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Forum Review

Oxidative Stress and Endothelial Nitric Oxide Bioactivity

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ABSTRACT

The endothelium plays an important role in the maintenance of vascular homeostasis. Central to this role is the endothelial production of nitric oxide (NO), synthesized by the constitutively expressed endothelial isoform of nitric oxide synthase. Vascular diseases, including hypertension, diabetes, and atherosclerosis, are characterized by impaired endothelium-derived NO bioactivity that may contribute to clinical cardiovascular events. Growing evidence indicates that impaired endothelium-derived NO bioactivity is due, in part, to excess vascular oxidative stress. This review outlines how different forms of oxidative stress can impact on NO bioactivity and discusses strategies to prevent oxidative stress-induced endothelial dysfunction. *Antioxid. Redox Signal.* 5, 181–194.

INTRODUCTION

It became apparent in the 1980s that the endothelium represents a central element in the control of vascular homeostasis, in part, through the production of nitrogen monoxide [nitric oxide (NO)] (for review, see 52). Vascular diseases, including hypertension, diabetes, and atherosclerosis, are characterized by impaired endothelium-derived NO bioactivity, and such impairment is thought to contribute to clinical events associated with vascular disease, including myocardial infarction and stroke (101). Oxidative stress in the vascular wall is a prominent feature of vascular disease (16), and convincing evidence indicates that impaired endothelium-derived NO bioactivity is due, in part, to excess oxidative stress (7). This review discusses the impact of oxidative stress on endothelial NO bioavailability and strategies to prevent oxidative stress-induced endothelial dysfunction that may have clinical benefit.

VASCULAR HOMEOSTASIS AND NO

The production of NO from the endothelium regulates vascular tone (72) and arterial pressure (105). The importance of endothelial-derived NO in vascular homeostasis is highlighted by observations that endothelial nitric oxide synthase (eNOS) gene knockout mice exhibit spontaneous hypertension, defective vascular remodeling, enhanced vascular thrombosis, and leukocyte interactions (28, 51, 71, 106).

The bioactivity of NO is due, in part, to its binding the heme group of guanylate cyclase in target cells (*e.g.,* platelets, smooth muscle cells), thereby increasing the cellular content of cyclic 3′,5′-guanosine monophosphate (cGMP) leading to activation of cGMP-dependent protein kinase and NO-mediated vasodilation and platelet inhibition (52). Under aerobic conditions, NO also acts via *S*-nitrosation of critical cysteine thiols on target proteins to modulate the activity of ion channels, protein kinases, caspase enzymes, and transcription factors (83). Formation of *S*-nitrosothiols is thought to represent a major route of NO storage, trafficking, and metabolism through which NO bioactivity is effected. For example, a recent study indicated that *S*-nitrosohemoglobin in red blood cells distributes NO bioactivity by matching blood flow to tissue oxygen demands (88).

REGULATION OF ENDOTHELIUM-DERIVED NO PRODUCTION AND BIOACTIVITY

In the endothelium, NO is produced constitutively by eNOS, a 135-kDa protein that consists of a C-terminal reduc-

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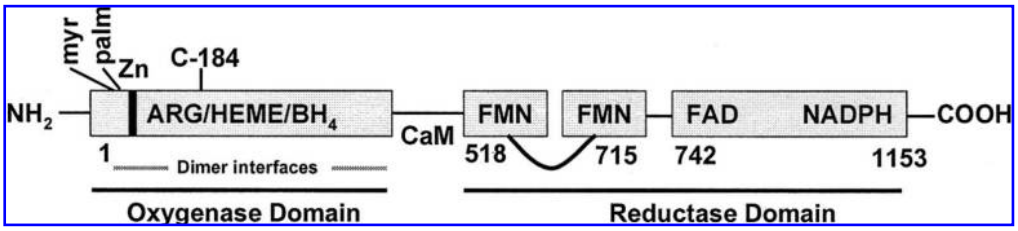

FIG. 1. Linear structure of eNOS. Areas involved in L-arginine (ARG), heme, tetrahydrobiopterin (BH_4), calmodulin (CaM), flavin (FMN, FAD), and NADPH binding sites are indicated. Also noted are sites of zinc incorporation (Zn), palmitoylation (palm), myristoylation (myr), and cysteine heme coordination (C-184).

tase domain linked by a regulatory calmodulin-binding site to an N-terminal oxygenase domain (Fig. 1). The catalytic action of eNOS involves the flavin-mediated transport of electrons from NADPH at the C-terminal reductase domain to the N-terminal heme, where O_2 is reduced and incorporated into the guanidino nitrogen of L-arginine to form L-citrulline and NO. Active eNOS requires formation of a homodimer, and most investigators have found that binding of calmodulin, heme, L-arginine and tetrahydrobiopterin (BH_4) influences dimer formation. Stabilization of the nitric oxide synthase (NOS) homodimer also appears to depend on the integrity of a zinc-thiolate cluster coordinated by critical cysteine residues in the oxygenase domain (46).

In the endothelium, eNOS is subject to transcriptional regulation (75) and various forms of co- and posttranslational regulation that include substrate and cofactor availability, enzyme acylation, subcellular localization to Golgi membrane and plasma membrane caveolae, protein–protein interactions (Table 1), and phosphorylation (Table 2) (for reviews, see 30, 36).

VASCULAR DISEASE, OXIDATIVE STRESS, AND ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is a poorly defined term that refers to a loss of normal homeostatic functions (*e.g.,* vasodilatation, platelet inhibition) often occurring early in the course of vascular diseases such as atherosclerosis, diabetes, and hypertension. One important manifestation of endothelial dysfunction is a reduction in endothelium-derived NO bioactivity that is an independent predictor of cardiovascular events in coronary artery disease patients (34). In theory, such a decrease in NO bioactivity could result from reduced NO production or inactivation of NO. There is considerable evidence for both of these situations in animal and human models of vascular disease.

Although oxidative stress is an imprecise term, it traditionally refers to a situation in which the generation of oxidants overwhelms antioxidant defense systems, resulting in oxidative damage to host tissue macromolecules. Oxidative stress may result from an increased production of oxidants and/or a decrease in antioxidant defenses. Oxidative stress is a characteristic of many vascular diseases, including atherosclerosis, diabetes, and hypertension (7, 16). Various stimuli have been proposed to promote vascular oxidative stress, including hypercholesterolemia, hyperglycemia, shear stress, angiotensin II, and proinflammatory cytokines (7, 37).

Diseased blood vessels from hypercholesterolemic rabbits produce substantial amounts of nitrogen oxides (NO oxidation products) despite the impairment in NO-dependent vascular relaxation (91). This finding suggested that NO production in vascular disease was not attenuated, but that NO was inactivated before reaching its cellular target. Subsequent studies have established that oxidative inactivation of NO frequently involves the superoxide anion radical ($O_2^{\cdot -}$). For example, hypertension, hypercholesterolemia, and atherosclerosis are associated with an increase in the steady-state flux of $O_2^{\cdot -}$ in the vascular wall (37), and $O_2^{\cdot -}$ reacts with NO at near diffusion-controlled rates ($k = 1.9 \times 10^{10}\ M^{-1}\ s^{-1}$) to produce the potent oxidant peroxynitrite ($ONOO^-$; reaction 1) (62).

$$O_2^{\cdot -} + {}^{\cdot}NO \rightarrow ONOO^- \qquad (1)$$

Inspection of rate constants for bimolecular reactions of NO indicate that $ONOO^-$ formation exceeds both NO autoxidation ($k = 2 \times 10^6\ M^{-1}\ s^{-1}$) and spontaneous $O_2^{\cdot -}$ dismutation ($k = 5 \times 10^5\ M^{-1}\ s^{-1}$). Moreover, the reaction of NO with $O_2^{\cdot -}$ is more rapid than either its reaction with enzyme-bound heme ($k = 10^2$–$10^6\ M^{-1}\ s^{-1}$) or the reaction of $O_2^{\cdot -}$ with superoxide dismutase (SOD) ($k = 2 \times 10^9\ M^{-1}\ s^{-1}$) (104). Thus, $ONOO^-$ formation is kinetically favored over other NO reactions and likely occurs whenever both NO and $O_2^{\cdot -}$ are present. As $ONOO^-$ inefficiently activates the soluble isoform of

TABLE 1. REGULATORY PROTEIN INTERACTIONS OF eNOS

Protein	*Action on eNOS activity*
Caveolin*	Binds to eNOS via its scaffolding domain to inhibit enzyme activity
G protein-coupled receptors*	Binds eNOS via the receptor's intracellular domain to inhibit activity
NOS inhibitory protein*	Binds eNOS at the N-terminal oxygenase domain to inhibit enzyme activity
Calmodulin*	Binds eNOS in the calmodulin binding domain to activate enzyme activity
HSP90*	Binds to the N-terminus of eNOS and activates enzyme activity
Dynamin-2*	Activates eNOS
Porin	Activates eNOS (110)

*See recent review articles (30, 36) and the references therein.

TABLE 2. KINASES AND PHOSPHATASES REPORTED TO MODULATE eNOS PHOSPHORYLATION STATUS

Enzyme	*Phosphorylation site(s)*	*eNOS activity*	*References*
Kinases			
Akt	$Ser^{1,177}$, Ser^{617}	Stimulatory	(18, 29, 90)
5′-AMP-activated kinase	$Ser^{1,177}$	Stimulatory	
	Thr^{495}	Inhibitory	(13, 131)
Protein kinase C	Thr^{495}, Ser^{116}	Inhibitory	(23, 63, 89)
Protein kinase A	$Ser^{1,177}$, Ser^{617}, Ser^{635}	Stimulatory	(90)
Calmodulin kinase II	$Ser^{1,177}$	Stimulatory	(23)
MAP Kinase	ND	Inhibitory	(3)
Phosphatases			
Protein phosphatase I	Thr^{495}	Stimulatory	(89)
Protein phosphatase 2A	$Ser^{1,177}$	Inhibitory	(89)
Calcineurin	Thr^{495}, Ser^{116}	Stimulatory	(39, 63)

MAP, mitogen-activated protein; ND, not determined.

guanylate cyclase (113), its formation effectively decreases NO bioactivity in the vascular wall.

Various studies have provided important evidence that direct inactivation of NO by $O_2^{\cdot-}$ is a mechanism of impaired NO bioactivity. For example, the addition of $O_2^{\cdot-}$ to vascular bioassay systems impairs NO-dependent vessel relaxation (38). Exogenous SOD improves the vascular relaxation response to endothelial-derived NO under both basal and acetylcholine-stimulated conditions (38). Perhaps more importantly, increasing vascular SOD activity afforded a significant improvement in NO-mediated arterial relaxation in atherosclerotic rabbits (94). Blood vessels with decreased Cu,Zn-SOD activity exhibit enhanced vascular $O_2^{\cdot-}$ production and impaired NO-mediated arterial relaxation (80). Finally, acute intraarterial infusion of ascorbate at concentrations that effectively prevent $O_2^{\cdot-}$ interaction with NO (53) improves endothelium-dependent relaxation in cardiovascular disease patients (44). In fact, patients that demonstrate the greatest improvement in NO bioactivity in response to ascorbate also exhibit the greatest extent of cardiovascular events, consistent with the notion that oxidative stress-induced endothelial dysfunction is clinically important (44). Collectively, the evidence outlined above substantiates the idea that increased vascular $O_2^{\cdot-}$ is intrinsically involved in the impairment of NO bioactivity.

VASCULAR SOURCES OF $O_2^{\cdot-}$

Vessels constitutively release $O_2^{\cdot-}$ with increased steady-state levels of the reactive oxygen species produced by diseased blood vessels (for review, see 37). Implicated sources of $O_2^{\cdot-}$ anion in the vascular wall under both normal and pathophysiological conditions include mitochondria, cytochrome P450-type enzymes, cyclooxygenase, lipoxygenase, NAD(P)H oxidase, xanthine oxidase, and NOS itself. In the context of vascular disease, the major sources of $O_2^{\cdot-}$ important in the modulation of endothelial-derived NO bioactivity appear to be NADPH oxidase, xanthine oxidase, and eNOS.

NAD(P)H oxidase (Nox)

Increasing evidence implicates the NADPH oxidases as an important source of $O_2^{\cdot-}$ in the vascular wall (37). Endothelial cells and smooth muscle cells constitutively express NADPH oxidase isoforms that belong to the Nox family of enzyme (37). This family of proteins includes the classical respiratory burst enzyme that is a membrane-associated heterodimer (flavocytochrome b_{558}) consisting of 91-kDa (gp91^{phox} or Nox2) and 22-kDa (p22^{phox}) components. Full activity of the neutrophil oxidase requires membrane translocation of the cytosolic proteins p47^{phox}, p67^{phox}, and Rac-1. The binding sites for NADPH, heme, and FAD are present in the Nox2 subunit that, in conjunction with p22^{phox}, mediates electron flow from NADPH to oxygen resulting in $O_2^{\cdot-}$ formation (Fig. 2).

Endothelial cells express mRNA and protein for all the components of the phagocytic enzyme. Recently, the four novel homologues of gp91^{phox} (Nox2) termed Nox1, Nox3, Nox4, and Nox5 have been cloned in nonphagocytic cell (67). Endothelial cells express low levels of Nox1, intermediate levels of Nox2, and higher levels of Nox4 (107). Gorlach and colleagues identified a gp91^{phox} containing NADPH oxidase in the arterial endothelium as a major source of $O_2^{\cdot-}$ that impaired NO bioactivity (35). A further link between the Nox family and increased vascular oxidative stress is supported by observations that the endothelium in vessels from p47$^{phox-/-}$ mice produce less $O_2^{\cdot-}$ in response to infusion of angiotensin II (68).

A recent study has reported that NADPH oxidase activity in endothelium consists of a preassembled intracellular complex associated with the cytoskeleton that exhibits a continuous, low output basal activity (76). The Nox enzyme family appears to mediate vascular cell signaling events important for the regulation of events in endothelial and smooth muscle cells, such as proliferation and migration (67). The NADPH oxidase activity in endothelial cells is increased by specific physiological and pathophysiolgical stimuli that include angiotensin II, oscillatory and cyclical strain, and proinflammatory cytokines (37). Further studies are required to understand the regulatory mechanisms and role of the different

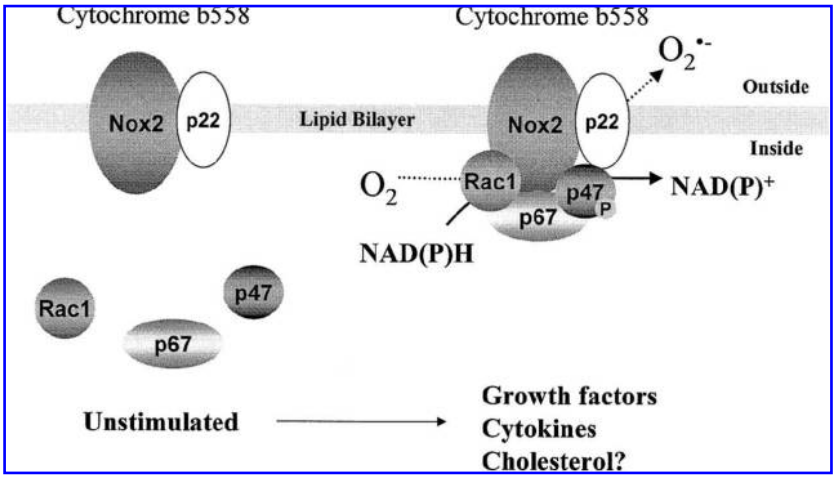

FIG. 2. Structure of classical leukocyte NADPH oxidase.

Nox isoforms in the vasculature. Importantly, the relative importance of the different Nox isoforms is likely to depend on the species, vascular bed, and cell type involved (*e.g.,* 116).

Recent evidence indicates that vascular NADPH oxidases are important for the increased $O_2^{\cdot-}$ production noted in vascular disease (47, 107, 111). For example, Sorescu and colleagues reported increased levels of Nox1 and Nox4 in smooth muscle and endothelial cells of human atherosclerotic lesions that were associated with increased $O_2^{\cdot-}$ production (107). The potential role of NADPH oxidases in atherogenesis has been examined using apolipoprotein (apo) $E^{-/-}$ mice crossbred with mice lacking either $gp91^{phox}$ (Nox2) or $p47^{phox}$. The lack of $gp91^{phox}$ had no effect on the extent of atherosclerosis in the $apoE^{-/-}$ model (61). Two studies have examined the extent of atherogenesis in $apoE^{-/-}/p47^{phox-/-}$ mice (2, 49). Both studies reported no difference between the extent of atherosclerosis in the ascending aortae of $apoE^{-/-}$ compared with $apoE^{-/-}/p47^{phox-/-}$ mice. However, one study (2) observed that mice lacking $p47^{phox}$ had significantly reduced atherosclerosis in the descending aorta when compared with mice deficient in apoE only. Similar findings have been reported in $apoE^{-/-}$ mice treated with antioxidant supplements (115), suggesting that redox modulation of atherogenesis may be dependent on the vascular site.

Xanthine oxidase

The molybdoenzyme xanthine oxidase is derived from the oxidation and/or proteolytic conversion of xanthine dehydrogenase. Xanthine oxidase catalyzes the metabolism of NADH, O_2, and hypoxanthine/xanthine to produce $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2). With respect to its role in vascular disease, pharmacological inhibitors of xanthine oxidase improve vascular function in hypercholesterolemic patients (10). One potential mechanism for these observations is derived from rabbits on a high-cholesterol diet that exhibit increased circulating levels of xanthine oxidase that bind to glycosaminoglycans on the endothelial cell surface and mediate $O_2^{\cdot-}$ production (126).

eNOS

In the presence of adequate amounts of substrate and cofactors, eNOS efficiently channels the transport of electrons from NADPH bound at the C-terminal reductase to the N-terminal heme for O_2 reduction and incorporation into the guanidine group of L-arginine to produce NO and L-citrulline. However, *in vitro* studies have demonstrated that eNOS under conditions of limiting L-arginine and/or BH_4 concentrations can exhibit NADPH oxidase activity to produce $O_2^{\cdot-}$, a process known as "NOS uncoupling" (119, 127) (Fig. 3). In this process, O_2 acts as the terminal electron acceptor (rather than L-arginine), resulting in the production of $O_2^{\cdot-}$. Two studies indicate that BH_4-depleted eNOS generates $O_2^{\cdot-}$ in a Ca^{2+}/calmodulin-dependent manner by the oxygenase domain via dissociation of the ferrous-dioxygen complex (119, 127). Therefore, the endothelial intracellular BH_4 concentration is an important determinant of the NO to $O_2^{\cdot-}$ ratio generated by eNOS (57). Likewise, eNOS interaction with heat shock protein 90 (HSP90) may also prevent eNOS uncoupling (103), although data to support this contention used geldanamycin, an inhibitor of HSP90 now known to redox-cycle to produce $O_2^{\cdot-}$ (17). Thus, defining the precise role of HSP90 in the control of eNOS uncoupling will require alternative strategies for HSP90 inhibition.

Considerable evidence now indicates that eNOS uncoupling represents an important mechanism of pathological vascular $O_2^{\cdot-}$ production that supports $ONOO^-$ formation from the simultaneous fluxes of $O_2^{\cdot-}$ and $^{\cdot}NO$ in the endothelium. A number of pathophysiologically relevant stimuli have been implicated in promoting eNOS uncoupling and $ONOO^-$ formation in endothelial cells *in vitro* such as native low-density lipoprotein (LDL) (102), oxidized LDL (121), high glucose (132), and ceramide (74). This phenomenon of eNOS-dependent $O_2^{\cdot-}$ production appears relevant to vascular disease as it has been documented in blood vessels derived from atherosclerotic mice (70), diabetic rats (47), and rats treated with angiotensin II (92). The precise mechanism(s) of eNOS uncoupling are not yet clear, although pharmacological studies indicate that protein kinase C is an important signal in this process (47, 92). Several reports have indicated successful restoration of endothelial function using BH_4 in human subjects with hypercholesterolemia, atherosclerosis, and cigarette smoking (3, 57, 108), suggesting that eNOS uncoupling is a feature of human endothelial dysfunction.

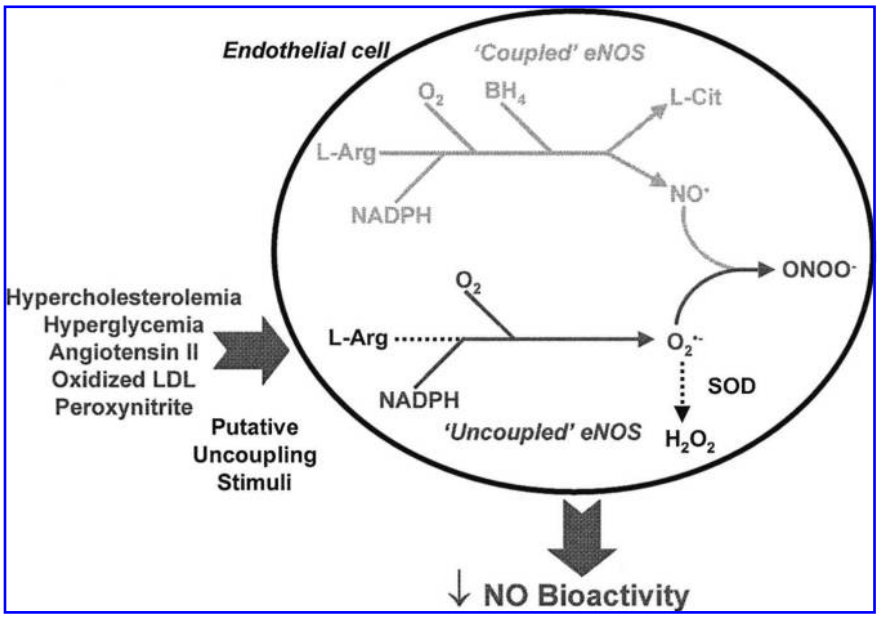

FIG. 3. Basic scheme of eNOS uncoupling.

Although less well developed at this stage, there are also data to suggest that mitochondria represent an important source of $O_2^{\cdot-}$ in vascular cells exposed to high concentrations of glucose (96). Another report indicates that cytochrome P450 2C9 may also represent a significant source of $O_2^{\cdot-}$ in coronary endothelial cells that can modulate vascular tone (24). The precise extent to which these and other systems contribute to the pathophysiological production of $O_2^{\cdot-}$ remains to be determined. It is likely that pathological $O_2^{\cdot-}$ production may depend upon the particular vascular disease, the specific blood vessel in question, or the stage of the disease process.

OTHER FORMS OF OXIDATIVE STRESS AND ENDOTHELIAL NO BIOACTIVITY

Although endothelium-derived NO bioactivity is dependent on the local vascular concentration of $O_2^{\cdot-}$, the full extent of endothelial dysfunction associated with vascular disease is only partially explained by this source of oxidative stress. For example, increasing vascular SOD activity only partially improves NO bioactivity (94), and the effect of SOD is dependent on the stage of vascular disease. Reducing the $O_2^{\cdot-}$ flux with SOD treatment improves NO bioactivity in the early (94) but not advanced stages of atherogenesis (55). Thus, it is important to consider other means by which oxidative stress may modulate NO bioactivity.

Lipid peroxidation

Vascular diseases such as atherosclerosis are characterized by increased levels of oxidized lipids in the vascular wall (109). The process of lipid peroxidation has potential consequences for NO bioactivity. Similar to its reaction with $O_2^{\cdot-}$, NO also rapidly undergoes radical–radical combination reactions with lipid peroxyl radicals ($LOO^{\cdot}$; $k = 2 \times 10^9\ M^{-1}\ s^{-1}$), resulting in the formation of lipid nitration products (98). This chemical reaction is responsible for NO catabolism by 15-lipoxygenase, an enzyme that generates lipid peroxyl radicals during its catalytic cycle. Such scavenging of NO by lipid peroxyl radicals limits the activation of guanylyl cyclase and impairs NO bioactivity (99).

Lipid peroxidation also has indirect implications for NO bioactivity. Vascular segments exposed to *ex vivo* oxidized LDL demonstrate impaired receptor-stimulated NO responses (64). This defect results from an interruption of G protein-dependent signal transduction that is due to oxidized lipids in the modified LDL particle (64). The extent to which this phenomenon contributes to endothelial dysfunction in human disease is not clear at the present time.

Oxidized lipoproteins

The oxidative modification hypothesis of atherogenesis states that oxidation of LDL is an important early event in the pathogenesis of the disease (16). As a consequence, many studies have examined the effects of oxidized LDL on eNOS and NO bioactivity. Exposure of vascular rings to oxidized LDL impairs endothelium-dependent relaxation (64). Chronic exposure of endothelial cells to copper oxidized LDL impairs NO bioactivity through various mechanisms, including increasing endothelial $O_2^{\cdot-}$ production (121), modulation of eNOS expression, and phosphorylation status (33, 48, 77), inhibition of Akt-dependent eNOS activation (12), displacement of eNOS from plasmalemmal caveolae (118), and reduction in eNOS substrate availability (121). High-density lipoprotein (HDL) antagonizes the adverse effects of oxidized LDL by donating cholesterol to the caveolae, thereby preserving caveolae structure and function and preventing the intracellular mislocalization of eNOS (118).

Although the precise oxidants responsible for LDL modification in early atherogenesis are not known with certainty, in-

creasing evidence implicates hypochlorous acid (HOCl) in this process (40). Exposure of endothelial cells to *in vitro* HOCl-oxidized LDL inhibits eNOS activity by inducing mislocalization of the enzyme away from the plasma membrane caveolae and Golgi membranes (97), cellular sites where eNOS is optimally activated (31).

Hydrogen peroxide

H_2O_2 is the dismutation product of $O_2^{\cdot -}$, a reaction that is also catalyzed by SOD (reaction 2).

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

H_2O_2 is a relatively nonreactive oxidant that freely diffuses through cell membranes and may travel several cellular diameters before reacting with targets such as thiols and heme. Diseased vessels produce increased levels of H_2O_2 (37), and this oxidant is also produced in significant concentrations by activated leukocytes. In fact, activated neutrophils at normal circulating concentrations have been estimated to produce 200–400 μ*M* H_2O_2 over a 60-min period (78). The importance of H_2O_2 for vascular disease is linked to growing evidence that it is required for specific cellular functions, such as the response to growth factors, cellular hypertrophy, and the induction of apoptosis (37). H_2O_2-mediated signaling has been linked to a number of cellular signaling events, such as intracellular Ca^{2+} transients, inhibition of protein tyrosine phosphatases, activation of protein kinases, and stimulation of transcription factor binding (37). A unified mechanism for H_2O_2-mediated signaling, however, remains elusive at this time.

There is evidence that H_2O_2 may participate in the regulation of vascular tone. Although H_2O_2 does not react with NO directly, it does induce relaxation of arterial segments in an endothelium- and eNOS-dependent manner (114, 128). As the vasorelaxant properties of H_2O_2 are eNOS-dependent, we examined the effect of H_2O_2 on eNOS activity in cultured endothelial cells (114). We found that H_2O_2 promoted Ca^{2+}-dependent eNOS activity and enhanced basal NO bioactivity by promoting a Src kinase- and phosphoinositide 3-kinase-dependent signaling pathway that resulted in the phosphorylation of the enzyme at $Ser^{1,177}$ and dephosphorylation at Thr^{495} (114). These changes in eNOS phosphorylation status have been shown to be important for eNOS activation in response to shear stress, vascular endothelial growth factor and bradykinin (18, 23, 29, 39). Similar to growth factors, H_2O_2-induced eNOS phosphorylation at $Ser^{1,177}$ was dependent on Akt activation. Importantly, a more recent study has shown that angiotensin II uses endogenous H_2O_2, derived from NADPH oxidase to promote eNOS activity in endothelial cells (9). These studies raise the possibility that endogenous H_2O_2 represents an important signal for eNOS activation induced by certain agonists.

H_2O_2 treatment also promotes chronic increases in eNOS activity by up-regulating eNOS expression via stimulation of mRNA transcription and enhancing of mRNA stability (20). These effects of H_2O_2 on eNOS have been linked to activation of Ca^{2+}/calmodulin kinase II and janus kinase 2 signaling pathways (8). The clinical relevance of such findings relates to observations that therapeutic agents such as cyclosporin A and doxorubicin increase eNOS expression in an H_2O_2-dependent manner (56, 79). Oxidant-induced eNOS up-regulation is consistent with observations that the eNOS promoter contains both an antioxidant response element and consensus sequences for the redox-sensitive transcription factors AP-1, nuclear factor-κB, and SP-1 (26). Taken together, the observations that H_2O_2 promotes both acute and chronic increases in eNOS activity suggest that eNOS-derived NO may represent a compensatory response to oxidative stress. Such speculation is consistent with data demonstrating that eNOS-derived NO protects an endothelial cell line against H_2O_2-induced toxicity (100).

Whereas treatment of endothelial cells with H_2O_2 alone can promote eNOS activity above basal levels, pretreatment of endothelial cells with H_2O_2 can inhibit agonist-stimulated NO bioactivity (54, 82). Exposure of feline cerebral arterioles to H_2O_2 impairs NO-mediated arterial relaxation in response to acetylcholine or authentic NO, an effect reversed by SOD suggesting that $O_2^{\cdot -}$ is involved (125).

A potential deleterious role of H_2O_2 on overall NO bioactivity is consistent with recent studies reporting reduced NO bioactivity in a mouse model of heterozygous cellular glutathione peroxidase deficiency (25), as this selenoenzyme is an important detoxification mechanism for H_2O_2. Glutathione peroxidase may also improve NO bioactivity by detoxifying lipid hydroperoxides and catalyze the decomposition of endogenous *S*-nitrosoglutathione, thereby liberating NO (27).

H_2O_2 can also act as endothelium-derived hyperpolarizing factor (EDHF). For example, H_2O_2 derived from eNOS is responsible for the relaxation of mouse small mesenteric arteries to acetylcholine (84). The mechanism whereby H_2O_2 functions as EDHF is derived from studies demonstrating that H_2O_2 induces polarization and relaxation of coronary arteries devoid of endothelium (6) and activates Ca^{2+}-activated K^+ channels in vascular smooth muscle cells (84). Burke and Wolin have provided evidence that H_2O_2 promotes smooth muscle cell relaxation via a mechanism dependent on the formation of catalase compound I that stimulates guanylyl cyclase to increase intracellular cGMP (6) (reactions 3 and 4).

$$\text{Catalase-Fe}^{3+} + H_2O_2 \rightarrow H_2O + \text{Compound I} \rightarrow \text{cGMP} \quad (3)$$

$$\text{Compound I} + H_2O_2 \rightarrow HO_2 + H_2O + \text{Catalase-Fe}^{3+} \quad (4)$$

This ability of H_2O_2 to act as an endogenous EDHF may explain the redundancy of endothelium-dependent relaxation caused by EDHF and NO (93). Therefore, SOD may play an essential role in preserving endothelium-dependent relaxation, not only by increasing the half-life of NO, but also by converting $O_2^{\cdot -}$ into H_2O_2.

The precise contributions of H_2O_2 versus NO to vasodilation are not known at this time. However, it is attractive to speculate that under certain pathologic conditions when BH_4 levels are limiting (57), the endothelium could switch over to H_2O_2 as a vasodilator. This paradigm was illustrated in a recent study using hph-1 mice, which display a 90% reduction in GTP cyclohydrolase activity (the rate-limiting enzyme for BH_4 synthesis) and reduced tissue BH_4 levels. In these mice,

arterial relaxation in response to acetylcholine was mediated by H_2O_2 derived from eNOS in the endothelium (14).

Peroxynitrite

$ONOO^-$, the reaction product of NO and $O_2^{\cdot -}$, is a strong oxidant capable of promoting oxidative damage, thereby propagating $O_2^{\cdot -}$-mediated oxidative stress. Recent studies indicate that $ONOO^-$ can promote endothelial dysfunction. $ONOO^-$ readily oxidizes BH_4 that would be expected to promote eNOS uncoupling (57). Indeed, Laursen and colleagues provided evidence that $ONOO^-$-mediated oxidation of BH_4 promotes eNOS uncoupling and endothelial dysfunction in aortic segments from $apoE^{-/-}$ mice (70). $ONOO^-$ has also been reported to induce eNOS uncoupling in the absence of BH_4 oxidation by promoting the oxidation of the Zn^{2+}-thiolate cluster present in the enzyme, resulting in Zn^{2+} release and destabilization of eNOS dimers (132). The uncoupling of eNOS by $ONOO^-$ may also involve changes in the enzyme phosphorylation status. Thus, $ONOO^-$ promotes eNOS phosphorylation at $Ser^{1,177}$ via stimulation of 5′-AMP-activated kinase (131), an event that stimulates electron flow through the enzyme (85). Therefore, by inducing oxidation of BH_4 and Zn^{2+}-thiolate cluster and promoting eNOS electron flow, $ONOO^-$ can act as a potent stimulus for eNOS-derived $O_2^{\cdot -}$ production.

Myeloperoxidase/HOCl

Myeloperoxidase (MPO) is a heme protein abundantly expressed in phagocytes, including polymorphonudear neutrophils, monocytes, and subpopulations of tissue macrophages. Upon phagocyte activation, MPO is secreted into both the extracellular milieu and the phagolysosome where it utilizes H_2O_2 and chloride ions to catalyze the formation of the two-electron oxidant, HOCl. Growing evidence implicates MPO in the oxidative events of atherosclerotic lesions. Human lesions contain active MPO and evidence of HOCl-oxidized proteins (15, 40). Immunoreactive MPO and HOCl-modified epitopes in atherosclerotic lesions are detected not only inside monocytes/macrophages, but also in endothelial cells (40, 81). Consistent with these data, MPO may bind to the endothelial surface and undergo transcytosis to the basal surface of the cell, where the enzyme remains catalytically active (1).

Increasing evidence suggests that both MPO and HOCl may represent important modulators of endothelial function during vascular disease. For example, HOCl treatment of endothelial cells inhibits receptor-dependent activation of NO production (54). Treatment of rat aortic rings with HOCl inhibits endothelium-dependent relaxations in a manner reversed by L-arginine (129). Such inhibition of relaxation may relate to the ability of HOCl to react with L-arginine to produce chorinated amino acid products that are effective inhibitors of eNOS activity (130). A recent *in vivo* study by Eiserich and colleagues indicates that MPO derived from degranulated leukocytes impairs endothelium-dependent relaxation responses in mice due to catalytic consumption of NO by substrate radicals produced during the MPO catalytic cycle (21). Together these findings suggest that MPO-catalyzed redox reactions are important for the impairment of NO bioactivity noted during vascular inflammation.

TREATMENT OF OXIDATIVE STRESS-INDUCED ENDOTHELIAL DYSFUNCTION

As enhanced vascular oxidative stress is an important mechanism of endothelial dysfunction, it is not surprising that many studies have attempted to normalize NO synthesis and bioactivity in the setting of vascular disease through the administration of antioxidants. Other strategies have included treatments with eNOS substrate and cofactors to enhance NO production or lipid-lowering agents to reduce native and oxidized LDL levels.

Ascorbic acid, BH_4, and glutathione

Ascorbate is an important aqueous extra- and intracellular antioxidant. Many studies have reported a consistent beneficial effect of acute and chronic ascorbate administration on the bioactivity of endothelium-derived NO in human subjects. Both intraarterial infusion and oral supplementation of ascorbate result in improved endothelial-dependent vasodilation in human patients with vascular pathologic conditions, including atherosclerosis, diabetes, hypertension, and cigarette smoking (*e.g.,* 41, 73). Initial speculation as to the mechanism(s) through which ascorbate improved NO bioactivity involved scavenging of $O_2^{\cdot -}$ as ascorbate effectively scavenges reactive oxygen and nitrogen species. However, the rate constant for the bimolecular reaction of ascorbate with $O_2^{\cdot -}$ is $\sim 10^5\ M^{-1}\ s^{-1}$, some 10,000-fold slower than the reaction of NO and $O_2^{\cdot -}$ (62). As such, one would predict that supraphysiological concentrations of ascorbate would be required to compete effectively with the reaction of $O_2^{\cdot -}$ and NO. This prediction has been confirmed using an *ex vivo* NO bioassay demonstrating that ascorbate concentration of $\sim$10 m*M* is required to prevent $O_2^{\cdot -}$-mediated impairment in NO bioactivity (53). Therefore, although $O_2^{\cdot -}$ scavenging may explain the effect of intraarterial infusion of supraphysiological concentrations of ascorbate (1–10 m*M*), another mechanism is required to explain the benefit afforded by physiological doses of ascorbate.

Studies in cultured endothelial cells have been helpful in elucidating the beneficial actions of ascorbic acid on NO bioactivity. Cultured cells are typically scorbutic as ascorbic acid added to commercially available culture media is most often oxidized. However, incubation of cultured endothelial cells with physiologic doses of ascorbate leads to time- and concentration-dependent ascorbic acid accumulation and enhanced NO bioactivity (45, 50). The mechanism for this observation appears due to increased cellular levels of BH_4 (50), perhaps through chemical stabilization of this NOS cofactor (45). Consistent with the notion that ascorbate acts via cellular BH_4 levels, supplementation with BH_4 improves endothelial function in human subjects with vascular disease (43, 57, 108). Furthermore, Heitzer and colleagues (43) have reported that although administration of BH_4 or ascorbate improves endothelium-derived NO bioactivity, the combination is not additive. These data raise the possibility that ascorbate and/or BH_4 is a limiting factor for endothelial NO production in vascular disease patients.

Glutathione, present in cells at millimolar concentrations, is a major determinant of the intracellular redox status. Studies have shown that treatment of human patients with L-2-

oxothiazolidine-4-carboxylic acid, an agent that selectively increases intracellular glutathione concentrations, improved NO bioactivity in the brachial artery (122). Similarly, intraarterial infusion of glutathione improves endothelial-dependent relaxation in response to acetylcholine (65). Together, the studies with ascorbate, BH_4, and glutathione indicate that the intracellular redox status of endothelial cells is an important determinant of NO bioactivity.

Vitamin E

Vitamin E, the major biological and chemical active form of which is α-tocopherol, represents an important lipid-soluble antioxidant. Similar to vitamin C, although vitamin E can scavenge $O_2^{\cdot-}$ ($k = 5 \times 10^3\ M^{-1}\ s^{-1}$), it is unlikely to compete with NO for $O_2^{\cdot-}$ *in vivo*. Despite this, increasing vascular vitamin E levels improves endothelial-dependent relaxation in experimental animal models of vascular disease (58). This beneficial action of vitamin E is not necessarily due to the ability of the vitamin to inhibit lipoprotein lipid oxidation (58). Instead, increasing tissue vitamin E levels may act by inhibiting protein kinase C-dependent promotion of $O_2^{\cdot-}$ production (60).

Whereas a consistent beneficial effect of vitamin E supplements for endothelial-dependent relaxation has been found in experimental animals, the situation in humans is contradictory. Vitamin E supplementation of hypercholesterolemic or coronary artery disease (CAD) patients improved acetylcholine and flow-mediated vasodilatation (42, 95). In contrast, other studies have reported no effect of vitamin E supplements for endothelial-dependent vasodilation in human patients with hypercholesterolemia, diabetes, or CAD (32, 87). The reasons for these mixed findings in animals and humans are unknown, but may reflect the stage of disease when intervention is applied. Intervention is typically applied in experimental animals early in the disease process, whereas human studies involve patients with established vascular disease. Therefore, a therapeutic benefit of vitamin E supplements for endothelial dysfunction in humans is not clear at this point.

Probucol is a lipid-soluble, lipid-lowering compound with antioxidant properties that has been shown to preserve NO bioactivity in animal models of vascular disease independent of its lipid-lowering properties (59). The precise mechanisms underlying this protective activity of probucol remain unclear, although probucol treatment consistently results in a reduction in the vascular $O_2^{\cdot-}$ flux in cholesterol-fed rabbit (59).

L-Arginine

Despite plasma (~100 μ*M*) and endothelial cell (millimolar range) L-arginine concentrations that clearly exceed the K_m for eNOS (~2 μ*M*), studies have reported that both dietary and intravenous administration of L-arginine increases NO bioactivity and endothelial-dependent relaxation in humans (19). The mechanisms behind the beneficial activity of L-arginine administration are unknown. One potential explanation for this "arginine paradox" relates to the observations that eNOS more avidly catalyzes the oxidation of recently transported L-arginine, and the L-arginine transporter (y+ or CAT1) is located in close proximity to eNOS present in the plasmalemmal caveolae (86). This paradox may also relate to the presence of endogenous inhibitors of NOS. For example, vascular disease is associated with increased levels of the eNOS inhibitor asymmetric-dimethylarginine (4), suggesting that increased L-arginine may effectively compete with asymmetric-dimethylargine for eNOS.

HMG-CoA reductase inhibitors

Hypercholesterolemia is a major independent risk factor for CAD and produces endothelial dysfunction due to a decrease in NO bioactivity (11). Inhibitors of the enzyme 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, are currently a mainstay of therapy for hyperlipidemia and CAD. These compounds reduce total and LDL cholesterol levels and mildly elevate circulating levels of HDL, effects thought to be responsible for their beneficial effects on cardiovascular disease. However, increasing evidence indicates that HMG-CoA reductase inhibitors have clinical benefit independent of the drug effects on circulating lipid levels (112). One such effect of HMG-CoA reductase inhibition is an improvement in endothelial function and NO bioactivity (117). Studies with endothelial cells indicate that HMG-CoA reductase inhibitors can increase eNOS activity by increasing eNOS expression (69), decreasing caveolin binding to eNOS (22), and enhancing the recruitment of HSP90 to eNOS to facilitate Akt-dependent phosphorylation of the enzyme (5, 66). Inhibition of HMG-CoA reductase may also improve NO bioactivity by decreasing vascular oxidative stress. For example, these compounds decrease endothelial $O_2^{\cdot-}$ production by decreasing protein kinase C- and Rac-dependent NADPH oxidase activation (123, 124). Withdrawal of HMG-CoA reductase inhibition is not without hazard as it produces impaired NO bioactivity due to increased vascular production of $O_2^{\cdot-}$ by a $gp91^{phox}$-containing NADPH oxidase (120). The precise mechanisms by which HMG-CoA reductase inhibitors improve NO bioactivity in human patients with vascular disease remain the topic of further study.

CONCLUSION

From the evidence outlined above, it is clear that oxidative stress and antioxidants are important determinants of NO bioactivity in the setting of vascular disease (Table 3). Moreover, increasing evidence now indicates that impairment of NO bioactivity via oxidative stress has prognostic implications for patients with vascular disease (34, 44). Although there is strong evidence that one mechanism for oxidative stress-induced endothelial dysfunction is increased $O_2^{\cdot-}$ production and NO scavenging, it is becoming increasingly apparent that different forms of oxidative stress can also impact NO bioactivity. Therefore, it is imperative to establish the mechanism(s) of impaired NO bioactivity in the setting of vascular disease in order to afford the effective design of therapeutic strategies aimed at improving NO bioactivity and reducing clinical cardiovascular events.

TABLE 3. REDOX CONTROL OF NO BIOACTIVITY

Redox factor	*Effect on NO bioactivity*
Oxidants	
$O_2^{\cdot -}$	↓
H_2O_2	↓ and ↑
Lipid peroxidation	↓
Oxidized LDL	↓
$ONOO^-$	↓
HOCl	↓
MPO	↓
15-Lipoxygenase	↓
Antioxidants	
SOD	↑
Glutathione peroxidase	↑
Ascorbate	↑
BH_4	↑
Glutathione	↑
Vitamin E	↑
Probucol	↑

See text for references.

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ABBREVIATIONS

apoE, apolipoprotein E; BH_4, tetrahydrobiopterin; CAD, coronary artery disease; cGMP, cyclic 3′,5′-guanosine monophosphate; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial isoform of nitric oxide synthase; HDL, high-density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; HSP90, heat shock protein 90; LDL, low-density lipoprotein; MPO, myeloperoxidase; NO, nitric oxide; NOS, nitric oxide synthase; $O_2^{\cdot -}$, superoxide anion radical; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase.

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