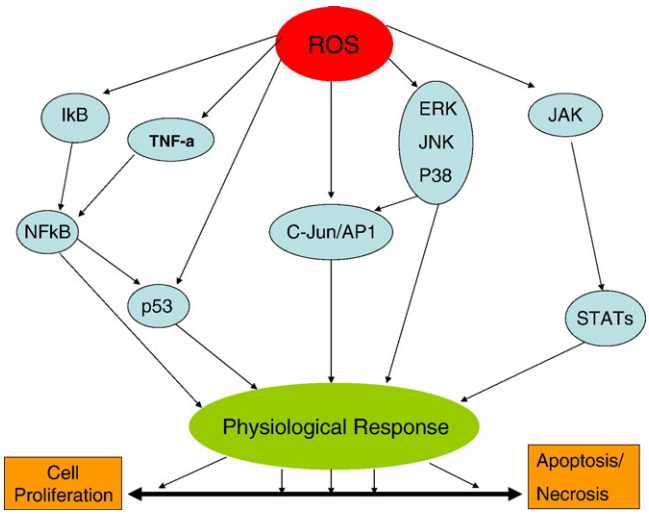

Fig. 2. Cellular response to oxidation.

One important downstream effect of JNK activation is activation of the transcription factor AP-1. Oxidative activation of JNKs promotes phosphorylation of serine residues 63 and 73 of the aminoterminal transactivation domain of c-Jun, part of the dimeric AP-1 transcription factor, and leading to transactivation of AP-1-regulated genes. JNKs have been observed to be activated in response to environmental stressors that also activate AP-1, such as radiation, UV light, and certain growth factors [21]. Members of the p38 family are activated in immune cells by inflammatory cytokines as well as by stimuli such as hormones, G-protein-coupled receptor ligands and stresses such as osmotic shock and heat shock. Given their role in regulating expression of cytokines, p38 kinases are thought to be involved in the pathogenesis of diseases such as asthma and some autoimmune conditions [11,25].

2.1.2. Gene expression

Although ROS coordinate and enhance activity of signal transduction cascades in response to extracellular stimuli, changes at the level of gene expression ultimately influences the decision of the cell to proliferate or undergo apoptosis. As mentioned above, JNKs promote phosphorylation and activation of the c-*Jun* component of the transcription factor AP-1, leading to up-regulated transcription of AP-1-regulated genes. In addition to AP-1, the transcription factor hypoxia-inducible factor 1 (HIF-1) provides another example of transcriptional regulation mediated by ROS. Activity of HIF-1 is controlled by changes in oxygen tension. Decreasing oxygen levels enhances formation of the active HIF-1 heterodimer complex, promoting transcription of HIF-1-inducible genes, such as those encoding erythropoietin, vascular endothelial growth factor (VEGF) and a tyrosine hydroxylase (TH) that participates in control of ventilation by the carotid body [21]. This indicates that changing levels of ROS may be influenced by oxygen levels in the cell. This may provide one way that cells could sense and adjust oxygen levels.

As another example, the transcription factor nuclear factor kappa B (NFκB), which participates in a wide variety of biological processes including inflammation, growth control, and apoptosis, was the first eukaryotic transcription factor shown to respond directly to oxidative stress in some cell types [21,28,29]. Although activity of this transcription factor is not strictly dependent on ROS, oxidative stress has been observed to be a strong activator of NFκB in some cell types. There are at least two ways by which NFκB activity is influenced by ROS levels. First, ROS enhance degradation of the NFκB inhibitor, IκB, resulting in increased levels of active NFκB in the nucleus and leading to increased NFκB DNA binding. Induction of IκB kinase α, which phosphorylates IκB, marking it for degradation by the proteasome, is observed in Jurkat cells after a moderate pro-oxidative shift in the intracellular redox state. ROS also can influence NFκB by direct action on the transcription factor itself. NFκB must be in a reduced form to bind DNA, and reducing agents such as dithiothreitol and β-mercaptoethanol have been observed to enhance NFκB DNA binding [30]. Increased NFκB activity results in increased transcription of NFκB-regulated genes, such as tumor necrosis factor receptor-associated factor 1 and 2 and the cellular inhibitors of apoptosis proteins, which all have antiapoptotic properties (reviewed in Pahl 1999 [31]) [32]. NFκB, however, also can activate proapoptotic genes such as Fas ligand and p53. While some studies have shown both prosurvival and proapoptotic effects of NFκB activation, most show a proapoptotic effect following oxidative injury [33–36]. The exact mechanisms that are physiologically responsible for the redox balance in cells and the relationship with cell survival and apoptosis is still an area which will benefit from further investigation.

2.2. Immune response modulation

ROS have also been observed to have important roles in proper functioning of the innate immune response, activation of the adaptive immune response, as well as down-regulation of inflammation and immune system activity. Disruption or dysregulation of immune system functions can lead to diseases characterized by inflammation, including atherosclerosis and cancer.

2.2.1. Innate immunity

During the initial response to an invading pathogen, activation of the innate immune response, characterized by generation of ROS within phagocytic cells such as macrophages and neutrophils, is a critical event in the initiation of phagocytosis and subsequent destruction of these microorganisms [21,37]. In the production of high levels of superoxide anions and hydrogen peroxide by NADPH oxidase, the “respiratory (or better, oxidative) burst” is required for destruction of engulfed pathogens. Defects in any of a number of phagocyte-based NADPH oxidase

enzymes has been shown to result in chronic granulomatous disease (CGD) which is characterized by increased susceptibility and mortality due to bacterial and fungal infections [38]. Engulfment of pathogenic bacteria can trigger this oxidative burst, mediated in part by bacterial lipopolysaccharide (LPS) activation of the transmembrane Toll-like receptor 4 (TLR4), during the host innate immune response. Stimulation of TLR4 by LPS also induces signaling pathways that activate NFκB and promote production of the inflammatory cytokine interleukin (IL) 8; both these activities can be blocked by antioxidants [39,40].

2.2.2. Molecular targets

Work by Liu et al. [41] suggests that the NADPH oxidase system is key in the generation of ROS following respiratory syncytial virus (RSV) infection. The authors also demonstrate that generation of ROS promotes activation of signal transduction pathways responsible for the production of inflammatory cytokines and chemokines, apparently by ROS-mediated inactivation of intracellular tyrosine phosphatases. This allows increased phosphorylation and activity of signal transducer and activator of transcription (STAT) 1 and 3, transcription factors that regulate expression of interferon regulatory factors 1 and 7. These regulatory factors, in turn, promote up-regulation of genes necessary for an effective antiviral response. In addition to ROS role in RSV infection, other groups have shown roles for virus-induced ROS activation in influenza, HIV, hepatitis B and hepatitis C infections (reviewed in Liu et al. [41]).

2.2.3. Adaptive immunity

ROS-mediated activation of ERK and p38 pathways required for LPS-induced cytokine production by macrophages has been well documented. Specific examples of a role for ROS in this process include the effects of ROS production on synthesis of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), IL6, IL1β, and IL8. Work by Yoshida et al. [42] showed that xanthine oxidase–derived superoxide anions were involved in TNF-α-induced production of IL6 in bronchial epithelial cells, although not in lung fibroblasts. Lowering of superoxide anion levels by addition of superoxide dismutase suppressed IL6 synthesis; pretreatment with the antioxidant dimethyl sulfoxide (DMSO) also inhibited TNF-α-induced IL6 production in a dose-dependent manner. Hsu and Wen [43] demonstrated that IL1β production by LPS-stimulated macrophages requires ROS; preincubation with the antioxidant *N*-acetylcysteine decreased IL1β levels by 40%. Superoxide anions generated by NADPH oxidase appear to be the key ROS mediators of this pathway, and downstream activation of the p38 pathway had a significant role in IL1β production. Work by DeForge et al. [39] used whole blood cells to demonstrate the role of ROS on LPS induction of IL8. IL8 levels also could be increased by direct stimulation of cells with hydrogen peroxide; both hydrogen peroxide and LPS-mediated stimulation of IL8 production were suppressed by DMSO. This regulation occurred at the level of both mRNA and protein synthesis. Similar results with respect to IL8 levels were obtained using cells from CGD patients, which are typically defective in NADPH oxidase activity. The authors propose that these results indicate that the generation of ROS by other systems, e.g., radicals generated during mitochondrial electron transport, has a role in inducing cytokine production.

2.2.4. Inflammation

The generation of ROS during the innate immune response also modulates apoptosis of neutrophils at the site of inflammation [21]. Failure to down-regulate these processes can lead to pathologically chronic inflammation. Several groups have shown that a sustained and robust oxidative burst is required for neutrophil apoptosis. Zhang et al. [44] demonstrated that control of apoptosis by ROS is achieved by ROS promotion of cleavage and activation of caspase 8, which, in turn, activates caspase 3, triggering apoptosis. Pretreatment of neutrophils with an NADPH oxidase inhibitor blocked caspase 8 processing and subsequent apoptosis; similarly, neutrophils from CGD patients deficient in NADPH oxidase activity fail to undergo apoptosis. ROS formation triggered by phagocytosis also inhibits the generally prosurvival ERK pathway. However, within the inflammatory environment, neutrophils are exposed to prosurvival growth factors such as granulocyte–macrophage colony stimulating factor (GM-CSF), which sustains activation of the ERK pathway. Conversely, GM-CSF can trigger an oxidative burst, down-regulating ERK. This balanced crosstalk between proapoptotic and prosurvival signals allows neutrophils to survive long enough to complete necessary phagocytosis but eliminates the neutrophils once they have reached the end of their useful lifespan. In a similar situation, most activated antigen-specific T lymphocytes must die after the immune response peaks to prevent autoimmunity and ensure T-cell homeostasis. ROS involvement in activated T-cell apoptosis has been demonstrated by Hildeman et al. [45], who showed that T-cell death was characterized by superoxide generation and that a superoxide dismutase mimetic could prevent T-cell apoptosis.

2.2.5. Adaptive response targets

ROS also provide a connection between the innate immune response and subsequent adaptive immune responses, both by themselves and by their effects on production of proinflammatory cytokines. In the initial stages of infection, antigen levels often are suboptimal for activation of T cells through coordinated binding to the T-cell receptor CD3 and the costimulatory receptor CD28. However, survival of the host often is dependent on initiation of an immune response even before optimal antigen levels are attained. Hehner et al. [46] demonstrated that exposure of T cells to macrophage-produced hydrogen

peroxide or L-lactate facilitates a shift in the glutathione/glutathione disulfide ratio in the cell, allowing activation of response even at low antigen levels. Physiologically relevant amounts could substitute for antigen binding to CD3 but not to CD28. This ROS-induced shift in glutathione/glutathione disulfide ratio also was accompanied by activation of the MAPKs JNK and p38, signaling intermediates activated by ROS. Thus, production of ROS and their subsequent action on T cells may allow an early and robust immune response even at low levels of stimulating antigens. Additionally, up-regulation of inflammatory cytokine expression and production by ROS, as discussed above, is necessary for subsequent activation of T cells by macrophages/neutrophils initially mobilized by the invading pathogen. TNF-α and IL1β, in particular, are necessary to optimally activate differentiation of naive T cells [37].

Thus, production of ROS ties the innate immune response to the adaptive immune response and is required for production of cytokines and other inflammatory molecules. Precise regulation of ROS production and activity is a key to down-regulation of the immune response; failure to do this can result in prolonged inflammation, which may be a factor in certain pathological conditions. Modulation of ROS levels by drugs or dietary intervention could have an impact on treatment of these conditions. As mentioned earlier, in a more general sense, additional work on the overall interaction of diet and drugs on the actual immune mechanism is absolutely necessary.

2.3. Reactive oxygen species in disease

Actions of ROS have been implicated in diseases including diabetes, Alzheimer's and other neurodegenerative diseases as well as atherosclerosis contributing to CVD and cancer, particularly in a setting in which ROS generation or detoxification has become unbalanced. A large amount of research interest has focused on the roles of ROS in cancer and CVD, and a great deal of effort has been exerted to determine if antioxidant supplementation can be beneficial in preventing or treating these various conditions. Consequently, understanding the specific molecular effects of ROS on various risk factors of CVD and cancer is paramount to assessing the role of antioxidants on ameliorating these diseases and may aid in reconciling the sometimes-contradictory data generated by various epidemiological studies. These studies are discussed in more detail in Ref. [15].

2.4. Carcinogenesis and cancer etiology

Cancer can be viewed as a number of distinct diseases, each defined by different genetic lesions, with certain characteristics shared by most cancers and cancer types. These "hallmark capabilities" necessary for tumorigenesis were described by Hanahan and Weinberg [47] as: (1) self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis [48]. ROS and reactive nitrogen species (RNS) participate in these processes, contributing to cancer progression but also playing important roles in endogenous defenses, working to eliminate and controlling the spread of transformed cells. Due to recent increases in the number of cancer patients using antioxidant supplements, a greater understanding of both the damaging and protective actions of ROS in carcinogenesis is crucial to further advances in cancer treatment. Data from large-scale studies concerning supplements in cancer prevention and treatment in humans are often equivocal, reflecting the dual roles of ROS/RNS in malignant diseases. This is discussed in more detail in another review [15].

2.4.1. Genetic alterations

Mutagenic alteration of DNA is an initiating event in carcinogenesis, and mutations affecting gene expression and/or function, particularly those of tumor suppressor genes and proto-oncogenes; this can lead to unregulated growth typical of cancer cells [49]. DNA damage can arise from direct interaction of ROS or RNS with nucleic acid bases or the DNA backbone or can be caused by reactive compounds generated by oxidized lipids. One of the easiest-assayed and, therefore, widely studied forms of oxidative DNA damage is the oxidation of guanine residues, resulting in 8-oxo-deoxyguanosine (8-oxo-dG). These lesions induce G:C to T:A transversions that have been observed in oncogenes and tumor suppressor genes (eg, p53) known to have significant roles in carcinogenesis. The reliability of commonly used methods to determine levels of DNA oxidation is imperfect and controversial, leading to questions about the importance of oxidatively damaged DNA in cancer and other pathologies [50]; there has been, however, recent focus on methodological refinement, which have improved the usefulness of this biomarker [51].

Elaborate mechanisms exist for the removal or repair of this damaged DNA, which would seem to imply a need to correct this damage and thereby prevent abnormal cell function. Nonetheless, increased amounts of oxidative DNA damage, particularly generation of 8-oxo-dG and 8-oxo-deoxyadenosine, has been associated with cancers including breast cancer [52], in chronic inflammatory conditions potentially leading to cancer, such as hepatitis and cirrhosis [53], and in gastric tissue from subjects with *Helicobacter pylori* infections [54].

2.4.2. ROS balance

Many groups have noticed increased ROS production in cancer cells that may be due to dysfunctional ROS generators and/or detoxifiers. Aberrant regulation of ROS/RNS levels is widely believed to have a role in carcinogenesis. Several groups have assessed a number of tumor cell lines in which abnormally high levels of hydrogen peroxide are produced [55]; other cell lines have been shown to have reduced catalase and glutathione peroxidase

levels, suggesting an inability to detoxify hydrogen peroxide [56]. These observed abnormalities in ROS/RNS metabolism also have functional implications, as several lines of research have demonstrated, that experimental manipulation of ROS levels in cells can affect tumorigenicity. An increase in superoxide dismutase leading to a decrease in superoxide anion and an increase in hydrogen peroxide levels has been correlated with malignancy in breast cancer cell lines [57]. Stable transfection with human catalase, lowering hydrogen peroxide levels, has resulted in a reversion of the malignant phenotype of MCF-7 breast cancer cells, indicated by an increase in doubling time, decreased 5-bromo-2-deoxyuridine incorporation and a decrease in anchorage-independent growth [57].

Another group has demonstrated that reestablishing stable expression of catalase could also revert the transformed phenotype induced in NIH3T3 cells by overexpression of NADPH oxidase (which generated high levels of superoxide anion). The unreverted cells were shown to be able to produce aggressive tumors in an athymic mouse model [58,59]. A Wistar-derived rat strain with an inherited overgeneration of hydroxyl radicals has increased oxidative damage to DNA, lipids and proteins and developed cataracts, emphysema, cardiomyopathy and preneoplastic liver foci at higher rates than wild-type rats [60,61].

2.4.3. Tumor metabolism and progression

Besides altered regulation of enzymatic systems involved in generation and detoxification of ROS, several physical characteristics of tumors can also be associated with generation of ROS, including glucose deprivation, hypoxia, and inflammation arising from infiltrating macrophages. Glucose deprivation, observed as a result of the tumor outgrowing its blood supply, has also been observed to induce oxidative stress in cultured MCF7 breast cancer cells, triggering MAPK activity in these cells but not in nontransformed cells [62]. Hypoxia can develop as tumors exceed their blood supply, and the chaotic angiogenesis observed in tumors drives cycles of hypoxia and reperfusion, generating ROS. Additionally, ROS themselves have roles in the regulation of genes involved in oxygen sensing (HIF-1) and angiogenesis (VEGF). Macrophage infiltration within the tumor results in ROS production, as occurs during inflammation, and activated macrophages can produce cytokines, such as TNF-α, that induce oxidative stress.

A cellular environment in which ROS production and detoxification is not properly controlled can lead, in turn, to improper regulation of downstream molecular events that impinge on proliferation, apoptosis and cell migration, all of which have effects on cancer progression. The impact of aberrant levels of ROS observed in malignant cells can be viewed in the context of normal ROS function, which includes mediating the levels and activity of numerous transcription factors and other gene products that can influence cancer cell growth, angiogenesis, invasiveness and metastasis. The role of ROS in signal transduction by the MAPKs (ERKs, JNKs and p38 family members) has been described, and the precise modulation of these pathways leads to the proper growth response to mitogens. In normal cells, exposure to higher levels of hydrogen peroxide or superoxide anion leads to increased expression of growth regulated genes and proliferation. The potential impact of increased ROS on these pathways in cancer cells can be seen in the response of some cancer cell lines to increased (but sublethal) levels of ROS. Sustained activity of MAPK in response to ROS is observed in HeLa cells [63], and the hyperphosphorylation of JNK and increased activation of AP-1 observed in MCF7 breast carcinoma cells is associated with increased proliferation [64]. Activation of ERK1/ERK2 has been seen in multidrug-resistant breast cancer cell lines in response to oxidative stress induced by glucose deprivation [62]. A review by Benhar et al. [65] highlights the roles of p38 and JNK in tumorigenesis for a number of cancer types, including effects on proliferation and cell cycle progression, while an earlier review addresses the activity of oncogenes and tumor suppressor genes [59].

2.4.4. Gene targets

Cancer researchers have identified numerous oncogenes and tumor suppressor genes; ROS can participate in promoting carcinogenesis mediated by these genes. Transformation by overexpression of oncogenic Ras leads to increased superoxide anion levels produced by Ras/Rac1-mediated activity of inducible NADPH oxidase. This generates high levels of superoxide anions that serve as substrate for processing to hydrogen peroxide by manganese superoxide dismutase (MnSOD) [66]; high levels of MnSOD activity have been observed in highly invasive metastatic tumors [67]. Increased hydrogen peroxide levels generated by MnSOD appear to cause an increase in AP-1 and/or NFκB activity, aiding tumor invasiveness by up-regulation of matrix metalloproteinases and allowing increased degradation of the subcellular matrix, which is a key step in metastasis [68]. Activated Ras/Rac1 may be required for invasiveness mediated by MnSOD activity because MCF-7 cells that overexpress MnSOD but do not have activated Ras/Rac1 do not show an increased invasiveness [69]. Another oncogene involved in development of many cancers is the tumor suppressor p53; its activity also is affected by ROS. In work described in Ohshima et al., excess nitric oxide in cells leads to an accumulation of p53 protein, but the ability of p53 to bind to its consensus DNA sequence is decreased and as a result, tumor suppression activity of p53 is lost [48,70]. The p53 protein appears to be modified by the highly reactive nitric oxide derivative peroxynitrite, produced by excess nitric oxide and superoxide anion. Evidence of p53 peroxynitrite modification has been seen in gliomas and is associated with decreased p53 DNA binding [71].

2.4.5. Cell cycle effects

Other events promoting carcinogenesis, such as inhibition of apoptosis, senescence and DNA repair as well as promotion of angiogenesis, are affected by ROS. Nitric oxide (NO) and its derivatives appear to contribute to DNA damage, although evidence that this is integral to transformation is conflicting [72–74]. Nitric oxide has been demonstrated to inhibit the enzymatic activities of DNA repair enzymes such as alkyl transferase and DNA ligase, resulting in increased genotoxic burden on the cell [75,76]. Nitric oxide also can inhibit caspase 3-like activity, interfering with apoptosis, which is designed to eliminate transformed cells [77]. Two other cancer hallmarks, uncontrolled angiogenesis and impaired senescence, also are affected by the actions of ROS on transcription factors and other cellular proteins. Changes in oxygen levels regulate activity of the transcription factor HIF-1; this, in turn, regulates expression of genes involved in angiogenesis such as erythropoietin and VEGF, thereby contributing to the abnormal angiogenic activity of some solid tumors [64]. ROS also have been shown to activate cellular telomerase in endothelial cells, delaying normal replicative senescence [78].

2.4.6. Cellular response

Tumor development represents an imbalance between cell proliferation, strictly controlled in healthy cells, and apoptosis, used to remove damaged or precancerous cells. Despite their roles in processes promoting abnormal proliferation and tumorigenesis, ROS also can promote apoptosis, in this case, helping to prevent proliferation and the spread of transformed cells. As part of their abnormal proliferation, cancer cells need to evade apoptosis normally triggered by high levels of oxidative stress and cellular damage.

2.4.7. Tumor resistance potentiation

Researchers have long observed that precancerous cells or cells in early stages of cancer are more sensitive to chemotherapy and radiation treatment, than are normal cells, which often work by generating oxidative stress and inducing apoptosis, but this sensitivity is lost as the cancer progresses. The increase in basal ROS levels observed in transformed cells contributes to this by “potentiation” of SAPK activity [59]. In potentiated cells, activation of the JNK and p38 pathways is enhanced, and induction of kinase activity is more sensitive to lower doses of stress stimuli than in nontransformed cells. As experimental evidence of the effects of potentiation, two cell lines, EGF-transformed NIH3T3 cells and A431 epidermoid cancer cells, which show elevated ROS levels and enhanced induction of JNK and p38 pathways, also showed greater sensitivity to apoptosis induced by the chemotherapeutic agent cisplatin. In contrast, the HT29 colon cancer cell line, which has lower levels of ROS and JNK and p38 activity in comparison to the A431 cells and transformed NIH3T3 cells, is resistant to the cisplatin-induced cell death observed in those other two cell lines [65]. The enhanced susceptibility of potentiated transformed cells to undergo apoptosis in response to either endogenous or exogenous stress stimuli may be advantageous to the organism because potentially cancerous cells would be eliminated when still at an early stage of transformation. As cancer progresses, many tumors become drug-resistant; in these tumors, an increase in activity of antioxidant defense systems, including malondialdehyde formation, superoxide dismutase, glutathione peroxidase and catalase, has been observed. This results in decreased ROS and SAPK levels, thereby interfering with the apoptotic response to chemotherapeutic agents [57]. Additionally, resistance to apoptosis can occur as the cell acquires further genetic lesions, e.g., inactivation of tumor suppressor genes or amplification of anti-apoptotic genes such as members of the Bcl-2 family. Overexpression of MAPK phosphatase-1, which down-regulates JNK and p38, has been observed in prostate, colon and other tumors, rendering these cells insensitive to death mediated via the SAPK pathways [65]. Thus, intracellular levels of ROS may be a key to the understanding of the ability of ROS to participate in both cancer promotion and cancer control or elimination. While cellular damage caused by ROS can be tumor promoting, in some transformed cells, elevated levels of ROS lead to enhanced SAPK activity, making the cells more sensitive to apoptosis elicited by cancer-fighting therapies.

2.4.8. Overall function of ROS

The multiple (and conflicting) roles of ROS in initiation, progression and metastasis of cancer are also reflected in results from clinical trials which investigated the use of antioxidants in cancer treatment and prevention. To achieve the proper balance between cell proliferation and cell death, the levels of ROS need to be precisely regulated; overzealous antioxidant supplementation could theoretically upset this balance and potentially lead to undesirable effects. As some chemotherapy drugs and radiation regimes kill cancer cells by generating high levels of ROS, antioxidant supplementation could conceivably interfere with some cancer treatments, and some evidence for this exists. However, there are also indications that antioxidant supplementation can help to counteract side effects of some chemotherapy and may allow tolerance of higher doses and longer-lasting treatment regimens. With respect to cancer prevention in healthy individuals, some studies have shown antioxidant supplementation to be protective; this effect may be more pronounced in high-risk populations [79]. The bottomline clearly suggests a cautious approach on antioxidant supplementation unless clear mechanistic studies can support intervention in specific cases backed by clinical data confirming the hypothesis as valid and of low risk.

2.5. Cardiovascular disease

This section is included to provide background relating to potential effects on the cardiovascular system, which have been noted in some recent antioxidant clinical

intervention studies. No review of antioxidants would be complete without consideration of potential side effects and the mechanisms behind them. Cardiovascular diseases (CVD), including atherosclerosis and its attendant vascular disorders, comprise the leading causes of death in developed countries [80,81]. Vascular insults such as those associated with cigarette smoking, diabetes mellitus, hypertension and hyperlipidemia can trigger an inflammatory response in vessels; atherogenesis is generally accompanied by chronic low-grade inflammation [21,82]. Establishment of this inflammatory state, mediated in part by ROS, results in damage to vascular endothelial and smooth muscle cells. Endothelial dysfunction, characterized by pathological changes in endothelial cell anticoagulant, anti-inflammatory and vasorelaxation properties, promotes recruitment of monocytes, macrophages, growth factors and cellular hypertrophy, all of which contribute to the formation of atherosclerotic plaques. ROS activity helps drive many of these processes.

2.5.1. Lipid oxidation

One of the most well-known participants in atherosclerotic plaque formation is oxidized low-density lipoprotein (oxLDL) [80]. Low-density lipoprotein (LDL) is the major cholesterol carrier in plasma, and elevated levels of circulating LDL are associated with increased risk for atherosclerosis; increased levels of oxLDL have been associated with hypertension in men [83]. Vascular trauma generates aberrantly high levels of both intracellular and extracellular ROS in the vasculature, thus leading to fatty acid and lipid peroxidation, particularly under conditions of hyperlipidemia [84]. Oxidized lipids can affect cell function by accumulating in the cell membrane, causing leakage of the plasmolemma and interfering with the function of membrane-bound receptors [85]. Additionally, the by-products of lipid peroxidation, such as unsaturated aldehydes and other metabolites, have cytotoxic and mutagenic properties. oxLDL itself has a specific role in the pathogenesis of atherosclerosis. Seminal work by Goldstein and Brown [86] determined that oxLDL is specifically recognized and taken up by receptors called “scavenger receptors” that are expressed on macrophages infiltrating sites of vascular inflammation. Uptake of oxLDL by macrophages results in the formation of lipid-filled macrophages called “foam cells,” which undergo apoptosis and contribute significantly to the architecture of the atherosclerotic plaque [87]. oxLDL effects on the vessel walls themselves include stimulation of cytokine and growth factor production and generation of endothelial dysfunction, including inhibition of endothelial cell vasodilator function, all of which contribute to atherosclerosis.

2.5.2. Vascular and molecular responses

Regulation of endothelial vasorelaxation and vasodilation properties provides an example of the direct effect of a free radical, in this case NO, on vascular function [85]. Improper regulation of these processes is characteristic of endothelial dysfunction, a key step in the pathogenesis of CVD including atherosclerosis, hypertension, and heart failure, and traditional risk factors for these diseases have been shown to predispose to endothelial dysfunction. In addition to changes in the anticoagulant and anti-inflammatory properties of the endothelium, endothelial dysfunction also encompasses impairment of vasorelaxation regulated by the endothelium. Endothelial-derived relaxing factor, which modulates vasodilation, was determined to be the reactive nitrogen species NO, and endothelial dysfunction can result from the loss of NO bioavailability in the vascular wall. NO can be inactivated by superoxide anion and stabilized by the addition of superoxide dismutase; deviations in the balance between superoxide anion and NO by cellular antioxidant defenses has been noted in some common disease states [88]. Increases in superoxide anion levels by NADPH oxidase activity leads to loss of NO, subsequently altering vasodilation and relaxation qualities of the endothelium and ultimately contributing to vascular disease [89,90].

2.5.3. Signal transduction and molecular targets

In addition to the direct effect of NO on vessel walls, ROS participate in the pathogenesis of atherosclerosis through their ability to serve as second messengers in signal transduction pathways and as effectors of gene transcription [91,92]. Up-regulation of leukocyte adhesion molecules, such as vascular adhesion molecule 1 and intracellular adhesion molecule 1, increased apoptosis of endothelial cells, activation of matrix metalloproteinases, and altered vasomotor activity all are affected by generation of intracellular ROS. These events mediate recruitment of inflammatory cells and their attendant production of inflammatory cytokines including TNF-α, IL1β and IFNβ [82]. Additionally, ROS levels influence the accumulation and hypertrophy of vascular smooth muscle cells (VSMC) observed in atherosclerotic, restenotic lesions and hypertensive vascular diseases [93]. Intracellular ROS may provide a molecular mechanism coordinating diverse extracellular stimuli (such as cigarette smoking, trauma and diabetes) to initiate multiple processes involved in atherogenesis [94]. Cytokines, hormones, mechanical forces and other pathophysiological stimuli known to influence development of vascular diseases can regulate the activity of vascular Nicotinamide adenine dinucleotide (NADH)/NADPH oxidase, an important ROS source in VSMC. Angiotensin II, thrombin, PDGF, transforming growth factor α and lactosylceramide, which promote proliferation of VSMC, have all been observed to increase activity of NADH/NADPH oxidase (reviewed in Cai et al. [85]). This increase promotes production of intracellular ROS, in particular, hydrogen peroxide and superoxide anion, which influences activity of intracellular kinases including the MAPK p38 (previously determined to be regulated by ROS) leading to VSMC proliferation and hypertrophy [95]. Studies have determined

that the NADH/NADPH oxidase is the predominant enzyme involved in generation of superoxide anion in VSMC as treatment with catalase or superoxide dismutase suppresses ROS generation and inhibits kinase phosphorylation and cell proliferation [91].

2.5.4. Apoptotic response

Although ROS have growth-promoting effects on VSMC and endothelial cells, ROS also can suppress growth and/or induce apoptosis. Increased activity of the tumor suppressor p53 leads to increased ROS levels in VSMC, which, in this case, results in growth inhibition or apoptosis [96]. The ability of cells to distinguish between ROS as a proliferative signal and ROS as a growth inhibitor or apoptotic signal may be controlled by both dosage and duration of ROS exposure. Studies have demonstrated that exposure of VSMCs to low oxidative stress for a short time period leads to growth promotion, while exposure to high levels of oxidative stress for a long duration leads to cell death; this may be a reflection of the cell's capacity to detoxify and defend against ROS [97,98]. Additionally, the species of ROS may be an important deciding factor with superoxide anion leading to cell growth and hydrogen peroxide to cell death.

2.6. Overarching Issues

The scientific community used to view the production of ROS in cells as harmful because aberrant production or incorrect regulation may lead to the development of potentially fatal disorders. With the link between ROS and these diseases established, scientists put much effort into antioxidant research. Cells use antioxidants in normal cellular processes to mediate the immune response, cell proliferation, and signal transduction, as well as for quenching excess ROS. The myriad of interactions between antioxidants and ROS has received a great deal of interest in the scientific community of late, as conflicting data has arisen linking dietary antioxidant supplementation to protection against cancer and CVD as well as increased mortality from those same conditions.

Studies in health professionals failed to show marked prevention potential for either cancer or heart disease, while studies in smokers showed an exacerbation. Coronary effects have been linked to antioxidants in several different studies, but there were a number of conflicting findings when the literature is taken in toto. There are a number of possible explanations for the conundrum and animal model studies have addressed a few of these in mechanistic detail.

A more critical issue is the interaction of antioxidants and orthodox cancer therapy. There is no clear picture as the myriad anticancer treatments act at many different points in the cell cycle, many affecting only specific processes in cell division. The individual antioxidants also are multifunctional and can only be lumped together as oxygen or ROS scavengers. Their other physiological properties may be just as important and make the premise that antioxidants a priori will either stimulate or inhibit standard cancer treatment highly premature without as yet unavailable supporting evidence. These topics are covered in more detail in a recent review [15].

2.7. Issues needing additional research emphasis

Several areas require additional study, including the examination of individual antioxidants and combinations in a variety of models and populations for specific effects; "speciation" both of the antioxidants and the models; the variation in effect with low, normal and high physiological dosages and formulations and the examination of subgroup hypersensitivity due to diet, disease state, and polymorphic differences in populations. This could include the use of transgenic and knockout animal models and the study of pure compounds as well as enriched dietary milieu. The effects of antioxidants on inflammatory response and other early preneoplastic changes is another area needing careful investigation.

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