Review Article

Molecular Characterization of Free Radical Function in Redox Signaling and Strategies to Reduce Oxidative Stress in Cardiovascular Diseases

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Abstract

Free radicals are molecules with an unpaired electron. Due to the presence of free electron, these molecules are highly reactive. At moderate concentrations, free radicals play an important role as regulatory mediators in signaling molecules in a number of normal biochemical and physiological processes. Although there are several sources of vascular reactive oxygen species (ROS), the enzyme nicotinamide adenine dinucleotide phosphate oxidase is emerging as a strong candidate for excessive ROS production that is thought to lead to vascular oxidative stress. The implication of oxidative stress in the etiology of several cardiovascular diseases suggests that strategically, nonpharmacological and pharmacological therapy represents a promising avenue for treatment.

Keywords: Cardiovascular diseases, free radical, oxidative stress, reactive oxygen species

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Introduction

In 1773, Lavoisier and Recherches de were the first to recognize that earth's atmosphere was composed of a vital substance ("air") that supported life.[1] As the key life-supporting element, oxygen was independently discovered by Priestly, in 1775,[2] and Scheele, in 1777.[3] Within a few years of these seminal findings, oxygen toxic side effects that did not support life were also discovered. This revelation was made by Lavoisier in 1785 by a simple experiment in which guinea pigs exposed to oxygen in a container showed congestion of the right heart as well as lungs and died before the oxygen was fully utilized.[4] More than two centuries ago, the good and bad facets of oxygen that are played out by its unique molecular structure were already known.[5] The structural configuration of oxygen molecule is diradical that can accept four electrons and the resultant one-step tetravalent reduction results in the formation of water, with concurrent production of ATP. Ironically, if these four electrons are added one at a time, partially reduced forms of oxygen or free radicals are produced.[6-8] Free radicals can be defined as reactive chemical species having a single unpaired electron in an outer orbit.[9] This unstable configuration creates energy that can initiate autocatalytic reactions so that molecules to which they react are themselves converted into free radicals.[10] Although reactive oxygen species (ROS) are more common in biological systems,[10] free radicals also include reactive nitrogen species.[11] ROS are produced both endogenously and exogenously.[12] The endogenous sources of ROS are the mainly by-products formed in the cells of aerobic organisms within mitochondria.[13] Additional endogenous sources are certain enzymes, neutrophils, eosinophils, and macrophages.[10,14,15] In addition, microsomes and peroxisomes are the sources of ROS, and microsomes are responsible for the majority of ROS produced *in vivo* at hyperoxia sites.[16,17] ROS can also be produced by a host of exogenous sources, such as xenobiotics, chlorinated compounds, environmental agents, metals (redox and nonredox), ions, and radiation.[10,16,18] In general,

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ROS commonly include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$).[19,20] It has been established that ROS can be both harmful and beneficial in biological systems depending on the environment.[21,22] At normal physiological levels, in phagocytic cells, ROS play a key role in cell-mediated immunity and microbicidal activity.[23,24]

In nonphagocytic cells, they are involved in a number of cellular signaling systems as well as in the induction or inhibition of cell proliferation.[25-27] The rate of ROS production in nonphagocytic cells is only about one-third of that of phagocytic cells.[28-31] In contrast, at very high concentrations, ROS are often associated with the principle of oxidative stress.[32] The term oxidative stress is used to describe the condition of oxidative damage to a wide range of cellular structures, as a result of an imbalance between free radical production and antioxidant defenses.[33] Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hyperoxia, toxins, and excessive exercise.[34,35] Moreover, harmful effects are balanced by the action of antioxidants, some of which are enzymes present in the body.[36] However, long-term oxidative stress despite the presence of the cell's antioxidant defense system, ROS have been implicated in the induction and complications of various cardiovascular diseases, such as atherosclerosis.[37,38]

The Role of Free Radicals in Redox Signaling

At normal physiological levels, free radicals are ideally suited to be signaling molecules because they are small and can diffuse short distances; there are several mechanisms for their production, and there are also numerous mechanisms for their rapid removal.[39] Furthermore, several enzymes which are involved in cell signaling mechanisms, such as guanylyl cyclase,[40] phospholipase C,[41,42] phospholipase A_2,[43-46] and phospholipase D,[47] are also potential targets of ROS. Ion channels too may be targets,[48,49] including calcium channels.[50] There are various examples of growth factors, cytokines, or other ligands that trigger ROS production in nonphagocytic cells through their corresponding membrane receptors. Such ROS production can mediate a positive feedback effect on signal transduction since intracellular signaling is often enhanced either by ROS or by a pro-oxidative shift of the intracellular thiol/disulfide redox state.[51] Signaling mechanisms that respond to changes in the thiol/disulfide redox state include AP-1 transcription factor in human T-cells, nuclear factor κB (NF-κB) transcription factor in human T-cells,[52] control of K^+ channel activity in the carotid body,[53] human insulin receptor kinase activity,[54] Src family kinases, JNK and p38 mitogen-activated protein kinase (MAPK) signaling pathways,[55] and signaling in replicative senescence.[56] Protein phosphorylation also plays a critical role in regulating many cellular metabolic processes in eukaryotes.

In particular, protein phosphorylation governs multiple signal transduction pathways.[57] Being a reversible and dynamic process, protein phosphorylation requires not only a PK but also a protein phosphatase (PP). Cellular target proteins are phosphorylated at specific cellular transduction sites (usually at serine/threonine or tyrosine residues) by one or more PKs, and the phosphates are removed by specific PPs. The extent of phosphorylation at a particular site can be regulated by changing the activity of either the PK or PP or both.[58] Among the extracellular signals, growth factor-dependent protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are of primary importance to mitogenesis, cell adhesion, cell differentiation, oncogenic transformation, and apoptosis.[41,54,59] There has been a growing body of evidence, suggesting that ROS modulate PTK and PTP activities directly.[59,60] ROS specifically H_2O_2 can be synthesized endogenously in certain cell types as a response to activation by specific cytokines or growth factors. This endogenous H_2O_2 then acts as a second messenger to stimulate PK cascades coupled to inflammatory gene expression or in control of the cell cycle.[61] The earliest convincing studies that implicated H_2O_2 as an endogenous messenger were performed by Sundaresan *et al*.[62] using, as a model system, vascular smooth muscle cells stimulated with platelet-derived growth factor (PDGF). PDGF receptor binding caused peroxide formation which could be inhibited by intracellular expression of catalase.[57] Catalase expression inhibited PDGF signal transduction by suppressing protein tyrosine phosphorylation. Antioxidants, particularly thiol-reducing agents such as N-acetyl-cysteine, could mimic the inhibitory effects of catalase and prevent redox activation of ligand-coupled PK cascades. Exposure to high concentrations of H_2O_2 or strong pro-oxidative changes in the intracellular thiol/disulfide redox state will generally lead to increased tyrosine phosphorylation in numerous proteins.[63-66] This effect is to some extent, albeit not exclusively, the consequence of the oxidative inhibition of PTPs. Massive inhibition associated with increased net phosphorylation of receptor tyrosine kinases is induced by various types of strong oxidative stress, including high doses of ROS, ultraviolet irradiation, or alkylating agents.[67-74] PTPs counteract the effect of PTKs and reset membrane receptors after ligand-induced autophosphorylation.[57]

The epidermal growth factor (EGF) receptor, for example, is normally dephosphorylated at all tyrosine residues in <1 min after ligand-induced autophosphorylation,[75] but this dephosphorylation is retarded by high concentrations of H_2O_2 on the order of 1 mM or other inducers of oxidative stress. A PTP was also shown to regulate the activation of the EGF receptor.[76] Reversible protein phosphorylation is the key biochemical event in most cell signaling pathways, and signal transduction involving ROS is no exception. Several reports have shown that MAPKs are activated by H_2O_2 in both animals[77-79] and plants,[80-82] which could lead to the modulation of gene expression. Whether H_2O_2 has a direct effect on MAPKs or activating upstream effectors needs to be established. On the other hand, H_2O_2 has also been shown to inhibit phosphatases, probably by the direct oxidation of cysteine in the active site of these enzymes.[79] The Janus kinase-signal transducers and

activators of transcription pathways in animal cells are also activated by H_2O_2, suggesting that H_2O_2 may transduce its message into the nucleus of cells by at least two transduction pathways.[83] It is now becoming apparent that the redox status inside a cell is crucial to the correct functioning of many enzymes and can be used to alter enzyme activity; thus, alteration of the redox status could act as a signaling mechanism.[84] One of the most important redox-sensitive molecules in this respect must be glutathione (GSH), which forms the GSH–GSSG couple. Certainly, H_2O_2 will have the effect of lowering the GSH content of cells and altering the redox status, and hence, propagation of a signal induced by H_2O_2 through this route is likely. It is suggested that enzymes such as ribonucleotide reductase and thioredoxin reductase, as well as transcription factors, might be among the targets for altered redox status. Not only does GSH act as an antioxidant, but it also can modulate the activity of a variety of different proteins via S-glutathionylation of cysteine sulfhydryl groups. The thioredoxin system also makes a significant contribution to the redox environment by reducing inter- and intra-chain protein disulfide bonds as well as by maintaining the activity of important antioxidant enzymes such as peroxiredoxins and methionine sulfoxide reductases.[52,85,86] Baeuerle *et al.* and Rogler *et al.*[87,88] showed that certain transcription factors of the NF-κB/rel family can be activated not only by receptor-targeted ligands but also by direct application of oxidizing agents (particularly H_2O_2) or ionizing radiation. Subsequently, several other PK cascades and transcription factors have been discovered to possess redox-sensitive elements.

The common paradigm in all redox-sensitive signal transduction pathways is the presence of intermediate PKs which are activated by phosphorylation of specific regulatory domains. For example, NF-κB is activated upon phosphorylation of an inhibitory subunit (IκB).[71,87]

Strategies to Reduce Oxidative Stress in Cardiovascular Diseases

Pharmacological inhibition of nicotinamide adenine dinucleotide phosphate oxidase

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been reported to be the major source of O_2^- in the vascular tissue.[89] However, there is a lack of effective inhibitors targeting the NADPH oxidase system. Although diphenyleneiodonium is frequently used, it can inhibit a broad range of flavin-containing enzymes. Recently, several pharmacological and molecular approaches to directly target the NADPH oxidase enzyme have been proposed. Apocynin, a methoxy-substituted catechol, has been used by Peruvian Indians as an anti-inflammatory agent. It acts by blocking the assembly of p47 phox into the membrane complex.[90] Another study suggests that apocynin decreases O_2^- production in rat and human vascular rings, increases nitric oxide production in cultured human endothelial cells, and improves endothelial function *ex vivo* in human arteries and veins, as well as arteries from WKY and SHRSP rats.[91] Interestingly, effects of apocynin in young WKY rats (low oxidative stress) were minimal when compared with effects in age-matched SHRSP rats (high oxidative stress). It has also been reported that administration *in vivo* of apocynin to deoxycorticosterone acetate-salt hypertensive rats decreased both vascular O_2^- production and blood pressure.[92] Although apocynin appears to be an effective NADPH oxidase inhibitor in the vascular tissue from both rats and humans, it needs to be present in relatively high concentrations to be effective. Rey *et al*.[93] have also considered disruption of the active NADPH oxidase complex as a means of reducing oxidative stress. They used a chimeric peptide (gp91ds-tat) designed to cross cell membranes and then inhibit p47 phox association with gp91 phox. Infusion of this peptide into mice significantly inhibited Ang-II-induced rises in blood pressure and vascular O_2^- production.

Another recently developed compound, S17834, a benzo-(γ)-pyran-4-one, has been shown to inhibit NADPH oxidase activity and O_2^- production and attenuate atherosclerotic lesions in apolipoprotein-E-deficient mice.[94] However, its exact mechanism of action remains to be elucidated. Several studies have suggested that 3-hydroxy-3-methyl glutaryl-CoA reductase inhibitors (statins) have inhibitory actions on O_2^- production from NADPH oxidase-independent of low-density lipoprotein (LDL) reduction.[95,96] It has been shown recently that both O_2^- and H_2O_2 production by vascular tissue and leukocytes are inhibited by simvastatin in Ang-II-infused rats.[97] Prevention of O_2^- production by statins may be linked to prenylation-dependent Rac translocation and NADPH oxidase inhibition.[98]

Pharmacological inhibition of the renin-angiotensin system

Ang II has been shown to be a potent stimulation of NADPH oxidase activity in the vascular smooth muscle, fibroblasts, endothelial cells, and cardiomyocytes. Infusions of Ang II have been shown to cause upregulation of the subunits of NADPH oxidase and increase O_2^- levels in animal studies.[99-101] There is accumulating evidence that Ang II is also an important stimulant of NADPH oxidase activity and O_2^- production in human.[102-104] In addition to its interactions with NADPH oxidase, Ang II has been shown to induce LOX-1 expression, the human endothelial receptor for oxidized LDL.[105] Thus, it is not surprising that angiotensin-converting enzyme (ACE) inhibition and Ang-II-receptor antagonisms may play a key role in reducing levels of oxidative stress. It has been commonly postulated since the Heart Outcomes Protection Study[106] that some of the beneficial effects of ACE inhibitors are independent of their effect on blood pressure. ACE inhibition as an antioxidant strategy has been suggested as part of the explanation for this. Consistent with this hypothesis, ACE inhibition has been shown to improve endothelial function in patients with coronary artery disease.[107] In addition, AT_1-receptor antagonists have been shown to be antioxidant and vasoprotective in patients with coronary artery disease,

again downregulating vascular NADPH oxidase expression.[108] Treatment with either an ACE inhibitor or an AT_1-receptor antagonist resulted in lower levels of vascular O_2^-.[109] It has been shown that calcium channel blockers, beta-blockers, and alpha-receptor blockers have antioxidant effects in conditions *in vitro*.

However, although a recent study by Baykal *et al.*[110] demonstrated a reduction in malondialdehyde and an increase in erythrocyte levels of superoxide dismutase (SOD) in hypertensives taking the ACE inhibitor ramipril or the AT_1-receptor blocker valsartan, no improvement in the antioxidant status was observed in patients taking amlodipine (calcium channel blocker), metoprolol (beta-blocker), or doxazosin (alpha-blocker).

Antioxidant dietary supplements

A wealth of data from epidemiological studies suggest that a greater intake of antioxidant vitamins, such as Vitamin E, Vitamin C, and beta-carotene, are associated with a reduced risk of cardiovascular disease.[111] Numerous animal studies support this hypothesis[112-114] as do a number of relatively short-term functional studies in human although many of these studies employed supraphysiological concentrations of vitamins. Vitamin E has been shown to decrease LDL oxidation[115,116] and to improve endothelial function.[117,118] Similarly, Vitamin C administration has been shown to improve endothelium-dependent vasodilation.[119,120] The exact molecular mechanisms underlying these beneficial effects are not fully understood, but some recent studies are beginning to elucidate potential pathways. Ulker *et al.*[121] reported that 24 h and exposure to Vitamin C (10–100 μM) or Vitamin E (100 μM) enhanced nitric oxide synthase (NOS) activity and attenuated NADPH oxidase activity in the rat aorta. It has been suggested that the Vitamin C-mediated increase in NOS activity could be related to alterations in tetrahydrobiopterin (BH4) levels.[122,123] Consistent with this hypothesis, long-term treatment of apolipoprotein-E-deficient mice with Vitamin C resulted in a decrease in levels of 7,8-dihydrobiopterin (BH2), an oxidized form of BH4, and an improvement in the ratio of BH4/BH2.[124] Despite strong evidence demonstrating antioxidant effects of Vitamins C and E in animals, and acutely in human, prospective randomized clinical trials have produced contrasting results. Of the larger trials, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico,[125] the Heart Outcomes Prevention Evaluation,[106] the Heart Protection Study,[126] and the Primary Prevention Project[127] failed to show any benefit. In contrast, the Cambridge Heart Antioxidant Study[128] and most recently the Antioxidant Supplementation in Atherosclerosis Prevention Study[129] report positive results. Data from these and some smaller trials have been elegantly summarized in an editorial by Jialal and Devaraj.[115] Numerous explanations have been proposed for the lack of observed benefit in the majority of randomized trials.

They include oxidant stress status of the participants and dose and combination of vitamins administered. Vitamins C and E reside in different cellular compartments, supporting the concept of combined therapy. Moreover, Vitamin E may be oxidized to form the tocopherol radical. This radical can enhance lipid peroxidation and needs to be converted back into the reduced form by other antioxidants.[130] Although the role of the antioxidant vitamins remains controversial, it is widely accepted that a "healthy diet" has an important role in the prevention of cardiovascular disease. Two recent studies emphasize this. In one randomized placebo-controlled trial in which participants were encouraged to increase fruit and vegetable consumption, both systolic and diastolic blood pressure was significantly lower in the intervention group.[131] In the second study, 6 weeks of "Mediterranean diet," but not oral Vitamin C, was shown to improve vascular function.[132] It is probable that antioxidant vitamins in the "healthy diet" act in synergy with other antioxidants, such as flavonoids and other phenolic compounds, to provide a better antioxidant environment than that achieved with vitamin supplementation alone. Recently, the beneficial effects of polyphenols, particularly from red wine, have received much attention.[133] Several studies have demonstrated antioxidant properties of red wine and purple grape juice.[134,135] It has also been suggested that red wine polyphenols could act to improve endothelial function by increasing endothelial NOS (eNOS) expression.[136] However, it must be remembered that other beverages, including beer and green tea, have been reported to have oxidative potential as having a range of foodstuffs ranging from olive oil to nuts.[137] Such data support the recommendation of a diet rich in fruits, vegetables, whole grain, oils, and nuts for cardiovascular protection.

L-Arginine supplements

Numerous studies in both experimental animals and human have shown that acute and chronic administration of L-arginine improves vascular function in hypercholesterolemia and other forms of cardiovascular disease.[138-140] The availability of L-arginine for reaction with eNOS should not be rate limiting as intracellular levels of L-arginine are in the millimolar range, whereas the K_m for the substrate is in the micromolar range. This apparent discrepancy is frequently referred to as the "L-arginine paradox." Explanations for this paradox include decreased O_2^- production, decreased transport of arginine into endothelial cells, increased levels of asymmetric dimethylarginine, and increased insulin release.[141]

Most recently, it has been suggested that translational control of NOS expression by arginine can explain the arginine paradox, at least for inducible NOS (iNOS).[142]

Thiol-containing compounds supplements

Over the years, a number of thiol-containing compounds have been used experimentally to inhibit LDL oxidation and reduce oxidative stress. Recent studies would support the continued investigation of such compounds. In glucose-fed rats, α-lipoic acid attenuated hypertension, insulin resistance, and oxidative stress,[143] and in another study, it was shown to lower blood pressure in spontaneously hypertensive rats.[144] In human, the classical sulfhydryl compound N-acetyl-cysteine

reduced cardiovascular events in patients with end-stage renal failure.[145]

Estrogen and hormone replacement therapy

Premenopausal women are at a lower risk of atherosclerosis and have a lower incidence of coronary heart disease and myocardial infarction than postmenopausal women or age-matched men.[146,147] Acute estrogen administration has been reported to improve vasoreactivity in healthy postmenopausal women.[148,149] Epidemiological studies suggested that hormone replacement therapy reduced morbidity and mortality associated with cardiovascular disease.[146] Innumerable animal studies have also shown favorable effects of estrogen on the cardiovascular system.[150,151] However, controversy exists over the mechanisms underlying the beneficial effects of estrogen. Some groups have cited decreased O_2^- production as a primary cause[151,152] and others increased expression of NOS by genomic or nongenomic pathways.[150,153-155] In addition, estrogens may activate the gene encoding cyclooxygenase and decrease production of the potent vasoconstrictor endothelin.[156,157] Surprisingly, against this background, data from recently published randomized prospective-controlled clinical trials failed to show cardiovascular benefit from hormone replacement therapy (Heart and Estrogen Progesterone Replacement Study,[158] Estrogen Replacement and Atherosclerosis,[159] and the Women's Health Initiative Randomized Controlled Trial[160]). Part of this apparent contradiction may relate to the cohorts studied. Most animal studies and most of the early observational studies used healthy cohorts which may not be representative of the general population. Women with existing cardiovascular disease may not show the same beneficial effects of estrogen on endothelial function as demonstrated in healthy cohorts.

In such women, the adverse effects of estrogen, such as the increase in triacylglycerol levels and C-reactive protein, may out weight the benefits.[158]

Pharmacological superoxide dismutase mimetics supplements

Endogenous O_2^- is dismutated to H_2O_2 by a family of SODs. In general, studies both *in vivo* and *in vitro* aimed at reducing oxidative stress by increasing levels of Cu/Zn SOD have proved disappointing. This may be because Cu/Zn SOD does not gain access to the appropriate cellular compartments. However, a number of SOD mimetics are available that cross the membrane and have proved more successful in decreasing oxidative stress and improving endothelial function.[161,162]

Pharmacological inhibition of xanthine oxidase

Xanthine oxidase has been proposed to be an important source of O_2^- in human.[102] The enzyme exists in two isoforms, xanthine oxidase and xanthine dehydrogenase. Activity of the former may be increased in ischemia-reperfusion injury and inflammation. Cardillo *et al*.[163] reported that the xanthine oxidase inhibitor oxypurinol improved endothelial function in hypercholesterolemic, but not hypertensive, subjects. More recently, another xanthine oxidase inhibitor allopurinol has been shown to improve endothelial function in Type II diabetes, congenital heart failure, and cigarette smokers.[164-166] However, it must be noted that the patient numbers in all these studies were low (11 patients or less).

Conclusion

Free radicals can be either harmful or helpful to the body. The concentration and location of ROS are the main determinants of their effect. Many data support the notion that ROS released from NADPH oxidase, myeloperoxidase, xanthine oxidase, lipoxygenase, and NOS. At normal physiological levels, free radicals ideally suited to be signaling molecules. Several enzymes which are involved in cell signaling mechanisms, ion channels, human insulin receptor kinase activity, Src family kinases, and JNK and p38 MAPK signaling pathways are also potential targets of ROS. There has been a growing body of evidence suggesting that ROS modulate PTK and PTP activities directly.

ROS production can mediate a positive feedback effect on signal transduction since intracellular signaling is often enhanced by ROS or by a pro-oxidative shift of the intracellular thiol/disulfide redox state. When an overload of free radicals cannot gradually be destroyed, their accumulation in the body generates a phenomenon called oxidative stress. This process plays a major part in the development of various cardiovascular diseases. A wealth of data from epidemiological studies suggests that greater intakes of antioxidant are associated with a reduced risk of cardiovascular disease. In the future, both nonpharmacological and pharmacological therapeutic strategy to increase the antioxidant capacity of cells may be used to fortify the long-term effective treatment. Further research is needed before this supplementation could be officially recommended as adjuvant therapy.

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Conflicts of interest

There are no conflicts of interest.

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