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Role of oxidative stress and nitric oxide in atherothrombosis

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1. ABSTRACT

During the last decade basic and clinical research has highlighted the central role of reactive oxygen species (ROS) in cardiovascular disease. Enhanced production or attenuated degradation of ROS leads to oxidative stress, a process that affects endothelial and vascular function, and contributes to vascular disease. Nitric oxide (NO), a product of the normal endothelium, is a principal determinant of normal endothelial and vascular function. In states of inflammation, NO production by the vasculature increases considerably and, in conjunction with other ROS, contributes to oxidative stress. This review examines the role of oxidative stress and NO in mechanisms of endothelial and vascular dysfunction with an emphasis on atherothrombosis.

2. INTRODUCTION

The generation of reactive oxygen species (ROS) is important in both normal physiology and in the pathogenesis of many diseases. The ROS include partially reduced forms of molecular oxygen, such as hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), lipid peroxides, and hypochlorous acid (HClO). Accumulation of ROS may be accompanied by the production of reactive nitrogen species (RNS), such as the highly reactive peroxynitrite anion, a strong oxidant formed by the reaction of $O_2^{-\bullet}$ and nitric oxide (NO). Under physiological conditions, cells defend themselves against ROS damage through antioxidants that remove free radical intermediates and inhibit oxidation. An imbalance between endogenous oxidants and antioxidants results in oxidative stress, a condition that contributes to vascular dysfunction and atherogenesis (1).

Table 1. Reactive Oxygen Species

Oxidant	Characteristics	Function
Superoxide Anion ($O_2^{-\bullet}$)	**One-electron reduction of O_2** Formed in many autooxidation reactions Electron transport chain NAD(P)H oxidase Mitochondrial respiration Glucose oxidase Xanthine oxidase Cytochrome P450s Cyclooxygenase Lipoxygenase Aldehyde oxidase Flavin dehydrogenase	Cause oxidative damage Alterations in gene transcription, posttranslational protein modification Changes in protein function and enzyme activites Release Fe^{2+} from iron-sulfur proteins and ferritin Inactivation of NO
Hydrogen Peroxide (H_2O_2)	**Two-electron reduction of O_2** Dismutation of $O_2^{-\bullet}$ Xanthine oxidase Glucose oxidase	Lipid soluble Freely diffuses across membranes
Hydroxyl Radical ($^{\bullet}OH$)	**Three-electron reduction of O_2** Formation via Fenton reaction Decomposition of peroxynitrite	Extremly reactive Attacks most cellular components Modification of amino acids, carbohydrates, lipids, nucleic acids
Organic Hydroperoxide (ROOH)	Formed by radical reactions with cellular components	Reaction with lipids and nucleobases
Alkoxy ($RO^{\bullet}$) and Peroxy Radicals ($ROO^{\bullet}$)	Oxygen-centered organic radicals Formed in presence of oxygen by radical addition to double bonds Formed by hydrogen abstraction	Lipid peroxidation
Hypochlorous Acid (HOCl)	Formed from H_2O_2 by myeloperoxidase	Lipid soluble Highly reactive Oxidizes protein side chains, including thiol groups, amino groups, and methionine
Peroxynitrite ($OONO^-$)	Formed in a diffusion-controlled reaction between $O_2^{-\bullet}$ and $NO^{\bullet}$ Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide	Lipid soluble Highly reactive Uncouple NOS by oxidizing cysteines involved in the zinc-thiolate cluster

Superoxide anion is generated by the one-electron reduction of oxygen by nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase, mitochondrial respiration, and other oxidoreductases, such as glucose oxidase and xanthine oxidase (2, 3). The effects of $O_2^{-\bullet}$ include oxidative damage, the mediation of signal transduction leading to altered gene transcription, posttranslational modification with changes in protein function and enzyme activity ('redox signaling'), and rapid inactivation of NO, leading to endothelial dysfunction. Alterations in both the rate of formation and the extent of scavenging of $O_2^{-\bullet}$ have been implicated in the vascular dysfunction observed in atherosclerosis, hypertension, diabetes mellitus, chronic nitrate tolerance, and postischemic myocardial dysfunction (4, 5).

In the endothelium, NO is synthesized by the Ca^{2+}-calmodulin-dependent nitric oxide synthase (eNOS), using L-arginine, O_2, and NADPH as substrates (6). Nitric oxide is membrane permeable and diffuses throughout the vasculature, promoting smooth muscle cell relaxation by activation of soluble guanylyl cyclase and modulation of cation channels, and, consequently, regulating vascular tone (7). Additional antiatherogenic actions of NO relate to inhibition of platelet function and inflammatory cell adhesion, promotion of fibrinolysis, and attenuation of smooth muscle cell proliferation (8). Nitric oxide and $O_2^{-\bullet}$ react in a diffusion-controlled process to produce peroxynitrite, which interacts directly with lipids, DNA and proteins, or indirectly through downstream radical-medicated mechanisms.

In the setting of cardiac risk factors and pathological conditions such as atherothrombosis, oxidative stress is associated with impaired NO bioavailability (9-11).Endothelial dysfunction represents the earliest stage in the atherosclerotic process, and also contributes to the pathogenesis of acute vascular syndromes by predisposing to plaque rupture and intravascular thrombosis. In this review, we present an overview of oxidative stress, NO synthesis, and biological chemistry, as well as the evidence linking the pathophysiology of endothelial dysfunction and athero-thrombosis.

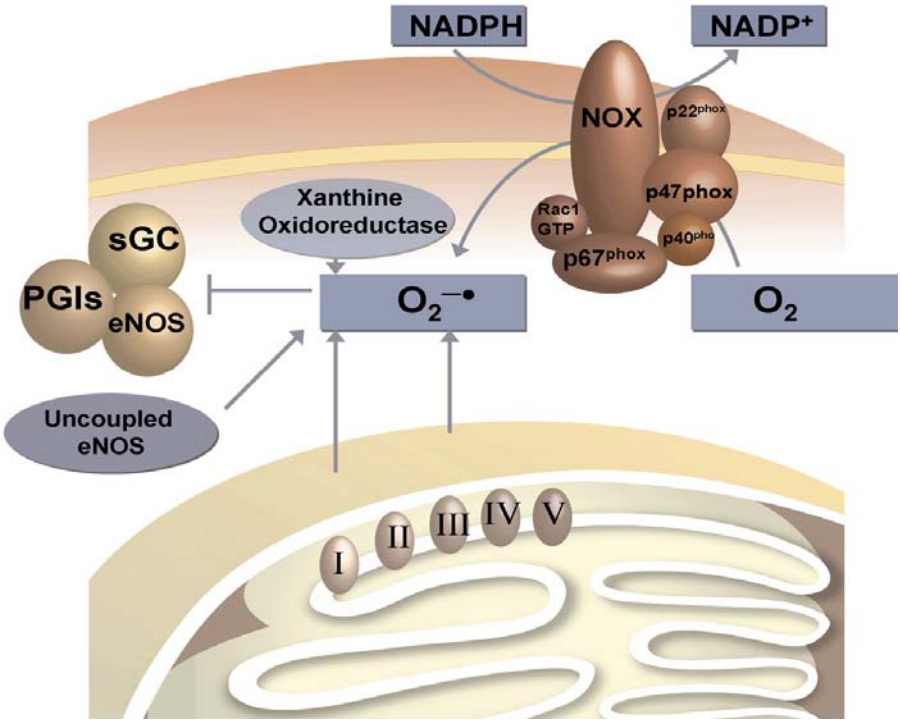

Figure 1. Source of Superoxide Anion. The membrane-associated NAD(P)H oxidase enzyme complex catalyze the one-electron reduction of molecular oxygen (O_2) using NAD(P)H as an electron donor, generating superoxide anion ($O_2^{-\bullet}$). The catalytic b_{558}-type cytochrome Nox subunit is bound to $p22^{phox}$ in the plasma membrane, and they stabilize each other. The cytosolic subunits shown may also be required for full and substained activation of the complex in vascular cells. Among the other sources of $O_2^{-\bullet}$ are the xanthine oxidoreductase enzyme system and 'uncoupled' eNOS. The mitochondrial electron transport chain produces $O_2^{-\bullet}$ by incomplete reduction of O_2, mainly at complex I (NADH coenzyme Q reductase) and complex III (ubiquinol cytochrome *c* reductase).

3. REACTIVE OXYGEN SPECIES

3.1. Sources of reactive oxygen species

During aerobic respiration, mammalian cells produce energy by reducing molecular oxygen (O_2) to water (H_2O). As a natural byproduct of normal metabolism, ROS play a regulatory part in cellular function (Table 1). These highly reactive molecules have the potential to interact with and irreversibly damage proteins, lipids, and DNA; antioxidant defenses modulate their steady-state flux, thereby limiting their toxicities.

A variety of enzymatic and non-enzymatic sources of ROS, particulary of $O_2^{-\bullet}$, are present in cells of the vasculature. Endothelial cells, vascular smooth muscle cells, adventitial cells, and fibroblasts contain forms of the membrane-associated NAD(P)H oxidase enzyme complex that catalyze the one-electron reduction of O_2 using NAD(P)H as an electron donor and the reduced form of b_{558}-type cytochrome, generating $O_2^{-\bullet}$ (Figure 1) (12-18). Stimulators of NAD(P)H oxidase includes agonists of G-protein-coupled-receptors, such as angiotensin II and endothelin-1; bradykinin; growth factors, such as thrombin, vascular endothelial growth factor, and platelet derived growth factor; and cytokines, such as tumor necrosis factor-alpha (TNF-alpha) (13, 19-23). Metabolic and mechanical factors, and nutrient deprivation can also increase NAD(P)H oxidase activity (24-26). NAD(P)H oxidase activity is increased during hypoxia-reoxygenation, reperfusion, prolonged exposure to nitroglycerin, and exposure to oxidized low-density lipoprotein (ox-LDL) (27-30). Activation involves modulation of membrane-bound non-phagocytic NAD(P)H oxidase (Nox) and $p22^{phox}$ subunit expression, and cytosolic regulatory subunits, namely $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and the small GTPase Rac1 (17, 31). Endothelial cells contain the isoforms Nox-1, Nox-2 ($gp91^{phox}$), Nox-4, and Nox-5; vascular smooth muscle cells express Nox-1, Nox-4, and Nox-5; and advential fibroblasts and vascular smooth muscle cells from resistance arteries contain Nox-2 (32-36). Acute increase in oxidase complex formation occurs secondary to post-translational modification of regulatory subunits (p47phox and Rac1) or an increase in the expression and abundance of component subunits.

Another source of vascular $O_2^{-\bullet}$ is the xanthine oxidoreductase (XOR) enzyme system, which consists of a ubiquitous metalloflavoprotein found in two forms, xanthine dehydrogenase (XD) and the posttrans-lationally modified form, xanthine oxidase (XO) (3). This enzyme system is expressed on the luminal surface

of the endothelium and catalyzes the oxidation of hypoxanthine to xanthine in normal purine metabolism. XD requires NAD^+ as an electron acceptor; whereas XO reduces O_2, thereby generating $O_2^{-\bullet}$. The conversion of XD to XO occurs either through the reversible thiol oxidation of cysteinyl residues or via an irreversible proteolytic cleavage of a segment of XD that can occur during hypoxia, ischemia, or inflammation (3). In addition, xanthine oxidase can directly donate two electrons to oxygen to produce H_2O_2. A marked increase in endothelial xanthine oxidase activity has been found following ischemia-reperfusion and hypoxia-reoxygenation (37). In hypercholesterolemic patients, the inhibition of xanthine oxidase activity with oxypurinol improved impaired vasodilation, suggesting $O_2^{-\bullet}$ production by this enzyme can substantially reduce bioavailable NO under certain pathophysiological conditions (38).

A third potential source of vascular $O_2^{-\bullet}$ in endothelial cells is the family of nitric oxide synthases (NOS) (39). The NOS enzymes are dimeric, calmodulin-dependent or calmodulin-containing cytochrome P450-like hemoproteins that combine reductase and oxygenase catalytic domains in one monomer, bear both FAD and FMN (flavin adenine nucleotide), and carry out a five-electron oxidation of one of the basic guanidino nitrogen atoms of L-arginine with the reductive aid of tetra-hydrobiopterin (BH_4), producing NO and L-citrulline (40). In the presence of suboptimal concentrations of L-arginine or of the cofactor BH_4, NOS is functionally 'uncoupled' and produces $O_2^{-\bullet}$ via one-electron reduction of molecular oxygen (41, 42). Similarly, peroxynitrite may also oxidize BH4 and thereby cause NOS uncoupling (43). Tetrahydrobiopterin repletion improves endothelial function in chronic smokers and augments NO bioactivity in normal, hypertensive, and hypercholesterolemic human subjects (44-46). Hypercholesterolemia is also associated with endothelial dysfunction in animal models as well as in human subjects (47-49). Treatment with L-arginine attenuates endothelial dysfunction in animal models; however, the ability of L-arginine to improve endothelial function in hypercholesterolemic or other human subjects is less clear, with some studies finding a benefit wherease others fail to show improvement in endothelial responses following L-arginine treatment (50-53). Peroxynitrite can also uncouple NOS by oxidizing cysteines involved in the zinc-thiolate cluster, releasing zinc and disrupting functional dimer stability (54).

Another mechanism of endothelial dysfunction that relates to eNOS substrate availability is the *in vivo* presence of NOS inhibitors, most importantly asymmetric dimethyl arginine (ADMA), a naturally occurring guanidino-substituted analog of L-arginine (55, 56). Elevated ADMA levels have been demonstrated in hyperhomocysteinemia, hypercholesterolemia, hypertension, atherosclerotic disease, and peripheral vascular disease, and correlate inversely with brachial artery flow-mediated vasodilation (57, 58).

Additional intracellular sources of ROS in the endothelium include an isoenzyme of cytochrome P450 (endothelium-derived hyperpolarizing factor synthase), cyclooxygenases, lipoxygenases, aldehyde oxidase, flavin dehydrogenases, and mitochondrial respiration (59-61). The mitochondrial electron transport chain produces $O_2^{-\bullet}$ by incomplete reduction of O_2, mainly at complex I (NADH coenzyme Q reductase) and complex III (ubiquinol cytochrome *c* reductase) (61-62). These mitochondrial sites may also be important participants in oxygen sensing vascular redox signaling (63). Increased mitochondrial $O_2^{-\bullet}$ generation in endothelial cells appears to be particularly prominent in diabetes, ischemia-reperfusion injury, and hypoxia-reoxygenation (64-65).

3.2. Effects of reactive oxygen species in the vasculature

At physiological pH, $O_2^{-\bullet}$ is both a free radical and an anion (pKa 4.8) (66). Owing to its charge, $O_2^{-\bullet}$ can be transported across biological membranes by anion channels, while its free radical character causes it to react with other radicals in the range of diffusion-controlled reactions (66). Its ability to participate in either electron-accepting oxidation reactions (with sulfydryl groups, ascorbic acid, NADPH) or electron–donating reduction reactions (with cytochrome *c*, metal ions) influences the vascular signaling system.

Superoxide anion reacts with itself to form H_2O_2 and O_2 by spontaneous (k = ~5 x 10^5 $M^{-1}\bullet$ s^{-1} at pH 7.4) and enzymatic dismutation reactions (Figure 2) (67). The spontaneous dismutation rate is second order with respect to $O_2^{-\bullet}$ concentration. Basal levels of H_2O_2 appear to regulate some signaling systems thought to participate in oxygen and redox sensing, or are reduced to H_2O by peroxiredoxins, glutathione peroxidases, or catalase (68). By the metal-catalyzed Fenton reaction, H_2O_2 forms the highly reactive $^{\bullet}OH$, which is the strongest oxidizing agent known and reacts with organic molecules at diffusion-limited rates. Hydroxyl radical can modify amino acids, carbohydrates, lipids, and nucleic acids (69). Theoretically, owing to its interaction with ferrous complexes, NO prevents the formation of $^{\bullet}OH$ by limiting the Fenton reaction (70, 71). Hydroxyl radical-induced endothelial injury inhibits both the production and activity of NO. Thus, NO and oxygen or oxygen-derived radicals can modulate NO bioactivity (72). Nitric oxide can be oxidatively inactivated to nitrite (NO_2^-) (in a third-order reaction with O_2 that is second-order in NO) and nitrate (NO_3^-), oxidation products that have been considered to be devoid of vasodilatory activity at physiological concentrations (73). Recent studies, however, suggest NO_2^- at physiological concentrations may function as a vasodilator, perhaps due, in part, to the nitrite reductase function of deoxyhemoglobin, or to the spontaneous reduction to NO under acidic, relatively hypoxic conditions in ischemic tissues (74-75).

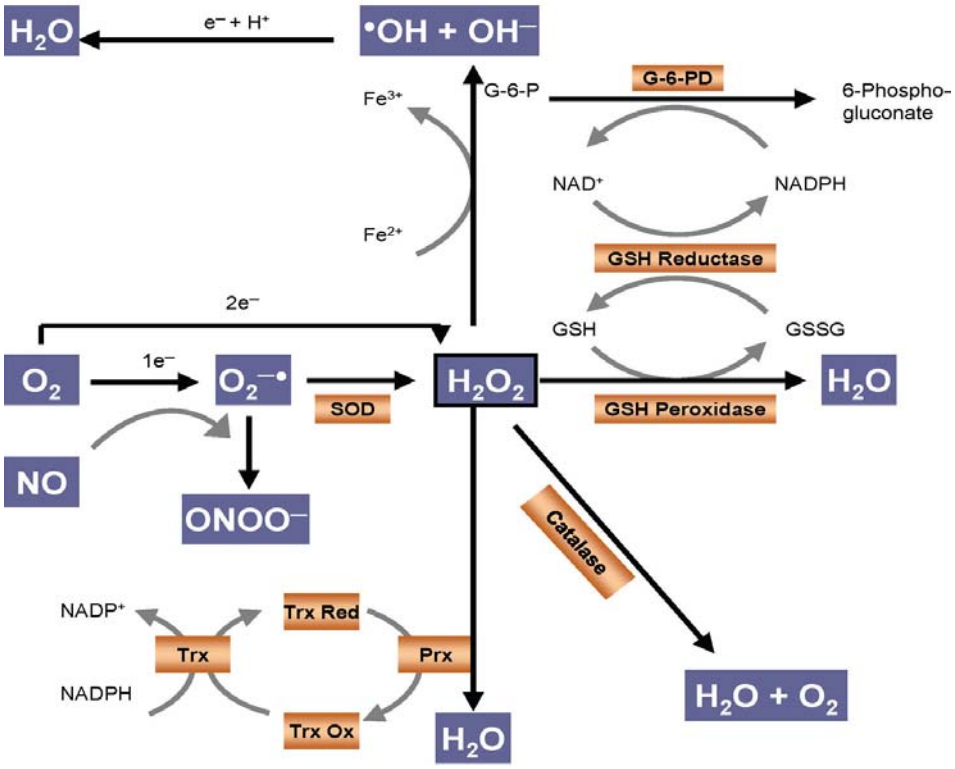

Figure 2. Biochemical Reactions of Reactive Oxygen Species. Superoxide anion reacts with itself to form hydrogen peroxide (H_2O_2) and oxygen by spontaneous (k = ~5 x 10^5 M^{-1} • s^{-1} at pH 7.4) and enzymatic dismutation reactions (k = ~2 x 10^9 M^{-1} • s^{-1}). Basal levels of H_2O_2 appear to regulate some signal transduction pathways, or are reduced to water (H_2O) by peroxiredoxins, glutathione peroxidases, or catalase. By the metal-catalyzed Fenton reaction, H_2O_2 forms the highly reactive hydroxyl radical ($^{\bullet}OH$), which is the strongest oxidizing agent known. Superoxide anion reacts also with nitric oxide (NO) to from peroxynitrite ($ONOO^-$) at the diffusion limit with a rate (k = ~6.7 x 10^9 M^{-1} • s^{-1}) that is faster than that of the superoxide dismutase (SOD) reaction.

Reactive oxygen species can potentiate vascular dysfunction. In small cerebral arteries, impairment of endothelial function by excessive production of free radicals during acute hypertension sets the stage for increased reactivity to vasoconstrictor stimuli and the production of contractile prostanoids (PGG_2, PGH_2) in endothelial cells as well as in smooth muscle cells via oxidation of the ferric heme of cyclooxygenase (76-78). In arteries obtained from diabetic animals, decreased endothelium-dependent relaxation appears to be linked to enhanced release of $O_2^{-\bullet}$ owing to excessive activation of arachidonic acid metabolism via cyclooxygenase (79, 80).

In angiotensin II-mediated hypertension, NAD(P)H oxidase activity plays an important role as a source of ROS and mediator of elevated blood pressure; both hypertension and NAD(P)H oxidase activation can be prevented by administration of the AT_1-receptor antagonist losartan (4, 81). Other studies have shown that the expression of angiotensin converting enzyme (ACE) is increased in atherosclerotic plaque, potentially resulting in increased local production of angiotensin II. ACE-inhibitors may act as antioxidants, in part, by limiting angiotensin II-mediated $O_2^{-\bullet}$ production, thereby decreasing blood pressure and improving vascular dysfunction (82, 83).

Reactive oxygen species may also modulate normal signaling pathways at multiple levels from membrane receptors and channels to various protein kinases and nuclear transcription factors (84, 85). An increase in $O_2^{-\bullet}$ production is associated with an increase in cytoplasmic Ca^{2+} by enhanced extracellular Ca^{2+} influx and intracellular inhibition of Ca^{2+} uptake by the sarcoplasmatic reticulum (86). In smooth muscle cells, $O_2^{-\bullet}$ can inhibit inositol 1,4,5-triphosphate-sensitive responses of the sarcoplasmic reticulum Ca^{2+}-ATPase, which inhibits Ca^{2+} uptake (87). An increase in Ca^{2+} influx is a consequence of the reaction between ROS and thiol groups of the L-type Ca^{2+}-channels or by reaction with thiol-containing cysteine-rich groups of protein kinase C (PKC) (88). Activation of PKC regulates L-type Ca^{2+}-channels by phosphorylation (89). An increase in intracellular Ca^{2+} concentration stimulates peroxynitrite formation, which may account for thiol group oxidation (nitrosation) of sarcoplasmatic reticulum cysteinyl side chains. In addition, ROS decreases voltage-dependent K^+ current density, possibly by oxidation of a methionine residue on the K^+ channel protein of coronary artery smooth muscle cells, leading to impairment of vascular function (90-91). Similarly, ROS also inhibits ATP-dependent K^+ currents in cardiac myocytes (92).

Table 2. Biological Antioxidants

Antioxidant	Characteristics	Function
Ascorbic Acid (Vitamin C)	Dietary sources Reduced form by maintained glutathione Reduction catalyzed by protein disulfide isomerase and glutaredoxins	Water soluble monosaccharide Free radical formation via Fenton reaction Protects vitamin E and GSH against oxidation
alpha-Tocopherol (Vitamin E)	Dietary sources	Lipid soluble Chain-breaking radical scavenger family Reaction with lipid radicals Modulation of several enzymes in signal transduction
Glutathione (GSH)	Synthesized from cysteine, glutamate, glycine Recycled by reduction of GSSG by glutathione reductase	Major low-molecular-weight cysteine-containing peptide Eliminating lipid hydroperoxides and H_2O_2 as a reducing co-substrate for glutathione peroxidases Involved in protein folding and ascorbate metabolism and generally preventing protein $^{-}$SH groups from oxidation and cross-linkage
Superoxide Dismutase (SOD)	Three isoforms Contain metal ion cofactors (copper, zinc, manganese, iron)	Convert $O_2^{-\bullet}$ to H_2O_2 and molecular oxygen
Catalase	Localized to peroxisomes	Reduction of H_2O_2 to H_2O using either an iron or manganese cofactor
Peroxiredoxins (Prx)	Three subgroups Redox-active cysteine oxidized to a sulfenic acid	Reduction of H_2O_2 to H_2O May remove thiyl radicals, causing $^{-}$SH group oxidation
Thioredoxin	Redox-sensitive signaling functions Expression induced by oxidative stress	Reduction of disulfides in protein, peptides, and GSSG Directly reducing ROS
Glutathione Peroxidase	Five isoforms	Reduction of H_2O_2 to H_2O Reduction of organic hydroperoxides Using GSH as a reductant Reduction of peroxynitrite
Heme Oxygenase	Two isoforms	Production of biliverdin and bilirubin Degradation of free heme Production of CO

Oxidant species control several other signaling mechanisms in the vasculature, including soluble guanylyl cyclases (sGC), prostaglandin production, cerulopasmin, myeloperoxidase, and tyrosine-kinase regulated systems (93-95). In smooth muscle cells, a prooxidant state promotes stimulation of mitogen activated protein kinases (p42/p44 MAPK or ERK 1/2) and tyrosine kinases (protein kinase B), which leads to cell growth and migration (13, 31). Stimulation of vascular smooth muscle cells by the mitogen platelet derived growth factor increases intracellular production of H_2O_2 and tyrosine phosphorylation; antioxidants, such as catalase and *N*-acetylcysteine, have been shown to modulate this activation (1, 96). In the vasculature, this signaling pathway leads to hypertrophy and intimal thickening contributing to the progression of hypertension, to an increase in adhesion molecule expression, and to the development of atherosclerosis (11).

4. ANTIOXIDANTS

A paradox of metabolism is that while the vast majority of complex life requires oxygen for its existence, molecular oxygen is a highly reactive molecule that can damage living organisms by conversion to its partially reduced forms, the ROS (97). Consequently, organisms contain a complex network of low-molecular-weight antioxidant molecules and specific antioxidant enzymes that modulate redox state and prevent oxidative damage of cellular components. In general, antioxidant systems either prevent ROS from being formed, or remove them before they can damage vital components of the cell (98). Nonenzymatic antioxidant molecules in endothelial cells include uric acid, ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), and glutathione (GSH), and are classified as hydrophilic or lipophilic (membrane-associated) (Table 2) (99).

Ascorbic acid is a water soluble monosaccharide that is maintained in its reduced form by GSH in a reaction that can be catalyzed by protein disulfide isomerase and glutaredoxins (100). Ascorbic acid scavenges ROS but, through its reductive effect on metal ions, can also contribute to free radical formation via the Fenton reaction (101-102). Ascorbic acid has a favorable redox couple that protects vitamin E and GSH from oxidation. In clinical studies, ascorbic acid supplementation improves NO-dependent vasodilation in human subjects with coronary artery disease, hypertension, hypercholesterolemia, and diabetes mellitus (103, 104).

Vitamin E is a lipid soluble, chain-breaking radical scavenger family of eight related tocopherols and tocotrienols, and is considered the most important antioxidant in cell membranes (105). Of the various forms, alpha-tocopherol has the highest bioavailability and protects cell membranes against oxidation by reacting with lipid radicals produced during lipid peroxidation chain reactions (105, 106). Oxidized alpha-tocopheroxyl radicals produced in this process may be recycled back to the active, reduced form through reduction by ascorbate, retinol, or ubiquinol (107). The function of the other forms of vitamin E are less well-studied, although gamma-tocopherol is a nucleophile that may react with electrophilic mutagens, and tocotrienols may have a specialized role in neuroprotection (106, 108). Vitamin E may also have nonantioxidant cell signaling functions by modulating the activity of several enzymes involved in signal transduction (109). Owing to the antioxidant properties of vitamin E, vitamin E may improve endothelial function and reduce cardiovascular risk. Despite an initial small study demonstrating a therapeutic benefit of vitamin E on reducing non-fatal myocardial infarction, more recent, placebo-controlled, large-scale trials of antioxidants have been disappointing and have found no clinically benefical effects of long term vitamin E supplementation (110-113).

Glutathione is the major low-molecular-weight cysteine-containing peptide found in most forms of aerobic life, and is synthesized in cells from its constituent amino acids (114-115). Glutathione has antioxidant properties as the thiol group in its cysteinyl moiety can be reversibly oxidized. In endothelial cells, GSH serves as a substrate for glutathione peroxidases to eliminate lipid hydroperoxides and H_2O_2, whereby it becomes oxidized to GSH disulfide (GSSG) (116). Depletion of endogenous GSH in ischemia-reperfusion and hypercholesterolemia may also alter the vascular wall's ability to detoxify peroxynitrite (117).

Important endothelial antioxidant enzymes include the superoxide dismutases (SODs), catalase, the thioredoxin system, peroxiredoxins, the glutathione peroxidases, and heme oxygenase. The SODs convert $O_2^{-\bullet}$ to H_2O_2 which is then reduced to H_2O by peroxiredoxins, catalase, or glutathione peroxidases; superoxide can also undergo spontaneous dismutation (k = ~5 x 10^5 M^{-1} • s^{-1} at pH 7.4). Vascular tissue contains three isoforms of SOD that enzymatically accelerate the dismutation of $O_2^{-\bullet}$ (k = ~2 x 10^9 M^{-1} • s^{-1}) (66, 118-121). SODs contain metal ion cofactors that, depending on the isoenzyme, can be copper, zinc, manganese, or iron. Lack of metal ion binding can lead to significant enzyme instability, resulting in reduced enzyme levels and activity (122). Naturally occurring mutations of SOD and H_2O_2-mediated oxidation have been shown to interfere with metal ion binding, thereby reducing enzyme function and contributing to a pathological state (123).

Healthy cells can control the intracellular formation of $O_2^{-\bullet}$ through the activities of cytosolic Cu,Zn-SOD (SOD 1) and mitochondrial Mn-SOD (SOD 2) (124). Cu,Zn-SOD expression is upregulated by shear stress and the cellular redox state (125). Mn-SOD expression is also redox-sensitive and can be induced by vascular endothelial growth factor via the activation of NAD(P)H oxidase. The extracellular form of Cu,Zn-SOD (EC-SOD or SOD 3) is produced by smooth muscle cells, tightly binds to heparan on the exofacial plasma membrane, and is particularly abundant in the interstitium of the arterial wall (126). Extracellular superoxide dismutase may be crucial for the vasodilating activity of extracellular NO by controlling the levels of extracellular $O_2^{-\bullet}$ and preventing the formation of peroxynitrite. The balance between the $O_2^{-\bullet}$-producing oxidases and SOD activities keeps basal $O_2^{-\bullet}$ concentrations below the range in which this species can directly interfere with vascular signaling (85).

Catalase is a highly catalytically efficient enzyme that reduces H_2O_2 to water using either an iron or manganese cofactor (127). Catalase is mostly limited to peroxisomes located adjacent to mitochondria, and is expressed in higher levels in smooth muscle cells than endothelial cells (128).

Peroxiredoxins (Prx) represent a unique type of peroxidase that catalyze the reduction of H_2O_2, organic hydroperoxides, as well as peroxynitrite (129). Mammalian cells express six isoforms of Prx, which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) based on the number and positions of Cys residues that participate in catalysis (130). All Prx enzymes share the same basic catalytic mechanism in which a redox-active cysteine (the peroxidatic cysteine) in the active site is oxidized to a sulfenic acid by the peroxide substrate and then is reduced by a thiol-dependent mechanism during the catalytic cycle. During catalysis, the active site cysteine is occasionally overoxidized to cysteine sulfinic acid (131). In fact, the susceptibility of this active site cysteine to irreversible oxidation accounts for the limited efficiency of peroxiredoxins in eliminating peroxides, which is generally confined to concentrations of H_2O_2 below 20 μM. The thioredoxin/thioredoxin reductase system regenerates the active cysteine site in peroxiredoxins (132, 133). Thioredoxins also reduce disulfides in proteins, peptides, and GSSG, as well as directly lower ROS levels through their conserved -Cys-Gly-Pro-Cys- active site sequence (132, 134). The active site disulfide in thioredoxin is itself reduced by the selenoprotein thioredoxin reductase and NAD(P)H (135). Thioredoxin also has redox-sensitive signaling functions through stimulation of DNA-binding of nuclear factor kappa B (NFkappaB (p50 subunit)), increasing activator protein-1 (AP-1) binding activity (redox factor-1 (Ref-1)), and binding to the MAPKK kinase ASK1 (132). Thioredoxin expression is induced by oxidative stress.

In contrast to the peroxiredoxins, the glutathione peroxidases (GPxs) catalyze the reduction of H_2O_2 and organic hydroperoxides to alcohols using GSH as a reductant (136). In addition, these selenoproteins have a unique GSH-dependent ability to catalyze the reduction of peroxynitrite (137). This class of enzymes includes GPx-1, which is ubiquitously expressed in cytosol and mitochondria of all cells; GPx-3, which is the most abundant extracellular antioxidant enzyme; and GPx-4 or

phospholid glutathione peroxidase, which is also widely expressed and reduces membrane phosholipid hydroperoxides. In addition, the glutathione *S*-transferases are another class of glutathione-dependent antioxidant enzymes that can inactivate lipid peroxides, although their importance in the vasculature is less well understood (114, 138).

Heme oxygenase has indirect antioxidant effects through degradation of free heme and the production of CO as well as biliverdin and bilirubin, which themselves have antioxidant properties (139). There are two isoforms of this enzyme, a constitutive heme oxygenase, HO-2, which is ubiquitously expressed in endothelial cells, and HO-1, which is induced in response to oxidative stress.

5. NITRIC OXIDE

5.1. Role of nitric oxide in the vasculature

Nitric oxide is an omnipresent signaling molecule with a short half-life that either acts within the source cell or diffuses from the source cell to affect adjacent cells (140). The targets of NO depend on the environment and on the quantity produced (141). Nitric oxide is an uncharged free radical composed of seven electrons from nitrogen and eight electrons from oxygen (142-144).

The local level of NO is determined by the balance between its rate of formation or exogenous production (e.g., cigarette smoke, medication), and its rate of inactivation (145). Nitric oxide is produced by a variety of mammalian cells, including vascular endothelium, neurons, smooth muscle cells, macrophages, neutrophils, platelets, and pulmonary epithelium (146). The physiological actions of NO range from modulating the vascular system (blood flow, inhibition of platelet adherence/ aggregation, angiogenesis), to regulating the immune system (cellular immunity by macrophage, neutrophil killing of pathogens, non-specific host defense) and controlling neuronal functions (neurotransmission, synaptic plasticity in the central nervous system, oscillatory behaviour of neuronal networks) (146-147).

Under physiological conditions, NO formation is stimulated by shear forces acting on the vascular endothelium generated by flowing blood or by agonist activation of endothelial receptors (148-149). Based on this stimulation, Ca^{2+} influx triggers the enzymatic activation of constitutively expressed endothelial membrane-bound NOS (cNOS; eNOS; NOS III).

Bacterial endotoxins and inflammatory cytokines, such as TNF-alpha and interleukins, activate the inducible NOS (iNOS; NOS II), which produces Ca^{2+}-independent NO at a rate 1,000-fold greater than that of eNOS (150). In the vasculature, iNOS can be induced in infiltrating macrophages and lymphocytes, endothelial cells, smooth muscle cells, or fibroblasts, whereas eNOS is predominantly produced in endothelial cells. In addition, there is a neural NOS (nNOS; NOS I) that primarily produces NO as a transmitter in the brain and in the peripheral nervous system, such as in non-adrenergic, non-cholinergic (NANC) autonomic nerves that innervate penile erectile tissue and other specialized tissues in the body to promote vasodilation (151).

5.2. Control of nitric oxide levels in vascular tissue

5.2.1. The bioreactivity of nitric oxide

After formation of NO by eNOS in the vascular endothelium, NO binds to the iron (III) hemes of cytochrome *c* oxidase in mitochondria, regulates certain transcription factors such as hypoxia-inducible factor-1 (HIF-1), or rapidly diffuses into the blood (143). In the vascular lumen, NO is readily scavenged by erythrocytes in which it reacts with the ferrous iron (Fe^{2+}) in the heme moiety of oxyhemoglobin to form met-hemoglobin and nitrate (NO_3^-) (144, 152). Nitric oxide also diffuses into the vascular smooth muscle cells adjacent to the endothelium where it modulates the activity of heme-containing soluble guanylyl cyclase. This enzyme dephosphorylates guanosine triphosphate (GTP) to produce cyclic guanosine 3',5'-cyclic monophosphate (cGMP), which activates K^+ channels and inhibits Ca^{2+} entry into smooth muscle cells by directly inhibiting voltage-gated Ca^{2+} channels, and activates protein kinases that phosphorylate myosin light chains and phosphorylate sarcoplasmic proteins, leading to the sequestration of Ca^{2+} in the sarcoplasmic reticulum (153-154). The reduction in cytosolic Ca^{2+} concentration affects phosphorylation of the regulatory myosin light chains and ultimately promotes smooth muscle cell relaxation (155).

Theoretically, NO can exist in three closely related redox forms: the free radical $NO^{\bullet}$; NO^+ or nitrosonium, resulting from a one-electron oxidation of $NO^{\bullet}$; and NO^- or nitroxyl anion, resulting from a one-electron reduction of $NO^{\bullet}$. Nitrogen oxide (NO^x) species react with oxygen-derived radicals, redox metals, and thiols (Figure 3) (156-157). Nitric oxide can be reduced to nitrous oxide (N_2O) or oxidized to nitrite (NO_2^-) (157). Nitrite can react rapidly with oxygen, yielding nitrogen dioxide radical ($^{\bullet}NO_2$), which exists in equilibrium with the potent nitrosating agents dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4); $^{\bullet}NO_2$ can either react with unsaturated lipids directly or participate in nitrosation reactions. Nitric oxide does not directly nitrosate organic molecules without a strong oxidizing cofactor to accept an electron, such as transition metals or $^{\bullet}NO_2$. Dinitrogen trioxide can nitrosate cysteinyl residues (*S*-nitrosation), forming *S*-nitrosothiols (RSNO), which are naturally found in cells and plasma (158). Owing to their relative stability, *S*-nitrosothiols are an important form of NO and may serve as a functional storage pool of bioavailable NO (73). In plasma, *S*-nitroso-albumin is an important NO adduct that may protect NO from inactivation in the oxidative extracellular milieu (158).

The dinitrogen species can also nitrosate (*N*-nitrosation) secondary amines to yield procarcinogenic *N*-nitrosamines (RNNO), and are involved in the formation of nitrotyrosine and *N*-nitrosotryptophan (159-161). These reactive nitrogen compounds can also nitrosatively deaminate deoxynucleosides and deoxynucleotides and promote mutagenic DNA strand breaks (162).

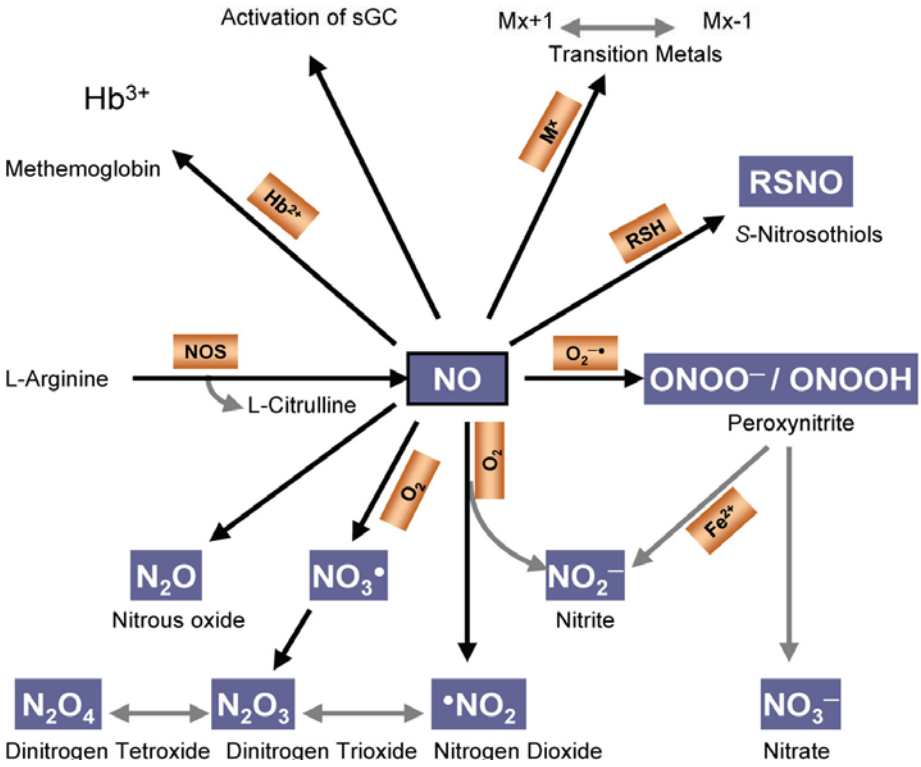

Figure 3. Common Chemical Reactions of Nitric Oxide. Synthesized from nitric oxide synthase (NOS), using L-arginine, nitric oxide (NO) reacts with a variety of targets, such as the ferrous iron (Fe^{2+}) in heme moieties.In hemoglobin (Hb^{2+}), the conversion to ferric iron (Fe^{3+}) forms methemoglobin (Hb^{3+}). Nitric oxide may also activate soluble guanylyl cyclase (sGC) and react with transition metals (M) to alter their valence (x). Nitric oxide also reacts with thiol groups (RSH) to produce *S*-nitrosothiols (RSNO). At rapid rates, NO reacts with superoxide anion ($O_2^{-\bullet}$) to form peroxynitrite ($ONOO^-$/ONOOH), which can form to nitrate (NO_3^-). Nitric oxide can be reduced to nitrous oxide (N_2O) or oxidized to nitrite (NO_2^-). Nitrite can react rapidly with oxygen, yielding nitrogen dioxide radical ($^{\bullet}NO_2$), which exists in equilibrium with the potent nitrosating agents dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4).

Biochemical studies in rat aortas reveal that *N*-nitroso compounds also exist in vascular tissue, but functional data suggest that RSNOs are more active by an order of magnitude than RNNO (163). Additional studies are necessary to understand the biological significance of various RSNO and RNNO species.

At low concentrations, intracellular NO may function as an antioxidant through termination reactions with lipid radicals ($L^{\bullet}$, $LO^{\bullet}$, $LOO^{\bullet}$), resulting in the formation of less reactive secondary nitrogen-containing products (LONO, LOONO) at near diffusion-limited rates (10^9 to 10^{11} $M^{-1} \bullet s^{-1}$) (142). LOONO can either decompose to caged radicals ($LO^{\bullet}$ $^{\bullet}NO_2$) with rearrangement of $LO^{\bullet}$ to an epoxide ($L(O)NO_2$), dissociate and react with additional NO, or hydrolyze to LOOH and NO_2^- (157). Thus, by these chemical actions, NO suppresses the generation of lipid-derived products that are chemotactic for monocytes, and can thereby be considered antiinflammatory and potentially antiatherosclerotic (164, 165).

5.2.2. Nitric oxide signaling properties

Other antiatherosclerotic actions of NO are its antithrombotic antiplatelet effects that contribute to maintaining vascular integrity and blood flow (166, 167). Produced by the intact endothelium, potent platelet inhibitors include prostacyclin, endothelial surface-bound ecto-AD(T)Pase (CD39), and NO, all of which affect platelet activation and function in differing, but complementary, ways (168). Nitric oxide diffuses into platelets and, similar to effects on vascular smooth muscle cells, stimulates cGMP production and activates cGMP-dependent protein kinases, resulting in a decrease in intracellular Ca^{2+} flux (169-171). Ca^{2+} is also an important second messenger in platelets, and its accumulation leads to the phosphorylation and activation of several Ca^{2+}-dependent enzymes that results in cytoskeletal rearrangement, shape change, release of storage granules, and platelet aggregation (168). Furthermore, cGMP, in part through the downregulation of PKC, regulates the desensitization of the thromboxane A_2 (TXA_2) receptor, a potent stimulator of platelet aggregation and vasoconstriction. By attenuating conformational changes in glycoprotein (GP) IIb/IIIa, cGMP controls the number and affinity of fibrinogen binding sites on the platelet surface and prevents the surface expression of the alpha-granule protein P-selectin, a mediator of platelet adhesion (172, 173). Nitric oxide may also inhibit platelet activation by cGMP-

independent mechanisms, such as its effects on non-specific cation channels (169).

In addition, through cGMP-dependent and –independent mechanisms, NO participates in the regulation of vascular smooth muscle cell migration, growth, and proliferation. Calcium promotes vascular smooth muscle cell proliferation, and, as in other cell types, NO decreases intracellular Ca^{2+} flux mediated by cGMP, promoting its antiproliferative action (174). In addition to regulating platelet and vascular smooth muscle cell function, NO has anti-inflammatory effects by controlling leukocyte responses through inhibition of cytokine-mediated cell adhesion molecule expression (175, 176).

6. ATHEROTHROMBOSIS

6.1. Impaired vascular nitric oxide bioavailability and oxidative stress

A dominant mechanism of impaired vascular NO bioavailability relates to its oxidative inactivation by $O_2^{-\bullet}$. A central feature of impaired endothelial function is the presence of ROS manifested by ox-LDL in hyper-cholesterolemia, glycoxidation products in hyperglycemia, redox-active compounds in tobacco smoke, and lipid peroxides in hyperhomocysteinemia owing to the homocysteine-dependent suppression of GPx-1 (177-179). Oxidized lipids can also be generated by metal-dependent Fenton oxidation; enzyme-catalyzed oxidation by lipo-xygenase (LOX) (180-182) or reaction with hypochlorous acid (HOCl) generated by myeloperoxidase; cell-dependent oxidation via a diversity of $O_2^{-\bullet}$ and H_2O_2-generating oxidases; and oxidation by NO-derived reactive nitrogen species such as $^{\bullet}NO_2$, nitryl chloride (NO_2Cl), and peroxynitrite (183-185).

Oxidative stress, particulary the oxidation of LDL, plays a key role at several steps of atherogenesis, according to the 'oxidative-modification hypothesis of atherosclerosis' (186-188). Initially localized in the vascular subendothelial space, LDL is oxidatively modified by endothelial cells, vascular smooth muscle cells, and monocytes. Macrophages within the vessel wall internalize ox-LDL via scavenger receptors, and develop into lipid-rich 'foam cells' (189-191). Evidence that LDL oxidation occurs *in vivo* is supported by the reaction of ox-LDL antibodies with atherosclerotic lesions (188, 192).

6.2. Inflammation

Oxidation of LDL and increased oxidative stress stimulates proinflammatory signals by transcription of genes sensitive to changes in cellular oxidant production, as well as by modulation of cell-signaling events (193). Following exposure to cytokines, such as interleukin-1beta or TNF-alpha, endothelial cells are activated to express vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin, all of which promote leukocyte binding. Moreover, the activated endothelium produces chemoattractants, such as interleukin-8 and monocyte chemoattractant protein-1, that facilitate monocyte recruitment and adhesion to the vessel wall and initiate early stages of atherosclerosis (194, 195). Superoxide anion and other ROS also promote transcription-dependent inflammatory processes, in part, controlled by the redox-sensitive transcriptional regulatory protein NFkappaB that is normally downregulated by NO under physiological conditions (196). NFkappaB is also important in pro-liferative signals involved in vascular smooth muscle cell growth, vascular remodeling, and atherogenesis. Evidence for the involvement of ROS in NFkappaB acitivation is provided by studies demonstrating its downregulation by antioxidants, such as catalase, SOD, and *N*-acetylcysteine (84).

The role of ROS in inflammation is supported by the fact that NAD(P)H oxidase-dependent $O_2^{-\bullet}$ production is found in shoulder regions of the atherosclerotic plaque (34). In this region, ROS also correlate with an increase in the proteolytic activity of matrix metalloproteinases (197, 198). In patients with acute coronary syndrome, atheroectomy specimens show increased levels of ROS compared to those from patients with stable angina, supporting a mechanistic role for ROS in plaque composition and behavior (199). In hypercholesteremic rabbits, *N*-acetylcysteine, a potent antioxidant, decreases matrix metalloproteinases-9 expression and activation, suggesting antioxidant therapy may be an effective means to stabilize plaques (197). In the same animal model, $O_2^{-\bullet}$ is increased in aortic tissue, and treatment with SOD attenuates impairment of endothelium-dependent relaxation (5, 200-201). Similarly, increased $O_2^{-\bullet}$ production is found in blood vessels from human subjects with coronary artery disease, hypercholesterolemia, and diabetes mellitus (202).

6.3. Peroxynitrite formation

In the atherosclerotic plaque, levels of NO can be elevated, generated typically after induction of iNOS (203). Exogenous cigarette smoke also yields high levels of NO that contribute to the formation of more potent secondary oxidants from $O_2^{-\bullet}$, most notably peroxynitrite (204). The formation of peroxynitrite from $O_2^{-\bullet}$ and NO occurs at the diffusion limit with a rate (k = ~6.7 x 10^9 $M^{-1} \bullet s^{-1}$) (120, 205) that is faster than that of the SOD reaction or of NO with heme compounds (206). Thus, NO can be considered a scavenger of $O_2^{-\bullet}$, even to the extent of acting as an antioxidant; in fact, NO is the only biological molecule that can kinetically outcompete SOD for $O_2^{-\bullet}$ (85, 207-209). In mitochondria, NO binds to cytochrome oxidase, inhibits mitochondrial respiration, increases $O_2^{-\bullet}$ production, and potentially augments peroxynitrite formation (210, 211). Significantly, peroxynitrite inactivates Mn-SOD, thereby increasing the flux of $O_2^{-\bullet}$ available to react with NO and establishing an autocatalytic spiral of increasing mitochondrial peroxynitrite formation (212, 213).

At physiological pH, peroxynitrite exists primarily as peroxynitrite anion ($ONOO^-$), a stable base (p*K*a of ~ 6.8 at 37°C) in *cis*-conformation. Its conjugate, peroxynitrous acid (ONOOH), comprises approximately 20% of total peroxynitrite at physiological pH. The *trans*-peroxynitrous acid is a strong oxidant and highly reactive, and, theoretically, may form an excited state that reacts like

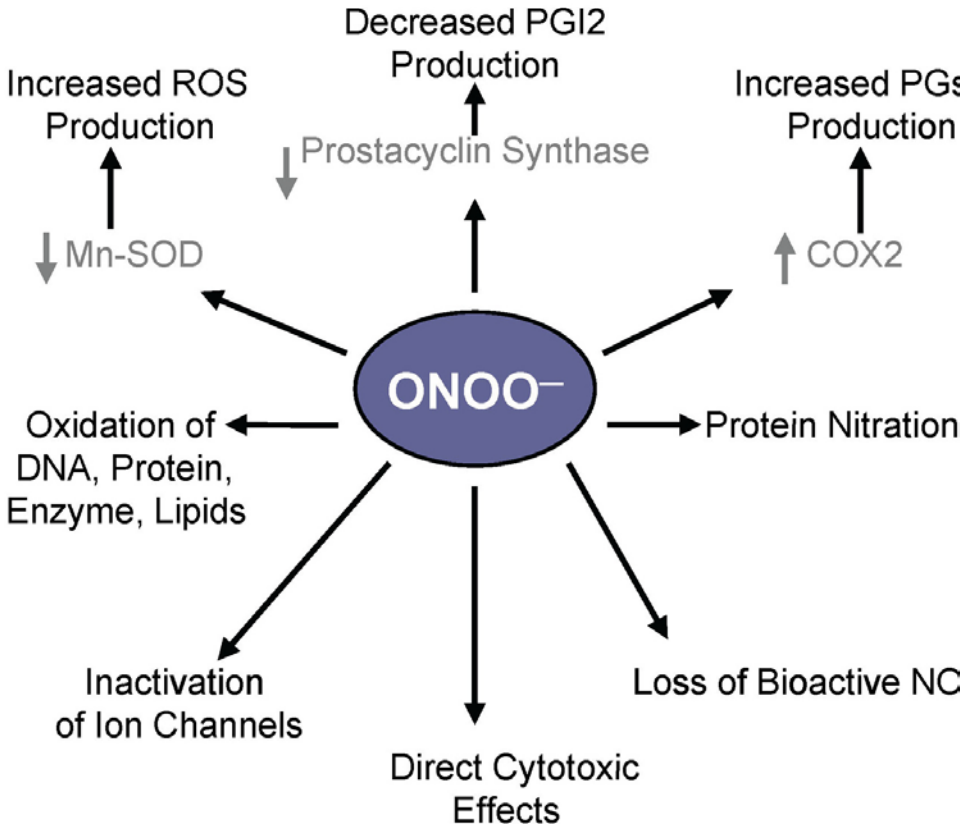

Figure 4. Biological Consequences of Peroxynitrite Formation**.** Peroxynitrite formation has two important biological consequences: loss of bioactive nitric oxide (NO) and direct cytotoxic effects. Peroxynitrite and its conjugate acid can oxidize a variety of biomolecules with the consequence of protein modification or inactivation of ion channels. Peroxynitrite inactivates Mn-SOD, thereby increasing the flux of superoxide anion ($O_2^{-\bullet}$) available to react with nitric oxide and establishing an autocatalytic spiral of increasing mitochondrial peroxynitrite formation.

hydroxyl radical. Peroxynitrous acid is also capable of rearranging to nitrate (214, 215). Peroxynitrite, unlike its precursor NO, is probably not an intercellular messenger molecule, although by modifying target proteins involved in signal transduction, peroxynitrite may have profound effects on cell signaling (216-217). Homolytic cleavage of peroxynitrite can produce $^{\bullet}OH$ and $^{\bullet}NO_2$ (205), which react together to form nitrate, to reform HOONO (cage return), or diffuse apart, leaving free radicals that can cause other oxidative events.

Peroxynitrite formation has two important biological consequences: loss of bioactive NO and direct cytotoxic effects (Figure 4). Peroxynitrite and its conjugate acid can oxidize a variety of biomolecules, including free thiols, such as cysteine, glutathione, or cysteinyl residues in protein; lipids; deoxyribose; guanine bases; methionine; and phenols (214, 215, 218-222). The consequences of these oxidations are protein modification, inhibition of mitochondrial respiration and of other enzymes, and lipid peroxidation (223). Peroxynitrite can also inactivate various ion channels, inhibit membrane Na^+/K^+ ATP-ase activity, and inactivate glyceraldehyde-3-phosphate dehydrogenase (224-226). The reaction with DNA to form 8-hydroxy-deoxyguanosine, single DNA strand breaks, and the activation of the DNA repair nuclear enzyme poly-ADP ribosyl synthetase with depletion of cellular NADH are also important cytotoxic effects of peroxynitrite (221, 227-229).

In the intracellular compartment, peroxynitrite may preferentially oxidize free thiols (RSH) such as glutathione in a second-order reaction, particularly with the anion form (RS^-), resulting in the formation of an intermediate sulfenic acid (RSOH), that then reacts with another thiol, forming disulfides (RSSR) (214, 230-231). Thiols may also be oxidized by the radicals formed from peroxynitrite, generating thiyl radicals ($RS^{\bullet}$); thiyl radicals may react with oxygen and promote oxidative stress or react with NO to form RSNO and promote transnitrosation of protein thiols (Protein-SNO). Peroxynitrite itself is not a direct nitrosating agent, but is involved in the nitrosation of thiols to yield RSNO or organic nitrates ($RONO_2$) (232-236). Peroxynitrite catalysis can be promoted by the presence of transition metals, SOD, or myeloperoxidase to produce hydroxyl anion (OH^-) plus nitronium cation (NO_2^+) (one-electron oxidation); NO_2^+ then reacts with phenolics to produce nitrophenols (237-238). The nitration of protein tyrosinyl residues to yield 3-nitrotyrosine is a stable byproduct left by the short-lived peroxynitrite *in vivo* (234). Tyrosine does not react directly with peroxynitrite;

rather, tyrosine nitration occurs through a radical mechanisms in which a hydrogen atom is first abstracted from the phenol ring to form a tyrosyl radical that quickly combines with $^{\bullet}NO_2$ to produce 3-nitrotyrosine. This reaction competes favorably with a secondary reaction in which tyrosyl combines with another tyrosyl radical to form dityrosine (239, 240). Immunodetectable 3-nitrotyrosine has been demonstrated in fatty streaks of coronary arteries of young autopsy subjects and in foam cells, vascular endothelium, and the neointima of advanced atherosclerosic lesions in older patients (241). Tyrosine nitration can inactivate enzymes (e.g., MnSOD) and disrupt tyrosine kinase signaling pathways by blocking tyrosine phosphorylation (212, 242-243).

The plasma thiol concentration is approximately one-tenth that of the cytosol; therefore, extracellular peroxynitrite will tend to react with non-thiol targets, such as LDL, to generate vasoconstrictive isoprostanes and reactive aldehydes (244). Peroxynitrite is unique as a lipid oxidant, producing lipid peroxyl radicals, and mediates peroxidation of diverse classes of lipids (e.g., purified fatty acids, neutral lipids, phospholipids, lipophilic antioxidants, and LDL lipids) forming conjugated dienes, malondialdehyde, lipid peroxides, lipid hydroxides, F_2-isoprostanes, and oxysterol products (219, 245-247). Peroxynitrite can also react with extracellular carbohydrates.

Cellular damage by peroxynitrite is augmented by its reaction with carbon dioxide radical ($CO_2^{\bullet}$) and formation of highly reactive free radicals. The concentration of carbon dioxide (CO_2) in the plasma is high (~1.3 mM), which is maintained by equilibrium with HCO_3^- (~25 mM). Consequently, in plasma the reaction rate of peroxynitrite with CO_2 will be ~60-fold greater than that with thiols (248-249). The result of this reaction is the formation of nitrosoperoxycarbonate anion ($ONO_2CO_2^-$) (k_2 = ~5 x 10^4 $M^{-1} \bullet s^{-1}$ at 37° C), which subsequently rearranges to form nitrocarbonate (248). Theoretically, nitrocarbonate can undergo hydrolysis to form carbonate radical (CO_3^{-}) and $^{\bullet}NO_2$ as a pair of caged radicals, which ~66% of the time recombine to CO_2 plus NO_3^- and ~33% of the time escape the solvent cage; it is these radicals that are believed to cause peroxynitrite-related cellular damage (250). Nitrocarbonate can also oxidize substrates via one- and two-electron transfers, and may also nitrosate substrates. *In vitro* studies have shown that CO_2 enhances tyrosine nitration by peroxynitrite (251).

Several approaches can be used to prevent or repair peroxynitrite-induced cellular injury. One can decrease the production of NO with NOS inhibitors, or decrease $O_2^{-\bullet}$ formation by increasing SOD levels or by inhibiting xanthine and NAD(P)H oxidases. Other approaches depend on enhancing the reduction of peroxynitrite to NO_2^- or rearrangement to NO_3^- by using organoselenium compounds, metalloporphyrin derivates, and peroxidases (252-255). Glutathione peroxidase-1, the most abundant selenocysteine-containing GPx, may play a role in the reduction of peroxynitrite (137). The relevance of this enzyme in protecting against oxidative stress has been demonstrated in cell culture, murine models of GPx-1 deficiency, and human subjects (179, 256-257). Compared to wild-type mice, heterozygous and homozygous GPx-1-deficient mice show an increase in plasma and aortic levels of isoprostane iPF_{2alpha}-III, a marker of oxidant stress. These GPx-1 deficient mice also have endothelial dysfunction and increased immunodetectable 3-nitrotyrosine in the aorta.

6.4. Platelet activation and thrombus formation

In response to blood vessel injury, platelets accumulate at the site, recruit other platelets, promote clotting, and form a hemostatic plug to prevent hemorrhage; in the course of their activation, platelets are a rich source of ROS, including $O_2^{-\bullet}$, H_2O_2 and LOOH. In addition to promoting endothelial dysfunction and atherogenesis, NO insufficiency combined with ROS accumulation predisposes to a platelet-dependent prothrombotic disorder. Reduction of NO and induction of $O_2^{-\bullet}$ formation cause an increase in intracellular Ca^{2+} levels in platelets. Increased Ca^{2+} levels activate cytoskeletal rearrangement, alpha- and dense-granule release, and overall platelet activation. Impaired NO bioavailability causes secretion of TXA_2 from the platelets, which consequently activates additional platelets in the microenvironment of the platelet plug. For hemostatic plug formation, platelets interact with one another by forming crosslinks through cell surface integrin receptors, thereby leading to aggregation. Fibrinogen, a bivalent molecule, augments aggregation by linking the GPIIb/IIIa receptors between activated platelets (258). Nitric oxide released from activated platelets regulates the recruitment of additional platelets to the growing thrombus (259). Platelet adherence to the endothelium requires the expression of P-selectin, a cell-surface protein found in endothelial cells and alpha-granules of platelets, which is normally down-regulated by NO (173, 260). In a study of platelet function from human subjects with a thrombotic disorder, NO failed to inhibit platelet P-selectin expression and platelet aggregation. The unresponsiveness of these platelets to NO was found to be associated with decreased GPx-3 activity (261). Glutathione peroxidase-3 is the only isoform in the GPx family that is found in the extracellular space, and is responsible for the majority of the peroxidase activity in plasmas (262). Addition of exogeneous GPx led to the restoration of NO's inhibitory effect on platelets in these patients' plasmas. Thus, GPx-3 maintains the bioavailability of NO by preventing the accumulation of ROS that are generated during the activation of the platelet second messenger cascade and may regulate subsequent conformational changes in cytoskeletal structure. In fact, H_2O_2 has been found to mediate changes in intracellular Ca^{2+} levels and affect Ca^{2+}-related mechanisms through sulfhydryl oxidation-dependent and –independent mechanisms within platelets (263). The modulation of intracellular Ca^{2+} levels by H_2O_2 occurs through stimulatory Ca^{2+} release from the dense tubular system and mitochondria within platelets, concurrent with inhibition of Ca^{2+} reuptake into the dense tubular system mediated by sarcoplasmatic reticulum Ca^{2+}ATPase (264, 265). The consequences of platelet activation and thrombus formation, as well as atherosclerotic plaque disruption (rupture or erosion), are key features of atherothrombosis.

7. CONCLUSION

Cardiovascular risk factors promotes the production of ROS, and an imbalance between endogenous oxidants and antioxidants results in oxidative stress, a condition that contributes to impaired NO bioavailability and vascular dysfunction. The generation of $O_2^{-\bullet}$ by NAD(P)H oxidase in vascular smooth muscle cells and by NAD(P)H oxidase or uncoupled eNOS in activated or dysfunctional endothelial cells can promote the production of peroxynitrite from the diffusion-controlled reaction between $O_2^{-\bullet}$ and NO. Oxidative stress characterized by lipid and protein oxidation in the vascular wall is considered an early event in atherogenesis. The oxidative respiratory burst from leukocytes that enter the vessel wall in the early inflammatory response to vascular injury and the production of oxidized arachidonate derivatives by activated platelets in the early thrombotic response to vascular injury represent key mechanistic determinants of atherothrombosis that reflect ROS flux.

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Note: Note that the International Union of Pure and Applied Chemistry (IUPAC) recommended names for peroxynitrite anion ($ONOO^-$), peroxynitrous acid (ONOOH), and nitric oxide (NO) are oxoperoxonitrate (1^-), hydrogen oxoperoxonitrate, and nitrogen monoxide respectively. The term, peroxynitrite, is used here to refer to the sum of both $ONOO^-$ and its conjugated acid, ONOOH.

Abbreviations: Activator protein-1, AP-1; angiotensin converting enzyme, ACE; asymmetric dimethyl arginine, ADMA; carbon dioxide radical, $CO_2^{\bullet}$; carbon dioxide, CO_2; carbonate radical, CO_3^-; cyclic guanosine 3', 5'-cyclic monophosphate, cGMP; flavin adenine nucleotide, FAD; ferron iron, Fe^{2+}; glutathione, GSH; glutathione disulfide, GSSG; glutathione peroxidase, GPx; glycoprotein, GP; heme oxygenase isoform, HO; hydrogen peroxide, H_2O_2; hydroxyl anion, OH^-; hydroxyl radical, $^{\bullet}OH$; hypochlorous acid, HOCl; hypoxia-inducible factor-1, HIF-1; lipooxygenase, LOX; low density lipoprotein, LDL; molecular oxygen, O_2; nicotinamide adenine dinucleotide (phosphate) oxidase, NAD(P)H; nitrate, NO_3^-; nitrite, NO_2^-; nitric oxide, NO; nitric oxide synthase, NOS; nitronium cation, NO^{2+}; nitrosoperoxy carbonate, $ONO_2CO_2^-$; nitryl chloride, NO_2Cl; non-adrenergic, non-cholinergic, NANC; non-phagocytic NAD(P)H oxidase; Nox; nitrogen, N_2; nuclear factor kappa B, NFkappaB; reactive oxygen species, ROS; reactive nitrogen species, RNS; soluble guanylyl cyclase (sGC); sulfenic acid, RSOH; superoxide anion, $O_2^{-\bullet}$; superoxide dismutase, SOD; peroxynitrite anion, $ONOO^-$; peroxynitrous acid, ONOOH; protein kinase C, PKC; tetrahydrobiopterin, BH_4; tumor necrosis factor-alpha, TNF-alpha; thiyl radical, $RS^{\bullet}$; thromboxane A_2, TXA_2; xanthine dehydrogenase, XR; xanthine oxidase, XO; xanthine oxidoreductase, XOR; water, H_2O.

Key Words: Oxidative Stress, Antioxidants, Endothelial Dysfunction, Atherothrombosis, Review

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