

# Oxidative pathways in cardiovascular disease Roles, mechanisms, and therapeutic implications

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## Abstract

Despite some recent declines, cardiovascular disease (CVD) remains the major cause of death in the United States and worldwide. Most recent advances in the treatment of CVD states have been produced by inhibition of mechanisms involved in disease progress. Many studies conducted in the last decade have illustrated increased biological oxidative pathways during CVD in animals and humans. Thus, increased production of reactive oxygen species may be a unifying mechanism in CVD progression, and antioxidants may have therapeutic value in this setting. In this review we address the following questions: Do oxidative mechanisms play a role in CVD? Where do the oxidants come from? What are the relevant oxidative events? What are the therapeutic implications? © 2001 Elsevier Science Inc. All rights reserved.

**Keywords:** Cardiovascular disease; Reactive oxygen species; Oxidants; Antioxidants; Nitric oxide; Peroxynitrite

**Abbreviations:** ACE, angiotensin-converting enzyme; AngII, angiotensin II; AP-1, activation protein-1; CHD, coronary heart disease; CHF, congestive heart failure; Cu/Zn-SOD, copper/zinc superoxide dismutase; CVD, cardiovascular disease; EC-SOD, extracellular superoxide dismutase; ERK, extracellular responsive kinase; FFA, free fatty acids; GSH, glutathione; IL, interleukin; JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MI, myocardial infarction; Mn-SOD, manganese-containing superoxide dismutase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NO, nitric oxide; NOS, nitric oxide synthase; ONOO<sup>-</sup>, peroxynitrite; oxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; SOD, superoxide dismutase; SR, sarcoplasmic reticulum; XO, xanthine oxidase; TNF, tumor necrosis factor.

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## 1. Introduction

Despite some recent declines, cardiovascular disease (CVD) remains the principle cause of death in both developed and developing countries, accounting for roughly 20% of all worldwide deaths per year. Combined, these conditions (hypertension, arrhythmia, coronary artery disease, myocardial infarction [MI], and cardiac failure) also represent the leading killer of males over the age of 45 and females over the age of 65 in the United States, and account for ~750,000 deaths annually (Hennekens, 1998). Since cardiac disease is typically progressive and often associated with inter-related disease states, these conditions also represent significant social costs requiring years of therapy and extensive health care costs. In 2000 alone, the total cost (direct and indirect) of CVD in the United States was estimated to be more than \$275 billion. As the population ages and costs rise, this annual estimate is expected to increase (American Heart Association, 2000).

Although these statistical data are intimidating, important advances in the understanding of CVD initiation and progression have emerged, and therapies continue to evolve as mechanisms are defined. Since CVD has been viewed historically as a hemodynamic disorder, traditional approaches for its management originally relied heavily on drug therapies that reduce fluid retention and/or systemic blood pressure or enhance cardiac contractility. These therapies were intended historically to alleviate symptoms of disease rather than addressing mechanistic processes. However, several agents used to control symptoms in the short term (such as diuretics and direct-acting vasodilators) have not been shown to affect long-term survival or disease progression in large clinical trials (Levinson et al., 1999). Thus, hemodynamic status apparently is not a sole determinant in disease progression. More recently developed approaches involve inhibition of pathways that contribute to disease progression. For example, inhibition of the renin–angiotensin system (via angiotensin-converting enzyme [ACE]

inhibition) has provided symptomatic improvement, as well as enhanced survival in long-term trials (Sharpe, 1999; Yusuf et al., 2000a). ACE inhibitors are now established as standard initial and maintenance treatment for congestive heart failure (CHF) in combination with diuretics, and newly developed angiotensin receptor antagonists may have similar value. Reduction in blood lipid profiles has also been shown to have value in reducing disease progression and enhancing long-term survival. Evidence of regression and delayed progression of coronary artery disease was observed in large-scale, randomized secondary prevention trials of lipid-lowering therapy during 1975–1996 (Brown & Zhao, 1999).

An emerging area in cardiovascular research is the apparent significance of biological oxidation mechanisms. Many recent studies have suggested that oxygen-derived free radicals may be important participants in a wide array of cardiac conditions, and several clinical trials evaluating the use of antioxidants as therapeutics either have already been conducted or are underway. In this review, we will address the following questions: *Do oxidative mechanisms play a role in CVD? Where do the oxidants come from? What are the relevant oxidative events? What are the therapeutic implications?*

## 2. Oxidative events in cardiovascular disease

Several recent studies have demonstrated that altered oxygen utilization and/or increased formation of reactive oxygen species (ROS) contribute to CVD progression. A brief and representative listing of evidence for oxidative mechanisms in these conditions is shown in Table 1.

The initial suggestions of oxidative mechanisms during CVD were described in the acute settings of ischemia/reperfusion injury and MI. These conditions are associated with a sudden reduction of coronary perfusion and oxygen availability, leading to altered myocardial metabolism, ROS production, and cell death. Interestingly, ROS production

Table 1  
Representative evidence of increased ROS production in CVD

Condition	Evidence	Reference
Hypertension	Vascular smooth muscle cell proliferation induced by ROS in vitro and in vivo AngII promotes oxidant production via NADH/NADPH oxidase	Griendling and Ushio-Fukai, 1998; Ushio-Fukai et al., 1998 Fukai et al., 1999
Coronary artery disease (atherosclerosis)	Superoxide production-mediated endothelial dysfunction Superoxide production-mediated endothelial dysfunction	Kanani et al., 1999 Britten et al., 1999
MI	Increased oxLDL Ischemia/reperfusion injury driven by ROS formation Oxidant-derived myocyte necrosis and/or apoptosis	Jimi et al., 1998 Ferrari et al., 1998 Anversa et al., 1998
Heart failure	Increased NO production induces cardiac dysfunction Cytokine-derived ROS induces cardiac apoptosis ROS-induced cardiac apoptosis and/or necrosis	Sawyer and Colucci, 1998 Ing et al., 1999; Pulkki, 1997 von Harsdorf et al., 1999; Taimor et al., 1999

and associated cellular damage is higher in cardiac tissue during tissue reperfusion relative to ischemic conditions.

The acute (and relatively severe) paradigm of cardiac ischemia and reperfusion has provided insight into the mechanisms of ROS-induced alteration of cardiac function and disease progression. In fact, it is now recognized that ROS may contribute to the progression of other more chronic cardiovascular conditions that are not related to acute oxygen deprivation. Supportive evidence of these phenomena is briefly listed in Table 1. In addition to cellular and/or tissue evidence of oxidative damage, elevated levels of oxidative stress markers are detected in several pathologic conditions of cardiovascular disorders, including hypertension, ventricular hypertrophy, atherosclerosis, and CHF (Carlos et al., 1998; Gokce et al., 1999; Harjai, 1999; Keith et al., 1998; Miller et al., 1998; Suzuki et al., 1998).

In summary, despite the diverse etiology of cardiovascular conditions, the enhanced production of ROS and altered oxygen utilization is apparently a common phenomenon and a participant in disease progression. Further understanding of the events that contribute to these changes, and the cellular adaptations involved, may provide new opportunities for rational therapeutic strategies.

### 3. Sources of reactive oxygen/nitrogen species during cardiovascular disease

The general phenomenon of increased ROS production in CVD is becoming increasingly evident, but the actual sources of these species and mechanisms involved may not be identical in all conditions. Important ROS in mammalian cells include superoxide anion ( $O_2^-$ ), hydroxyl radical ( $OH^\cdot$ ), and hydrogen peroxide ( $H_2O_2$ ). Recent investigations have suggested that the reactive nitrogen species peroxynitrite ( $ONOO^-$ ) also plays an important role in cardiovascular dysfunction. As described in Sections 3.1–3.3, there are several potential contributors to cellular ROS and  $ONOO^-$  increases during CVD, but the major sources of oxidants can be different in various settings, such as in acute ischemia/reperfusion (e.g., MI) and chronic conditions (e.g.,

heart failure). While direct evidence of ROS-induced cardiac injury during hypoxia or ischemia/reperfusion in humans is lacking (due to inadequate methodology), many studies have shown increases in biomarkers of oxidant production and/or decreases in antioxidant capacity during myocardial ischemia (Buffon et al., 2000; Miwa et al., 1999). Furthermore, the administration of antioxidants reduces cardiac cell injury and dysfunction in acute MI (Singh et al., 1996), coronary angioplasty (Rajakumar et al., 1999), and open heart surgery that mimics myocardium ischemia/reperfusion (Fabiani et al., 1993).

#### 3.1. Uncoupling of mitochondrial electron transport

This is a classical mechanism of intracellular oxidant production. Under normal physiological conditions, oxygen is essential for mitochondrial oxidative phosphorylation reactions and for production of ATP. The potentially toxic species (i.e., ROS) are formed intracellularly during mitochondrial electron transport, and are controlled by intracellular antioxidant defense. The lack of oxygen supply by either hypoxia (reduction of arterial oxygen partial pressure, but sufficient perfusion) or ischemia (reduction or interruption of coronary blood flow) disrupts mitochondrial electron transport chain, resulting in an accumulation of toxic metabolites, acidosis, ATP depletion, intracellular  $Ca^{2+}$  overload, mitochondrial membrane depolarization, matrix swelling, and cell death (Lemasters et al., 1997). Interestingly, it is now well established that although restoration of blood flow prevents progression of ischemic cell necrosis, it also causes “reperfusion injury” in surviving cells (Ambrosio & Tritto, 1999). This phenomenon is associated with a massive production of ROS due to the resumption of oxygen supply to mitochondrial respiration. Several experimental hypoxia or ischemia/reperfusion models, both in vitro and in vivo, have suggested that injury in the myocardium is caused by oxygen radicals from mitochondrial electron transport (Lesnefsky et al., 1997; Vanden Hoek et al., 1997, 1998). The generation of large amounts of ROS can overwhelm the intracellular antioxidant defense network, causing activation of neutrophils, lipid peroxidation,

protein modification, and DNA breaks (Ambrosio & Tritto, 1999; see Section 4).

### 3.2. Immune cell infiltration and cytokines

It is now well established that many immune cells produce free radicals as a means of host defense and pathogen killing. As such, infiltration of activated immune cells into cardiac muscle is a potential mechanism of cardiac oxidant production. The ROS burst during post-ischemic reperfusion is accompanied by cell death and tissue injury, which can trigger an acute inflammatory response. The expression of immune cell chemoattractants and surface cell adhesion molecules leads to the infiltration of the tissue by immune cells (particularly neutrophils). These activated neutrophils may cause more damage to the tissue by the secretion of several mediators, including ROS, proteolytic enzymes, and pro-inflammatory cytokines. It has been shown that inhibition of neutrophil migration reduces reperfusion cardiac injury in feline (Buerke et al., 1994) and ischemic human hearts (Fabiani et al., 1993). Furthermore, clinical studies in cardiopulmonary bypass (which mimics global cardiac ischemia/reperfusion) patients have demonstrated that neutrophil activation, leukocyte–platelet aggregation, and cytokine release from cardiac tissue are the primary contributors of an acute inflammatory reaction during post-ischemic reperfusion (Zahler et al., 1999). These findings suggest that neutrophils (and other immune cells, including monocytes/macrophages) are key participants in at least the early events in acute hypoxia/ischemia. This mechanism may be an early response to tissue injury, and may further accentuate cardiac dysfunction and remodeling. It is important to note that immune cell infiltration during chronic settings of cardiac disease is less well-established.

### 3.3. Induction of oxidative enzymes

While “escape” of ROS from the mitochondria has been a classical mechanism of intracellular oxidation, many recent studies suggest that non-mitochondrial sources may be equally or perhaps more important in some cardiovascular conditions. Activation and/or induction of cytosolic oxidases (such as NADH/NADPH oxidase, xanthine oxidase [XO], and nitric oxide synthase [NOS] isoforms) has been demonstrated during several physiological stress conditions. The potential significance of these processes is described in the following sections.

#### 3.3.1. Xanthine oxidase

Under normal physiological conditions, XO is a key enzyme in the purine degradation pathway. The enzyme generates the final product, uric acid (excreted by urine), and the byproduct, superoxide anion. Under normal physiological conditions, this enzyme is localized almost exclusively in the liver and the mucosa of the small intestine.

However, excess ROS production, during chronic hypoxia or in the presence of increased inflammatory cytokines, can enhance XO activity and its release into the plasma. It has been demonstrated that significantly increased XO activity (and superoxide production) in the mesenteric tissue may be responsible for escalating vascular tone in an animal model of essential hypertension (Suzuki et al., 1998). Recent studies suggest that the elevated levels of circulating XO can be concentrated several-fold in the vascular tissue, and may be a significant participant in endothelial dysfunction in hypercholesterolemic rabbits and atherosclerotic humans (Houston et al., 1999).

In addition to plasma increases in XO, induction of XO in cardiac tissues may also contribute to excess production of superoxide. It is important to note that the abundance and activity of XO, as well as endogenous antioxidant defense in the myocardium, is species-specific (Janssen et al., 1993). For example, XO mean activity (mU/g protein) is considerably higher in mice (33), rats (28.5), and guinea pigs (14.4) than in cows (3.7), rabbits (0.59), humans (0.31), and pigs (<0.1) (de Jong et al., 1990). Despite the differences in basal XO levels, it has been shown that pretreatment with the XO inhibitor allopurinol improved cardiac function in isolated ischemic/reperfused rat and rabbit hearts (Brown et al., 1988; Terada et al., 1992) and in human trials during coronary bypass surgery (Bochenek et al., 1990; Castelli et al., 1995; Coghlan et al., 1994). Thus, XO may play a role in some conditions of cardiac oxidative stress, and the clinical value of its inhibition awaits further large-scale trials.

#### 3.3.2. NADH/NADPH oxidase

The enzyme NADPH oxidase generally is found in phagocytic cells. It plays an important role in nonspecific host defense during infection by generating a large quantity of superoxide (millimolar). Recently, it has been demonstrated that vascular NADH/NADPH oxidase significantly contributes to superoxide production in all components of the vasculature, i.e., the endothelium, the medial smooth muscle, and the adventitia (Bayraktutan et al., 1998; Di Wang et al., 1999). Participants in cardiac disease such as angiotensin II (AngII) may activate superoxide production in vascular tissue via this enzyme. This enhanced production of superoxide has been implicated in the pathogenesis of AngII-induced vascular hypertrophy and endothelial cell dysfunction (Griendling et al., 1994; Rajagopalan et al., 1996; Ushio-Fukai et al., 1998; Wattanapitayakul et al., 2000a).

#### 3.3.3. Nitric oxide synthases

It recently has been recognized that several cytokines (e.g., interleukin [IL]-1 $\beta$ , IL-6, interferon- $\gamma$ , and tumor necrosis factor [TNF]- $\alpha$ ) and growth factors (e.g., insulin-like growth factor-1 and transforming growth factor- $\beta$ 1) are pro-oxidants, and elevated levels are commonly found in the plasma of patients with heart disease (Djurovic et al., 1999;

Ruotolo et al., 2000; Torre-Amione et al., 1996; Ueda et al., 1999). Increased nitric oxide (NO) production via induction of NOS has been suggested as a major mechanism by which cytokines mediate cardiac contractile dysfunction and development of heart disease (Sawyer & Colucci, 1998; Schulz et al., 1995; Wildhirt et al., 1995).

The cytokine-induced increases in oxidants are regulated by various stimuli in several cell types engaged in tissue repair and restoration of homeostasis, as well as immune response. For example, increased cytokine release was found in acute hypoxia followed by reperfusion and in myocardium stunning. These cytokines enhance the expression of a variety of cell adhesion molecules (e.g., intercellular adhesion molecule-1, vascular cell adhesion molecule, and monocyte chemoattractant protein-1) in the myocardium, leading to transient leukocyte sequestration and its transmigration to the areas of cardiac injury (Kacimi et al., 1998; Matsumori et al., 1997). In addition to acute activation of cytokines in MI, elevated plasma levels of pro-inflammatory cytokines, along with high oxidase activities, are commonly observed in a more chronic pathologic condition of the cardiovascular disorders such as in heart failure patients (Katz et al., 1994; Leyva et al., 1998; Milani et al., 1996; Torre-Amione et al., 1996). Recent studies suggest that the heart per se is capable of synthesizing biologically active TNF- $\alpha$ , which may be responsible for the progression of heart diseases (Bergman & Holycross, 1996; Kapadia et al., 1995).

Additionally, other potent oxidants can be generated from reactions of NO with other ROS. For example, NO avidly reacts with superoxide anion at diffusion-limited rates to form the potent oxidant ONOO<sup>-</sup> (Beckman, 1996). The presence of ONOO<sup>-</sup> and its biological marker 3-nitrotyrosine has been associated with several oxidant-related pathologic conditions, including the atherosclerotic lesion, endothelial cell dysfunction, ischemia/reperfusion injury, MI, and heart failure (Bauersachs et al., 1999; Buttery et al., 1996; Kooy et al., 1997; Wattanapitayakul et al., 2000a). Clearly, the actions of NO in vivo may be governed not only by production capabilities, but also by the setting and chemical environment in which it is formed. Reactions of ONOO<sup>-</sup> with pathologically relevant molecules have been implicated in oxidative-related cardiovascular disorders (see Section 4). Additionally, we recently have found evidence that the ONOO<sup>-</sup> biomarker 3-nitrotyrosine (free amino acid) is toxic to endothelial cells (Mihm et al., 2000).

#### 4. Consequences of cardiac oxidative events

##### 4.1. Cardiovascular dysfunction

**Not only are oxidants associated with CVD, they actually may mediate some aspects of cardiac and vascular dysfunction.** For example, in both experimental and pathologic

conditions, impaired vascular function and decreased cardiac performance are mediated by ONOO<sup>-</sup> and other ROS (Bouloumié et al., 1997; Ferdinandy et al., 1999; Matsuura & Shattock, 1991a, 1991b; Miller et al., 1998; Rubanyi & Vanhoutte, 1986; Terada, 1996). ROS have direct impact on myocardial function through inhibition of the sarcoplasmic reticulum (SR) Ca<sup>2+</sup> pump in the cardiac contraction–relaxation cycle (Matsubara & Dhalla, 1996; Temsah et al., 1999). Cardiac depression (cardiac stunning) during ischemia/reperfusion and tissue damage in acute MI was prevented by using superoxide dismutase (SOD) and the antioxidant, vitamin E (Carrasquedo et al., 1999; Nagel et al., 1997). Benefits from inhibition of superoxide production have also been shown in cytokine-induced cardiac dysfunction (Cheng et al., 1999).

Pro-inflammatory cytokines and immune cell activation are modulators of cardiovascular function by a variety of mechanisms, including the generation of oxygen-derived free radicals and the production of NO (Cheng et al., 1999; Ing et al., 1999). Elevated plasma and tissue levels of the cytokine TNF- $\alpha$  are commonly observed in severe cardiac depression, along with increased NO production (Fukuchi et al., 1998; Torre-Amione et al., 1996; Wildhirt et al., 1995). In contrast, it has been suggested that NO plays a protective role in ischemia and reperfusion by quenching the effects of superoxide (and other ROS) on mitochondrial Ca<sup>2+</sup> homeostasis (Hotta et al., 1999).

NO is important in reducing ischemia/reperfusion-induced adhesion of monocytes to post-ischemic endothelium and in inhibiting induction of pro-inflammatory cytokine and adhesion molecule synthesis (Grisham et al., 1998). Endothelial-derived NO plays a key role in the local regulation of vasomotor tone and the prevention of thrombus formation. Studies from our laboratory and others have suggested that reduction in the bioavailability of NO and endothelial cell dysfunction may be initial events in atherosclerosis and cardiovascular disorders (Ferrari et al., 1998; Lyons, 1997; Wattanapitayakul et al., 2000a). Additionally, endothelial cell dysfunction is one of the earliest events in the pathogenesis of myocardial reperfusion injury, and NO plays an important role in cardioprotection during reperfusion by directly enhancing coronary blood flow and by preventing adhesion of immune cells (Kupatt et al., 1996). The early decline in coronary NO release occurs simultaneously with the oxygen-derived free radical burst observed in the ischemic/reperfused heart (Zahler et al., 1999), suggesting that superoxide-induced reductions in NO may play a role in disease initiation and/or progression.

##### 4.1.1. Too much or too little nitric oxide?

Involvement of NO in CVD is different in specific organs/tissues and pathologic environments. Depending on tissue availability, NO can act as a “good guy” (inhibit platelet aggregation, prevent immune cell infiltration, maintain vascular tone, etc.) or a “bad guy” (induce cardiac dysfunction, activate apoptosis, generate a potent oxidant

ONOO<sup>-</sup>, etc. [see Sections 4.1 and 4.2]). Since NO is highly regulated (i.e., produced and acts locally), its bioavailability is dictated by the surrounding chemical environment (e.g., destroyed by superoxide or protected by SOD or antioxidants) and the amounts produced (e.g., induction of enzyme NO synthases). Thus, the net concentrations of NO at the tissue level may predict its protective effects or toxic effects. Furthermore, several recent investigations suggest that genetic polymorphisms of the NOS3 isoform (also known as endothelial NOS) occur in humans and that these molecular variations may play a role in NO control and CVD risk (Wattanapitayakul et al., 2000b).

#### 4.2. Cell death

**Two types of cell death—necrosis and apoptosis—are both implicated in the oxidative-related cell loss in cardiovascular tissue.** Generally, necrotic cell death is associated with inflammatory cell infiltration and subsequent collagen deposition and scar formation, while apoptotic cell death is differentiated by ultrastructural and biochemical features, such as cytoplasmic and nuclear condensation, formation of the membrane-bound apoptotic body, and DNA fragmentation (~ 180 bp). Although recognized since 1972, the apoptotic phenomenon has only been described in CVD over the last decade. It appears that cardiac and vascular cell loss observed during the remodeling process occurs in the absence of necrosis. This event may be exclusively controlled via apoptotic signaling pathways. Discussed in the following sections is the involvement of ROS in both types of cell death in the models of CVD.

##### 4.2.1. Necrosis

Prolonged ischemic conditions may cause irreversible myocardial cell injury and cell death via necrosis. Transmigration of immune cells from the vasculature into the myocardium results in the release of toxic mediators that induce myocardial cell dysfunction and necrotic cell death (Grisham et al., 1998). Unlike apoptosis, necrosis generally does not appear in more chronic oxidative conditions, but occurs primarily in prolonged ischemic conditions (Taimor et al., 1999).

##### 4.2.2. Apoptosis

Recently, the recognition of a different cell death phenomenon, “apoptosis,” has become of major clinical interest. It accounts for a great proportion of cell death associated with MI and/or myocardial ischemia/reperfusion. Cell loss through apoptosis contributes to the impairment of cardiac performance, and also plays an important role in the myocardial and vascular remodeling processes. Induction of apoptosis is implicated in atherogenesis and cardiac dysfunction. Not only ROS per se, but also their oxidative products and other secondary messenger molecules generated by ROS can trigger the programmed cell death. For example, oxidized low-density lipoprotein (oxLDL) has

been shown to induce DNA fragmentation and apoptosis in macrophages (Kinscherf et al., 1998). The antioxidant N-acetylcysteine prevented apoptosis induced by oxLDL, ceramide, TNF- $\alpha$ , and H<sub>2</sub>O<sub>2</sub> (Kinscherf et al., 1998; Insette et al., 2000).

ROS-induced cardiac apoptosis is mediated through several signaling systems, including intracellular Ca<sup>2+</sup>, cytokines, lipid oxidation, and proto-oncogene activation. The perturbation of intracellular Ca<sup>2+</sup> homeostasis by the cellular redox state can aggravate free radical reactions and can activate endonucleases via activation of caspases—key enzymes in the apoptotic pathway. Additionally, many recent studies have demonstrated that the intrinsic degree of oxidant production regulates cellular susceptibility to apoptosis through both p53-dependent and p53-independent pathways (Lotem et al., 1996; von Harsdorf et al., 1999). Different ROS activate distinct signaling pathways for programmed cell death in cardiac myocytes (von Harsdorf et al., 1999).

It has been shown that the saturated fatty acids palmitate and stearate induce apoptosis in neonatal rat myocytes (de Vries et al., 1997). Both palmitate and stearate are precursors of de novo synthesis of ceramide—a second messenger of the sphingomyelin signaling pathway. This signaling pathway is initiated by the hydrolysis of the plasma membrane sphingomyelin to ceramide. Ceramide and sphingolipid metabolites are involved in the antiproliferative responses and apoptosis in several cell types, including cardiac myocytes. Several stimuli known to trigger sphingolipid-induced apoptosis include TNF- $\alpha$ , interferon- $\gamma$ , ionizing irradiation, and ischemia/reperfusion (Sparagna & Hickson-Bick, 1999). The cytokine TNF- $\alpha$  is pro-apoptotic via several signaling pathways, including activation of the Fas ligand and the binding of TNF to the death domain of TNF receptors. Binding of TNF- $\alpha$  and the Fas ligand to their receptors results in degradation of sphingomyelin to ceramide, which mediates apoptosis through induction of c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) (see Section 4.6).

NO can mediate both pro-apoptotic and anti-apoptotic signals, depending on the level of oxidants/antioxidants and predominant regulatory pathways endowed in the cell types (Kim et al., 1999; Hotta et al., 1999). For example, it appears that NO-induced apoptosis in vascular smooth muscle cells is mediated by cyclic GMP accumulation, while intracellular elevation of cyclic GMP, in response to NO activation, inhibits apoptosis in PC12 cells and hepatocytes. The formation of ONOO<sup>-</sup> from the interaction of NO and superoxide may account for the pro-apoptotic effects of NO. ONOO<sup>-</sup> is a potent oxidant that induces DNA fragmentation and p53-dependent apoptosis. In addition, the accumulation of the tumor suppressor protein p53 is a crucial and early event in NO-mediated apoptosis (Kim et al., 1999).

In summary, ROS-induced changes in cellular signaling and gene expression, leading to apoptosis, involve multiple

pathways. There is not one unifying pathway in all pathologic conditions of CVD. Rather, it is a complex and intertwined process, requiring several selective participants in each pathologic condition or cell type.

#### 4.3. Altered endogenous antioxidant defenses

Oxidative stress is a reflection of excess intracellular concentrations of oxidants, such as  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ , as well as antioxidant defense molecules such as glutathione (GSH). The tripeptide GSH (consisting of glutamate, cysteine, and glycine) is important for cellular defense against ROS toxicity. It is the key cellular reductant to maintain the redox state of cysteine–thiol linkages in proteins. The intracellular levels of oxidized GSH (GSSG) is increased by the metabolism of  $\text{H}_2\text{O}_2$  and by GSH peroxidase, but decreased by extracellular GSSG export and GSH reductase. Depletion of the protective form of GSH by excess amounts of ROS leads to increased protein oxidation at the cysteine–thiol linkage. This modification has significant impact on protein conformation and protein function. Additionally, these alterations can occur in important proteins that function as receptors, enzymes, or signal transducers, thus impairing normal cellular processes. Additionally, GSH participates in maintaining ascorbic acid (vitamin C), an antioxidant, in its reduced form (the active form).

Low levels of GSH are associated with a number of disease conditions known to generate high amounts of ROS, such as observed in atherosclerosis, heart failure, diabetes, neurodegenerative disorders, and acquired immunodeficiency syndrome. Increased oxidative stress in CHF patients is associated with increased GSH peroxidase and decreased plasma antioxidant vitamins, e.g., vitamins C and E (Keith et al., 1998).

In addition to GSH, the most important endogenous antioxidant defense against superoxide is the enzyme SOD. Three isoforms of SOD have been cloned and identified: mitochondrial manganese-containing SOD (Mn-SOD), cytosolic copper/zinc SOD (Cu/Zn-SOD), and extracellular SOD (EC-SOD). Cu/Zn-SOD plays an important role in protecting NO from destruction by superoxide in the endothelium (Harrison, 1997).

An important question is whether the long-term consequences of oxidative stress are due to excess production of ROS or depletion of antioxidant defenses. While a myriad of evidence supports increased ROS production during CVD, depletion of endogenous chemical antioxidants and some reductions in antioxidant enzyme capacity have also been reported (albeit, at a less frequent rate). For example, reductions in cardiac antioxidant enzyme function, including GSH peroxidase, catalase, and SOD isoforms, have all been observed in a variety of animal models of cardiac disease (Singal et al., 1993; Kapoor et al., 1997; Lin et al., 1997; Haramaki et al., 1998). Interestingly, the potent oxidant  $\text{ONOO}^-$  can inactivate the Mn-SOD via

nitration of a specific tyrosine residue at its active site (Yamakura et al., 1998; MacMillan-Crow & Thompson, 1999), further promoting ROS availability. Whether deficient enzyme function is consistently involved in human cardiac disease is not clear, but recent studies also suggest that genetic variation in enzyme expression may be an important contributor to disease risk. For instance, polymorphic variations in the Mn-SOD isoform have been linked to increased risk of dilated cardiomyopathy (Hiroi et al., 1999). Several recent studies have also suggested that a high degree of EC-SOD genetic variant is associated with coronary artery disease and other risk factors (Wang et al., 1998, 1999). Clearly, excess production of ROS is not the only predictor of CVD risk; depletion of antioxidant reserve is also critical. Further research in the areas of defining oxidant and antioxidant balance during disease and the participation of genetic variations in the antioxidant defense network is warranted.

#### 4.4. Altered lipid and protein metabolism/function

##### 4.4.1. Lipid metabolism and lipid peroxidation

Lipids, both in free and bound forms, are vulnerable and represent one of the immediate targets of ROS. The overall effects of lipid peroxidation include diminishing membrane fluidity, increasing membrane permeability, destabilizing membrane receptors, and inducing immune response to altered phospholipids. Described in the following sections are three major lipid categories that contribute to the pathogenesis of CVD.

**4.4.1.1. Long-chain free fatty acids.** Elevated plasma levels of free fatty acids (FFA) are implicated in many pathologic conditions, as observed in myocardial ischemia, diabetes, hyperlipidemia, and cardiac hypertrophy (Sparagna & Hickson-Bick, 1999). Under normal physiological conditions of the heart, FFA are the preferred source of energy generated via  $\beta$ -oxidation within the mitochondrial matrix. FFA are estimated to account for 60–70% of the oxygen consumption for energy production (Grynberg & Demaison, 1996). However,  $\beta$ -oxidation cannot proceed under oxygen-deprived conditions, such as in hypoxia or ischemia, where FFA and their metabolites become harmful. When FFA metabolism is inhibited, metabolic intermediates are accumulated and incorporated into cell membranes, i.e., sarcolemma, SR, and mitochondrial membrane. The intermediates of lipid metabolism interfere with membrane integrity and the function of membrane-bound enzymes (by altering their conformations and affecting ion pumps). High levels of FFA and their metabolites impair  $\text{Ca}^{2+}$  homeostasis and ion gradients, which may lead to cardiac arrhythmias during ischemia/reperfusion (Hendrickson et al., 1997).

**4.4.1.2. Membrane lipids.** In addition to oxidation of FFA in the cytosolic compartment, ROS also react with mem-

brane-bound lipids leading to “lipid peroxidation.” Generally, reperfusion injury occurs when oxygen from the recirculating blood is exposed to reactive intermediate compounds formed during the ischemia. The interactions of those compounds lead to the generation of oxygen free radicals and highly active molecules, such as the superoxide anion, hydroxyl radical, and hydrogen peroxide. These ROS further react with polyunsaturated lipids in membranes generating lipid peroxidation products that can inhibit protein synthesis and alter enzyme activities. Oxidation of polyunsaturated fatty acid moieties of membrane phospholipids can cause membrane disintegration, mitochondrial dysfunction, and  $\text{Ca}^{2+}$  overload.

It has been shown that altered cellular redox state and increased lipid peroxidation are associated with the transition of cardiac hypertrophy to heart failure (Dhalla et al., 1996). Elevated levels of plasma lipid peroxides were also observed in CHF patients (Keith et al., 1998; Díaz-Vélez et al., 1996).

**4.4.1.3. Lipoproteins.** It is widely accepted that hypertriglyceridemia and abnormal lipoprotein profile are associated with an increase in cardiovascular risk. **Polyunsaturated fatty acid residues in lipoproteins are chemically vulnerable to free radical oxidation.** Unlike the superoxide anion, other oxidizing species, such as the hydroxyl, peroxy, and alkoxy radicals, are capable of entering the hydrophobic membrane interior and initiating free radical chain reactions (Maxwell & Lip, 1997). Therefore, endogenous antioxidants in the hydrophilic cellular compartment cannot prevent propagation of the carbon-centered radical and oxidation reaction, which leads to long-chain lipid (hydro)peroxidation. **These unstable peroxides are degraded rapidly and form secondary products that are toxic to cells. These adducts can interact with LDLs, resulting in oxLDLs.** These modified LDLs abnormally affect cellular recognition and are chemotactic for circulating monocytes and toxic to endothelial cells (Diaz et al., 1997). After monocytes enter the arterial wall, they differentiate into macrophages and take up oxLDLs due to their immunogenicity. The unregulated uptake of LDLs leads to the formation of foam cells, and ultimately results in fatty streak—the first phase of an atherosclerotic lesion. Studies in isolated human endothelial cells has also shown cytotoxic effects of oxLDLs mediated by both GSH depletion and the GSH-independent pathway (Therond et al., 2000). oxLDLs promote production of several cytokines, immune cell chemoattractant proteins, and growth factors. In addition, they increase platelet aggregation, which aggravates the lesion and causes arterial wall thickening (Zhao & Xu, 2000; Maeno et al., 2000). The discovery of novel oxLDL receptors—oxLDL receptor-1 and lectin-like oxLDL receptor-1—has provided a more mechanistic insight into oxLDL-induced atherogenesis. Expression of these receptors in vascular cells is sensitive to cytokine activation (Minami et al., 2000; Kume et al., 2000). Circulating oxLDLs may enhance the progress of

atherosclerosis via regulation of the redox-sensitive transcription factor natural factor (NF)- $\kappa$ B through ligand–receptor binding (Cominacini et al., 2000). **These data suggest that oxLDLs are not merely a by-product of lipid oxidation, but may act as a signaling molecule and a key participant in the initiation and progression of atherosclerotic disease.**

#### 4.4.2. Post-translational protein modifications

In addition to their direct impact on cardiac function, ROS can oxidize amino acid side chains and the protein backbone, leading to protein–protein cross-linking and protein fragmentation (Berlett & Stadtman, 1997). As a consequence of these modifications, signaling proteins may not function properly, which may lead to organ malfunction and even cell death. Two major protein derivatives have been used as markers of ROS-mediated protein modifications: protein carbonyl derivatives and protein nitrotyrosine derivatives. The presence of carbonyl groups in proteins has been referred to as ROS-mediated protein oxidation, while nitrated tyrosine residues have been identified as a product of peroxynitrite-dependent nitration ( $\text{ONOO}^-$ , mainly derived from the interaction of  $\text{O}_2^{\cdot-}$  and NO). Elevated levels of nitrated proteins are observed in cardiac tissue from animals and patients under various conditions of excess oxidant production, including ischemia/reperfusion, CHF, and myocardial sepsis (Flesch et al., 1999; Kooy et al., 1997; Lopez et al., 1997; Luoma et al., 1998). However, it is not clear whether protein nitration/oxidation causes cardiovascular dysfunction or if it simply represents excess oxidant production.

#### 4.5. Altered signal transduction

Recent studies have demonstrated that ROS may indeed act as signal transduction molecules. For example, cellular  $\text{H}_2\text{O}_2$  was transiently increased upon activation of platelet-derived growth factor (Sundaresan et al., 1995). Several signaling pathways are affected by the platelet-derived growth factor-induced increase in  $\text{H}_2\text{O}_2$  concentration, because of the effect of  $\text{H}_2\text{O}_2$  on tyrosine phosphorylation, mitogen-activated protein kinase (MAPK) activation, DNA synthesis, and chemotaxis. Additionally,  $\text{H}_2\text{O}_2$  activates hypertrophic and apoptotic signaling pathways in cardiac myocytes (Chen et al., 2000). ROS activate a wide variety of cellular signaling molecules and pathways, including  $\text{Ca}^{2+}$ , protein tyrosine kinases, serine/threonine kinases, and phospholipases (Kamata & Hirata, 1999). Components of cell signaling pathways known to be associated with ROS activation are discussed in Sections 4.5.1 and 4.5.2.

##### 4.5.1. $\text{Ca}^{2+}$ signaling

$\text{Ca}^{2+}$  is a second messenger that regulates a broad array of biological processes in cardiac tissue, including contraction, neurotransmission, gene expression, and cell growth. Two sources of  $\text{Ca}^{2+}$ —influx across the sarcolemmal

membrane and from the SR of cardiac cells—are critical for excitation–contraction coupling in cardiac tissue. Increases in ROS during ischemia/reperfusion induce excessive intracellular  $\text{Ca}^{2+}$  accumulation (Tani, 1990; Kaneko et al., 1994). Suggested mechanisms of oxidant-induced  $\text{Ca}^{2+}$  influx from extracellular to intracellular space include increased membrane ATP-dependent  $\text{Ca}^{2+}$  binding, activation of  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channels, changes in  $\text{Na}^{+}$ ,  $\text{Na}^{+}/\text{Ca}^{2+}$  exchangers and  $\text{Na}^{+}/\text{K}^{+}$ -ATPase activities, and stimulation of the adrenergic system (Chakraborti et al., 1998). Additionally, an increase in cytosolic  $\text{Ca}^{2+}$  is transiently enhanced by the inhibition of the  $\text{Ca}^{2+}$  pump of the SR, resulting in passive movement of SR  $\text{Ca}^{2+}$  to the cytosolic space (Suzuki et al., 1997). Other cellular components, such as the mitochondria and  $\text{Ca}^{2+}$ -binding proteins, have also been suggested as sources of oxidant-induced  $\text{Ca}^{2+}$  release.

#### 4.5.2. Protein phosphorylation

Protein phosphorylation regulates a wide array of cellular signaling pathways that carry signals through different functional proteins, including enzymes, receptors, transcription factors, and contractile elements (Suzuki et al., 1997). Generally, oxidant-related stimulation of protein phosphorylation acts through the enzymes that regulate the balance of phosphorylation (kinases) and dephosphorylation (phosphatases) of specific amino acid residues of signaling proteins. The overall increases in protein phosphorylation generally reflect stimulation of the signaling pathway. Two well-defined subtypes of protein phosphorylation are discussed in Sections 4.5.2.1 and 4.5.2.2.

**4.5.2.1. Tyrosine phosphorylation.** Most growth factor receptors are transmembrane tyrosine kinases, with the exception of transforming growth factor- $\beta$  and insulin-like growth factor-II. These receptor kinases are sensitive to ROS stimulation, leading to activation of downstream signal transducers, such as the protein kinases of the MAPK cascade, phospholipase C, protein kinase C, and phosphatidylinositol-3-kinase (see the next section) (Kamata & Hirata, 1999; Yamamoto et al., 2000).

**4.5.2.2. Serine/threonine phosphorylation.** The MAPK superfamily of protein serine/threonine kinases is a group of enzymes that are involved in the regulation of cell growth, proliferation, and differentiation, as well as oxidative responses. There are three major subfamilies of MAPKs, including the extracellular responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs, also known as SAPKs), and the p38-MAPKs. The ERKs appear to be associated with cell growth and differentiation, while the JNKs and the p38-MAPKs are involved in responses to cytotoxic insults. In the heart, the ERKs are primarily activated by G-protein-coupling receptor agonists, such as  $\alpha$ -adrenergic receptor agonists, AngII, and endothelin-1, leading to activation of the phospholipase C cascade and ultimately activation of protein kinase C (Choukroun et al.,

1998; Yamazaki et al., 1998). The release of ROS, pro-inflammation cytokines, and humoral factors during ischemia/reperfusion activates ERKs, which are known to be responsible for cardiac hypertrophy in the non-ischemic zone of the heart to compensate for myocyte loss (ventricular remodeling). Additionally, JNKs and p38-MAPKs are activated during global cardiac ischemia and after incubation of  $\text{H}_2\text{O}_2$ , TNF- $\alpha$ , or IL-1 $\beta$  with cultured myocytes (Sugden & Clerk, 1997, 1998). Inhibition of p38-MAPK attenuated ischemia-induced cardiac apoptosis and decreased TNF- $\alpha$  production in cardiac tissue of experimental animals and humans (Cain et al., 1999; Ma et al., 1999; Mackay & Mochly-Rosen, 1999).

#### 4.6. Altered gene expression

It has become increasingly evident that ROS are more than simply cellular toxicants and that they may be important modulators of cellular gene expression patterns. For example, redox cycling of cysteinyl residues is one of the important mechanisms of ROS-regulated activity of transcription factors and signaling molecules (Dalton et al., 1999). Disruption (reduction) or formation (oxidation) of disulfide bonds play a central role in determining a protein conformation. Conformation is critical for proper protein–protein and protein–DNA interactions, which, in turn, drive specific signal transduction pathways.

ROS-induced alterations in gene expression are mediated by activation of transcription activators, such as nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) and activation protein-1 (AP-1). Changes in early response genes, such as *egr-1*, *hsp70*, *c-fos*, *c-jun*, and *c-myc*, are detected within 30 min after a hypertrophic stimulation (Sen & Packer, 1996). Additionally, re-expression of fetal genes, such as  $\beta$ -major histocompatibility complex,  $\alpha$ -skeletal actin, and atrial natriuretic peptide, was also observed within 6–12 hr (Hefti et al., 1997). ROS-induced increases in intracellular  $\text{Ca}^{2+}$  homeostasis may be a significant initial step in the activation of NF- $\kappa\text{B}$  (Sen & Packer, 1996). Furthermore, lipid peroxidation products can activate NF- $\kappa\text{B}$ . Thus, ROS-induced alterations in gene expression are a consequence of changes in cellular signaling pathways through modifications of enzyme activities and alterations of molecular structures of biomolecules (e.g., proteins, lipids, glycoproteins, nucleotides).

At least two important transcription factors, NF- $\kappa\text{B}$  and AP-1, are controlled by the intracellular redox state. These redox-regulated transcription factors bind to several promoter regions of genes that are directly responsible for the pathogenesis of atherosclerosis, complications of diabetes, cancer, and acquired immunodeficiency syndrome (Sen & Packer, 1996). Transcription activator NF- $\kappa\text{B}$  is important in inflammatory responses since it regulates a number of cytokine genes and their receptors, including TNF- $\alpha$ , IL-1, IL-2, and major histocompatibility complex Class I. NF- $\kappa\text{B}$ -binding sites were also found in a variety of cell adhesion

Table 2  
Antioxidant supplements in CVD

Study/Trial	Study population	Antioxidant dosage and duration of study	Outcome	Reference
<i>Completed small and large-scale observational studies:</i>				
Vitamin C and risk of death from stroke and CHD in the elderly	730 men and women 65 years and over	1973–1974 Department of Health and Social Security nutritional survey Duration: 20-year follow-up	Significant lower risk of stroke in the highest vitamin status No association between vitamin C status and risk of death from CHD	Gale et al., 1995
The First National Health and Nutrition Examination Survey	11,384 adults from the United States	Vitamin C Duration: > 10 year	Significant lower risk of death from CHD	Enstrom et al., 1992
Nurses' Health Study	87,245 female nurses	Vitamin E ( $\geq 30$ IU/day) Duration: 8 years	Significant lower risk of CHD	Stampfer et al., 1993
Health Professionals Follow-up Study (HPFS)	39,910 male health professionals	Vitamin C (1162 mg/day) <sup>a</sup> Vitamin E (>60 IU/day) $\beta$ -carotene (13.5 mg/day) <sup>a</sup> Duration: 4 years	No benefit on CHD Significant lower risk of CHD Significant lower risk of CHD in smokers	Rimm et al., 1993
Dietary antioxidant vitamins and death from CHD in postmenstrual women	34,486 postmenopausal women	Vitamin A (>20,000 IU/day) Vitamin C (>390 mg/day) Vitamin E (>35 IU/day) Duration: 7 years	No benefit from vitamin A or C Significant lower mortality risk of CHD	Kushi et al., 1996
<i>Primary prevention trials:</i>				
Chinese Cancer Prevention Trial	29,584 men and women	Combination: $\beta$ -carotene (15 mg/day), vitamin E (30 mg/day), and selenium (50 $\mu$ g/day)	Reduction in risk of stroke	Blot et al., 1993
Alpha-Tocopherol, Tocopherol, Beta Carotene Cancer Prevention (ATBC)	29,133 male smokers	Vitamin E (50 IU/day) $\beta$ -carotene (20 mg/day) Combination Duration: 5–8 years	No benefit on risk of CHD; increased risk of death from hemorrhagic stroke Increased mortality from ischemic heart disease	The Alpha-Beta Carotene Cancer Prevention Study Group, 1994
$\beta$ -Carotene and Retinol Efficacy Trial (CARET)	18,314 male smokers and asbestos workers	Combination: $\beta$ -carotene (30 mg/day) and retinyl palmitate (25,000 IU/day) Duration: 4 years	Increased risk of CVD	Omenn et al., 1996
U.S. Physicians' Health Study (PHS)	22,071 male physicians	$\beta$ -carotene (50 mg/day) Aspirin (325 mg/day) Combination Duration: 12 years	No benefit on risk of CVD	Hennekens et al., 1996
Skin Cancer Prevention Study	1805 skin cancer patients	$\beta$ -carotene (50 mg/day) Duration: median of 4.3 years	No benefit on risk of CVD	Greenberg et al., 1996
The Western Electric Study	1843 men (40–55 years old)	Dietary $\beta$ -carotene (1.24–53.45 mg/day) and vitamin C (39.0–101.3 mg/day)	The risk of stroke was lower in the highest quartiles of dietary $\beta$ -carotene and vitamin C	Daviglus et al., 1997

Effect of B-group vitamins and antioxidant vitamins on hyperhomocysteinemia	509 men (30–49 years old) with plasma homocysteine $\geq 8.34 \mu\text{M}$	Duration: 30-year follow-up B-group vitamin (1 mg folic acid, 7.2 mg pyridoxine, 0.02 mg cyanocobalamin) and/or antioxidant vitamins (150 mg vitamin C, 67 mg $\alpha$ -tocopherol, 9 mg $\beta$ -carotene), or placebo	B-group vitamins: significantly decreases plasma homocysteine ( $\sim 30\%$ ) with or without antioxidant vitamins No benefit from antioxidant vitamins	Woodside et al., 1999
Women's Health Study	39,876 women (45 years or older)	$\beta$ -carotene (50 mg on alternate days) Duration: (median of 2.1-year treatment and 2-year follow-up)	No statistical significant differences in overall incidence of CVD (MI, stroke, and deaths from cardiovascular causes)	Lee et al., 1999
<i>Secondary prevention trials:</i> Cambridge Heart Antioxidant Study (CHAOS)	2002 men and women with coronary artery disease	Vitamin E (400 or 800 IU/day) Duration: median of 510 days (3–981 days)	Significant decrease in risk of MI Apparent increase in cardiovascular death	Stephens et al., 1996
The Heart Outcomes Prevention Evaluation (HOPE)	1511 men and women at high risk for cardiovascular events	Vitamin E (400 IU) Duration: mean = 4.5 years	No significant effects in deaths from cardiovascular causes, MI, and stroke	Yusuf et al., 2000b
<i>Clinical outcome studies:</i> Incidence of Restenosis after Percutaneous Transluminal Coronary Angioplasty (PTCA)	100 patients with PTCA	Vitamin E (1200 IU/day) 4 months post-percutaneous transluminal coronary angioplasty	No significant differences	DeMaio et al., 1992
Post-angioplasty Restenosis Prevention	119 patients	Vitamin C (500 mg/day) Duration: 4 months post-angioplasty	Significant reduction of restenosis incidence	Tomoda et al., 1996
Indian Experiment of Infarct Survival Study	125 patients with suspected acute MI	Combination: vitamin A (50,000 IU/day), vitamin C (1000 mg/day), vitamin E (400 IU/day), and $\beta$ -carotene (25 mg/day) Duration: 28 days post-MI	Significant reduction in infarct size and post-MI complications	Singh et al., 1996
Multivitamins and Probucol Study (MVP)	317 patients	Probucol (1000 mg/day) Combination of probucol and multivitamins Duration: 1 month pre-angioplasty and 6 months post-angioplasty	No benefit on prevention of restenosis after angioplasty	Tardif et al., 1997
Cholesterol Lowering Atherosclerosis Study (CLAS)	146 subjects with previous coronary artery bypass graft surgery	Colestipol/niacin (drug) or vitamin E ( $\geq 100$ IU/day) Drug plus vitamin E Drug plus vitamin C ( $\geq 250$ mg/day) Duration: 4 years	Significant less IMT progression in the vitamin E group No benefit No benefit on IMT	Azen et al., 1996

IMT, intima-medial thickness; multivitamins include  $\beta$ -carotene, 1000 mg/day; vitamin C, 1000 mg/day; and vitamin E, 1400 IU/day.

Note: 1 mg  $\beta$ -carotene = 600 IU.

<sup>a</sup> Median intake of the quintile that showed significant effect.

molecules, such as colony-stimulating factor-1, monocyte chemoattractant protein-1, and vascular cell adhesion molecule-1 (Suzuki et al., 1997).

AP-1 proteins are a family of leucine zipper transcription factors that specifically regulate gene expression upon binding to cis-acting transcriptional control DNA element. AP-1 genes are inducible by a broad range of stimuli, including ROS. ROS act as mediators of transcriptional regulators in signal transduction processes, leading to cell proliferation and transformation (Sun & Oberley, 1996). Examples of AP-1 transactivators are immediate-early response gene families, such as homodimers of c-jun proteins or heterodimers of c-fos and c-jun. The activity of AP-1 is regulated at transcriptional, post-transcriptional, and post-translational levels. Activation of c-fos/c-jun transcription is promoted by oxidants, cytokines, and growth factors. Redox regulation of AP-1 binding appears to occur at the post-translational level. Oxidants can modify the redox state of cysteine residues located in the DNA-binding domain of each fos/jun protein that are crucial to their DNA-binding activity and subject to redox regulation. Under oxidative stress, the loss of redox regulation by cysteine residues in AP-1 proteins inhibits their binding to DNA, whereas treatment with reducing agents, such as NADPH, GSH, or  $\beta$ -mercaptoethanol, increases AP-1 DNA binding (Sun & Oberley, 1996).

The field of oxidant-related stress genes and signaling is rapidly expanding, and a great deal of new insight into cellular responses to physiological stress has emerged. The regulation of transcriptional activators by oxidants and reductants is complex, and the ultimate biological effects are dependent upon the net results of interactions between these redox-mediated transcriptional activators.

## 5. Therapeutic implications

In general, the current “conventional” therapies for CVD include drugs that primarily affect vascular tone, cardiac contractility, fluid status, or lipid levels. However, given emerging evidence that oxidative processes are involved in cardiac and vascular disease, it seems reasonable to suggest that antioxidant therapeutic strategies may have value. Below is a brief review and current perspective regarding antioxidants for CVD management.

### 5.1. Currently employed pharmaceuticals

Interestingly, several of the most prominent pharmaceuticals used in cardiovascular medicine are already known to have some antioxidant properties that may contribute to their long-term efficacy. For example, the first commercially available ACE inhibitor, captopril, contains a sulfhydryl moiety in its chemical structure. While this has been linked to a unique side effect of patient coughing, the sulfhydryl structure has also been suggested as a chemical scavenger of

free radicals that may contribute to captopril's actions (Anning et al., 1997; Mittra & Singh, 1998). Several recent reports also suggest that the long-term value of AngII inhibition for CVD is mediated by nonhemodynamic actions rather than acute vasorelaxant effects per se. It has been recognized that AngII itself is a stimulator of cardiac and vascular oxidative pathways via induction of NADH/NADPH oxidase (see Section 3.3). Therefore, inhibitors of the renin–angiotensin system (ACE inhibition or AngII receptor antagonism) may actually serve as indirect antioxidants by blocking this pathway, and some aspects of their long-term efficacy may be related to this effect. ACE inhibition recently has been shown to improve endothelial function in patients with coronary artery disease or its risk factors (TREND; Trial on Reversing Endothelial Dysfunction) (Schlaifer et al., 1999). While the mechanism of this clinical benefit is unclear, inhibition of AngII-mediated superoxide production may be a protective mechanism associated with preservation of endothelial health (Rajagopalan et al., 1996; Laursen et al., 1997).

The recently introduced vasodilating  $\beta$ -blocker carvedilol has also been shown to possess antioxidant effects in vitro and in vivo. This agent has emerged as a valuable therapeutic drug for long-term survival enhancement in heart failure, for prevention of diabetes-related renal failure, and in long-term hypertension control. The antioxidant actions of carvedilol (and its primary metabolite) significantly contribute to cardioprotective effects in hypertension and CHF (Moser & Frishman, 1998; Feuerstein et al., 1998). Carvedilol administration significantly increased the resistance to LDL oxidation in patients with essential hypertension (Maggi et al., 1996). Inhibition of ROS in the myocardium may prevent the consequences of oxidative damage, such as cardiac remodeling, activation of transcription factors, and apoptosis (Feuerstein & Ruffolo, 1998; Feuerstein et al., 1998).

### 5.2. L-arginine

Following the recognition of the important preventive role of NO in cardiovascular disorders (i.e., increases blood flow, inhibits key processes involved in atherogenesis), its natural precursor, L-arginine, has received attention as a therapeutic agent. As described in Section 3, the bioavailability of NO is known to be inversely related to the presence of other ROS. One approach to augment NO bioavailability is through enhanced provision of NOS substrate arginine. Clinical studies have also shown that L-arginine supplement improved coronary and peripheral blood flow in healthy and heart failure patients (Lerman et al., 1998; Rector et al., 1996). Although L-arginine supplement has shown some therapeutic value, its low efficacy has become a drawback in patient compliance. The lowest effective dosage ranged from 6 to 8 g/day (up to 20 large capsules/day), whereas the maximum effective dose is 18–20 g/day (Tenenbaum et al., 1998). In addition to the large

dose required, L-arginine has a bitter unpleasant taste. Therefore, the ongoing development of an L-arginine-enriched nutrient bar (the HeartBar<sup>®</sup>) may promote the use of this potentially valuable nutrient (Cooke, 1998). While there has been some suggestion that this supplementation may have value, large-scale, double-blind, placebo-controlled trials have not been conducted, and the available small scale data suggest only minor efficacy.

### 5.3. Antioxidant vitamin supplements

#### 5.3.1. General aspects

Having recognized the significance of ROS in the pathogenesis of CVD, several antioxidants from normal and dietary supplements have been proposed to prevent or reduce disease risk (see Table 2). The term cardiovascular “nutriceuticals” has been introduced to describe natural agents that have been shown scientifically, through clinical trials, to enhance cardiovascular health (Cooke, 1998). The discussion in the following sections is limited to three important exogenous antioxidants—vitamin C (ascorbic acid), vitamin E ( $\alpha$ -tocopherol), and  $\beta$ -carotene (provitamin A)—because little information is available on the benefits of other potential nutritional antioxidants in human subjects.

The potential value of antioxidant vitamin supplements has become an area of interest for cardiovascular and other disease management. Table 2 shows completed large-scale trials and some small clinical outcome studies of the effects of antioxidants on CVD. In the last decade, several studies have been conducted using highly variant experimental designs, populations, vitamin supplements, and/or combinations. In general, targeted therapy in high-risk populations (secondary prevention trials) have shown more consistent value for antioxidants than large-scale primary prevention trials.

**5.3.1.1. Vitamin C.** Vitamin C is the primary antioxidant water-soluble vitamin that can directly scavenge singlet oxygen, superoxide, and hydroxyl radicals. The Food and Nutritional Board of the Academy of Sciences (Washington, D.C., USA) now proposes the new recommended dietary allowance for vitamin C as 120 mg/day (previously 60 mg/day), based on new biochemical, molecular, epidemiologic, and clinical data (Levine et al., 1999).

Hydrophilic antioxidants (e.g., ascorbic acid and GSH) appear to be the first line of antioxidant defenses against reperfusion damages during the return of blood flow, while lipophilic antioxidants (e.g., ubiquinol 9 and vitamin E) play an important role in protecting the integrity of cellular membranes from oxidative damage at later times (Haramaki et al., 1998). Several factors are known to contribute to changes in the plasma concentration of antioxidant vitamins. For instance, a significant decrease in plasma vitamin C is associated with the presence of unstable coronary syndrome in patients with coronary artery disease (Vita et al., 1998), while there was no significant association of plasma levels

of vitamin C and increased risk of CVD mortality in healthy nonsmokers (Loria et al., 2000). Serum ascorbic acid concentrations were associated with decreased prevalence of angina only in subjects who consumed alcohol (Simon et al., 1999).

In addition to the antioxidant activity of vitamin C, recent studies have shown that it activates NOS in endothelial cells (Heller et al., 1999; Huang et al., 2000). A single dose of oral vitamin C supplement (2 g) reduces arterial stiffness and platelet aggregation in healthy subjects (Wilkinson et al., 1999), and it improves endothelial function in CHF and angina patients (Hirashima et al., 2000; Hornig et al., 1998). Studies in young smokers demonstrated that a single dose of oral vitamin C supplement (2 g) immediately improved endothelial function, but had no beneficial long-term effect (1 g daily, 8 weeks) (Raitakari et al., 2000). Dietary vitamin C has also been shown to protect oxLDL-induced smooth muscle cell apoptosis in vitro (Siow et al., 1999). Additionally, administration of ascorbic acid increases the efficacy of estrogen in inhibiting LDL oxidation (Hwang et al., 2000).

**5.3.1.2. Vitamin E.** Vitamin E appears to be the most effective lipid soluble antioxidant in biological systems (Nagel et al., 1997). It inhibits lipid peroxidation and regenerates reduced vitamin C and GSH. By protecting myocardial membranes and inhibiting the oxidation of lipoproteins, vitamin E inhibits membrane peroxidative damage and atherogenesis (Upston et al., 1999).

The cardioprotective effect of oral vitamin E against oxidative damage has been well recognized in both animal studies and clinical trials (Carrasquedo et al., 1999; Dhalla et al., 1996; Nagel et al., 1997). While endothelial dysfunction may be an initiating event in CVD, recent investigations have shown that plasma levels of  $\alpha$ -tocopherol correlate well with endothelial health (Kinlay et al., 1999). The decreases in the plasma levels of lipophilic antioxidants, such as  $\alpha$ - and  $\beta$ -carotene, and vitamin E have been suggested as indicative of atherosclerosis in patients with coronary heart disease (CHD) (Kinlay et al., 1999; Kontush et al., 1999). It has been shown that administration of vitamin C or vitamin E restored endothelial function in patients with CHD (Gokce et al., 1999; Ito et al., 1998; Motoyama et al., 1998) and patients with abnormal lipoprotein profile (Kugiyama et al., 1999). Therefore, vitamin E and/or vitamin C may be important in preserving endothelial function in atherosclerotic disease.

**5.3.1.3.  $\beta$ -Carotene and vitamin A.** The antioxidant properties of  $\beta$ -carotene and vitamin A have been demonstrated by their ability to quench singlet oxygen and to interrupt generation of ROS at a very early stage (Nagel et al., 1997; Palace et al., 1999). Most of the provitamin A carotenoids absorbed across the brush border are packaged into chylomicrons and transported into the plasma in lipoprotein particles. Their uptake, cellular distribution,

and metabolism vary widely among species. Thus, studies from animal models cannot be extrapolated to humans. Despite the fact that the antioxidant mechanisms involved are not well understood, some epidemiological studies in humans provide supportive evidence of their antioxidant value (Table 2).

### 5.3.2. Antioxidant supplements in cardiovascular disease

Although inconsistent, the observational epidemiologic studies provide data suggesting that consumption of foods rich in antioxidant vitamins reduces the risk of developing heart disease (Table 2). The available data from large-scale, randomized, controlled trials are also inconclusive. Among the lipid-phase antioxidants studied, vitamin E shows the most preventive benefits on CHD, while  $\beta$ -carotene shows very little benefit or even harmful effects. Both vitamin E and  $\beta$ -carotene (and vitamin A) are carried within LDL particles. However, the apparent superior preventive effects of vitamin E may be explained by its higher efficiency in protecting LDLs from oxidation in vitro and in vivo (Tribble, 1999; van het et al., 1999).

While oxidative processes have been consistently demonstrated in CVD, the use of antioxidants has not been shown to be consistently effective in all clinical trials. Several issues are likely to be important.

For example, conflicting outcomes from the studies of antioxidant intake may reflect the type of preparation used and bioavailability. Since antioxidant supplements fall into the dietary supplement category, quality control of the products is not regulated by the United States Food and Drug Administration. Vitamin E provides an important example of how vitamin isomers present in commercially available preparations affect bioavailability.  $\gamma$ -Tocopherol is the predominant tocopherol that accounts for 70% of the total vitamin E intake in the United States, but its antioxidant activity and steady-state concentration in plasma and tissue is only 10–20% of the  $\alpha$ -tocopherol isomer (Cohn, 1997).

Other factors also influence plasma levels and bioavailability of each antioxidant. For example, plasma levels of  $\beta$ -carotene may not reflect the absorbability differences between natural and supplemental sources (van het et al., 1999). In a study of the effect of antioxidant vitamins on death from CHD in postmenopausal women, Kushi et al. (1996) found no association between vitamin E supplements (>250 IU/day) and mortality rate, whereas an inverse correlation was observed for food-derived vitamin E (>10 IU/day). Additionally, alterations in vitamin E metabolism and antioxidant defenses in pathologic states can influence the bioavailability of tissue vitamin E. For example, tissue levels of vitamin E vary in patients with different manifestations of atherosclerosis, such as in aortic occlusion, aneurysm, and peripheral occlusive disease (Killion et al., 1996).

Dosage differences may account for the incongruent outcomes of the vitamins studied (Table 2). Based on kinetic studies of vitamin E transport in humans, it is recommended

that the amount of vitamin E required for maintaining a steady-state level is 135–150 IU/day and that the minimum amount effective for protecting against LDL oxidation is 40 IU/day (Weber et al., 1997). It has been suggested that a 200 IU/kg/day dose of vitamin E for 5–10 days is required to achieve a 2-fold increase of the vitamin in rat myocardium (Machlin & Gabriel, 1982). Levels of vitamin E decreased in the infarcted myocardium of rats receiving vitamin E supplements for 2 weeks, although plasma levels of the vitamin remained unchanged (Palace et al., 1999).

Using antioxidant vitamins in combination may also be important in maximizing antioxidant defenses through synergistic effects and replenishment of antioxidant reservoirs in both aqueous and lipid phases. For instance, in addition to its aqueous phase antioxidant property, vitamin C serves as a reducing agent in the regeneration of antioxidant vitamin E (and vice versa) from the  $\alpha$ -tocopheroxyl radical. A study in human subjects shows that elevated plasma levels of supplemental ascorbic acid are inversely correlated with susceptibility of lipoproteins to oxidation, while vitamin E concentrations in LDL particles were unchanged (Harats et al., 1998). Niki et al. (1995) demonstrated that during the oxidation of fatty acids, in the presence of both vitamin E and  $\beta$ -carotene, vitamin E was consumed first, while  $\beta$ -carotene was spared and rapidly used after depletion of vitamin E. Despite inconsistent data from human studies (see Table 2), the antioxidant vitamin C may have value when optimal plasma/tissue concentrations are achieved. Combination therapy with vitamin C and other antioxidants may provide a better antioxidant defense in body tissues.

In summary, some evidence from cohort studies suggests benefits of antioxidants for CVD. However, these benefits have not been demonstrated consistently, and additional randomized, placebo-controlled trials are needed. Several trials are underway, including continuation of Physician's Health Study, the Supplementation en Vitamines et Minéraux Antioxydants (SU.VI.MAX), Women's Health Study, Heart Protection Study, Women's Antioxidant Cardiovascular Study (WACS), etc. Furthermore, standardization and optimization of antioxidant use based on pharmacokinetic considerations are warranted. Several ongoing large-scale trials will further define the role of antioxidants in the prevention and treatment of CVD.

## 6. Conclusions

CVD is prevalent and represents huge costs to the United States health care system. Most recent successes in cardiovascular drug therapy have been shown for agents that abrogate mechanisms of disease progression (renin-angiotensin pathway antagonists, lipid-reducing agents, adrenergic antagonism) rather than short-term symptom relief. Despite diverse etiologies, ROS are unifying components in most forms of cardiac and vascular dysfunction

and disease. Several pro-oxidant pathways have been identified, and these oxidation mechanisms may be important in the structural and functional changes that occur during the initiation and/or progression of CVD. This consistency has provided the rationale for antioxidant trials in humans, but data currently available, using vitamin supplements, have been less than convincing. In general, secondary prevention trials and clinical outcome studies have shown more consistent benefit than primary prevention trials. Several ongoing trials will provide further insight regarding pharmacokinetic and pharmacologic aspects of antioxidants for CVD. Additional mechanistic investigations regarding the molecular and biochemical aspects of oxidation pathways will also be valuable. It is likely that such pursuits will lead to a better understanding of these biological phenomena, and hopefully will provide new opportunities for therapeutic interventions.

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