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Comprehensive Invited Review

Redox Control of Endothelial Function and Dysfunction: Molecular Mechanisms and Therapeutic Opportunities

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Abstract

The endothelium is essential for the maintenance of vascular homeostasis. Central to this role is the production of endothelium-derived nitric oxide (EDNO), synthesized by the endothelial isoform of nitric oxide synthase (eNOS). Endothelial dysfunction, manifested as impaired EDNO bioactivity, is an important early event in the development of various vascular diseases, including hypertension, diabetes, and atherosclerosis. The degree of impairment of EDNO bioactivity is a determinant of future vascular complications. Accordingly, growing interest exists in defining the pathologic mechanisms involved. Considerable evidence supports a causal role for the enhanced production of reactive oxygen species (ROS) by vascular cells. ROS directly inactivate EDNO, act as cell-signaling molecules, and promote protein dysfunction, events that contribute to the initiation and progression of endothelial dysfunction. Increasing data indicate that strategies designed to limit vascular ROS production can restore endothelial function in humans with vascular complications. The purpose of this review is to outline the various ways in which ROS can influence endothelial function and dysfunction, describe the redox mechanisms involved, and discuss approaches for preventing endothelial dysfunction that may highlight future therapeutic opportunities in the treatment of cardiovascular disease. *Antioxid. Redox Signal*. 10, 1713–1765.

I. Introduction

Oxidative stress, traditionally defined as an imbalance between oxidants and antioxidants that favors the former, is a prominent feature of vascular disease states including atherosclerosis, diabetes, and hypertension (64, 202, 457, 480). Original interest in the role of oxidative stress in vascular disease stems from the "oxidative-modification hypothesis" of atherogenesis (454). This hypothesis proposes that a critical initiating event of atherogenesis is the oxidation of low-density lipoprotein (LDL). Despite evidence that oxidatively modified LDL is detected in animal and human atherosclerotic lesions, direct support for LDL oxidation causing atherosclerosis is scarce [reviewed in detail by (457)]. In recent times, it has become increasingly apparent that oxidative reactions, in addition to oxidative modification of lipoproteins, are important for the initiation and progression of vascular disease. Of note, the generation of reactive oxygen species (ROS) by cells of the blood vessel wall is currently an area of intense research focus. Diseased arteries isolated from human patients show increased production of the superoxide anion radical ($O_2^{\bullet -}$), derived primarily from vascular smooth muscle and endothelial cells (193, 446, 449). Several enzymatic sources appear responsible for the elevated production of ROS noted in vascular disease: NADPH oxidase, xanthine oxidase, mitochondria, and "uncoupled" endothelial nitric oxide synthase (eNOS) (82, 480). This increased flux of ROS modulates the function and phenotype of vascular endothelial and smooth muscle cells that together contribute to vascular dysfunction. The current review focuses on the redox processes that influence endothelial function and dysfunction. Excellent reviews are available that discuss the importance of redox events for the control of vascular smooth muscle cell function (92, 489).

The endothelium represents a central element in the control of vascular homeostasis through maintaining the synthesis of endothelium-derived nitric oxide (EDNO) from eNOS. Convincing evidence now supports that endothelial

dysfunction apparent in various vascular disorders, including hypertension, diabetes, and atherosclerosis, is due, in part, to elevated vascular ROS production (64, 480). Thus, ROS can directly react with and inactivate EDNO, promote protein dysfunction, and stimulate aberrant cell signaling, actions that participate in the initiation and progression of endothelial dysfunction. Importantly, endothelial dysfunction is associated with an increased risk of acute clinical events, myocardial infarction, and stroke (180, 212, 291). As such, significant interest is found in defining the oxidative mechanisms involved. This article revises current knowledge on how ROS mediate cell signaling, discusses the endothelial sources of ROS, outlines ways through which different oxidants affect EDNO bioactivity, and highlights strategies that ameliorate oxidative stress–induced endothelial dysfunction that may ultimately provide clinical benefit.

II. Redox Signaling

The generation of ROS is inevitable for aerobic organisms. Originally, ROS were considered to be random and destructive agents produced as unnecessary byproducts of aerobic metabolism or components of the innate immune defense against microorganisms. The initiation of ROS formation requires electrons (e^-), frequently generated by the mitochondrial electron-transport chain or by NADPH oxidase, that mediate the univalent reduction of molecular oxygen (O_2) to form $O_2^{\bullet -}$, which is subject to spontaneous or enzyme-catalyzed dismutation into hydrogen peroxide (H_2O_2) (Reaction 1).

$$O_2 + 2\ e^- \rightarrow O_2^{\bullet -} + O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2 \quad [1]$$

Although the term ROS refers to all reactive species derived from the one-electron reduction of O_2, it is important to note that different ROS exhibit distinct chemical properties that have important implications from their biologic actions. For example, $O_2^{\bullet -}$ contains a single electron in its outer bonding orbital, meaning it is a free radical with a relatively short biologic half-life. Although capable of mediating one-electron oxidation reactions, $O_2^{\bullet -}$ represents a stronger reductant than oxidant (60). Also, $O_2^{\bullet -}$ is charged and thus unsuitable for crossing biologic membranes in the absence of specific anion transporters. In contrast, H_2O_2 is an uncharged, two-electron oxidant that has a longer half-life in biologic systems and is capable of diffusing across cellular membranes, characteristics suitable for the oxidant to act as a cell-signaling molecule (see later).

The ROS produced through the concerted action of NADPH oxidase and myeloperoxidase (MPO) in activated phagocytes has been considered toxic and destructive agents necessary for the killing of microbes. However, recent studies support that proteases and not ROS act as the antimicrobicidal agents (413). Instead, ROS appear to act as signaling molecules that stimulate protease release (413). Similarly, macrophage-derived ROS exhibit an immune signaling role by influencing T-cell selection, maturation, and activation status (170). These important findings add to the rapidly growing body of evidence that reduction and oxidation (redox) reactions control cell-signaling pathways that govern an array of physiologic processes, including cell growth, transformation, senescence, and apoptosis. This has focused interest on exploring the underlying basis through which redox reactions exhibit the specificity necessary to control divergent biologic responses.

It is now appreciated that an array of biologic stimuli, including growth factors, cytokines, hormones, and neurotransmitters, stimulate a transient burst of endogenous ROS or reactive nitrogen species (RNS) production in nonphagocytic cells, including vascular smooth muscle and endothelial cells (82, 92, 145, 319, 416, 504). Endogenous ROS and RNS with signaling potential are frequently synthesized by NADPH oxidase, mitochondria, or NOS and act by reversibly altering the function of a variety of target proteins, including phosphatases, kinases, small GTPases, transcription factors, ion channels, structural proteins, and metabolic enzymes (Table 1) (82, 145, 319, 416, 504). As such, sublethal concentrations of ROS or RNS are now recognized as ubiquitous and physiologic intracellular messengers. Accordingly, exposure of cells to low, nontoxic concentrations of exogenous H_2O_2 modulates cell proliferation and migration. For example, the proliferation of cultured human endothelial cells or tube formation by bovine aortic endothelial cells is enhanced by exposure to low concentrations of H_2O_2 (1–10 μM) (400, 557). Conversely, selective removal of endogenous H_2O_2 by overexpression of the peroxide catabolic enzyme catalase inhibits endothelial cell proliferation (562) and angiogenesis in an *in vivo* dermal wound-healing model (420). Exposure of cells to select ligands (*e.g.*, growth factors, cytokines, angiotensin II) or hemodynamic force (*i.e.*, shear stress or cyclic strain) induces cellular H_2O_2 production that stimulates cell signaling. Sundaresan (464) first showed that selective removal of endogenous H_2O_2 through the overexpression of catalase in vascular smooth muscle cells blocked platelet-derived growth factor (PDGF)-induced ROS production, PDGF-receptor tyrosine phosphorylation, Erk1/2 activation, plus cell proliferation and migration (464). Vascular endothelial growth factor (VEGF) is required for vasculogenesis and angiogenesis under physiologic and pathologic settings. Stimulation of endothelial cells with VEGF induces the production of NADPH oxidase–derived H_2O_2 that is important for activation of the tyrosine kinase activity of VEGF receptor-2 and subsequent downstream signaling events important for endothelial proliferation and migration and hence angiogenesis (94, 497).

A. Molecular targets of redox signaling

Essential to characterizing the importance and scope of action of redox cell signaling is the identification of the cellular targets that sense and transduce the redox signal. Different redox-active species react with selected cellular targets to modulate cell signaling. Thus, NO binds to the heme group of an array of enzymes to modulate their activity (*e.g.*, binding of NO to the heme of sGC activates the enzyme and this signals the vasorelaxation of blood vessels; see Section III.A.). Similarly, $O_2^{\bullet -}$ reacts with iron–sulfur clusters of key cellular proteins to alter their activity [*e.g.*, destabilization of the iron–sulfur cluster of mitochondrial aconitase by $O_2^{\bullet -}$ inhibits the enzyme activity, resulting in the inhibition of mitochondrial respiration (164, 165)]. Another important aspect underlying redox signaling is the reversible, covalent modification of specific cysteine thiol residues that reside within active and allosteric sites of proteins, which results in alteration of protein function (Fig. 1). Various redox modifica-

Table 1. Examples of Proteins Subject to Redox Control

Protein class	*Protein*	*Thiol redox modification*	*Effect on protein function*
Phosphatase	PTP1B	Oxidation	Inhibition
	SHP2	Oxidation	Inhibition
	LMW-PTP	Oxidation	Inhibition
	PTEN	Oxidation	Inhibition
	MAPK phosphatase	Oxidation	Inhibition
Protein kinase	Src	Oxidation	Activation
	PKA	Oxidation	Activation
	PKG	Oxidation	Activation
	PKC	Oxidation	Activation
	IKK	*S*-Nitrosylation	Inhibition
	JNK	*S*-Nitrosylation	Inhibition
	Akt	*S*-Nitrosylation	Inhibition
Small GTPase	Ras	*S*-Nitrosylation/*S*-Glutathiolynation	Activation
Transcription factor	AP-1	Reduction	↑ DNA binding
	NF-κB	Oxidation	↑ Nuclear export
		Reduction	↑ DNA binding
	Nrf2	Oxidation	↑ Nuclear export
		Reduction	↑ DNA binding
	P53	Reduction	↑ DNA binding
Ion channels	TRP	*S*-Nitrosylation	Activation
	SERCA	*S*-Glutathiolynation	Activation
Antioxidants	Thioredoxin	*S*-Nitrosylation	Activation
	Peroxiredoxin	Oxidation	Inhibition
Enzymes	GAPDH	Oxidation	Inhibition
	sGC	*S*-Nitrosylation	Inhibition
	eNOS	*S*-Nitrosylation	Inhibition
	Hsp90	*S*-Nitrosylation	Inhibition

For references, see text.

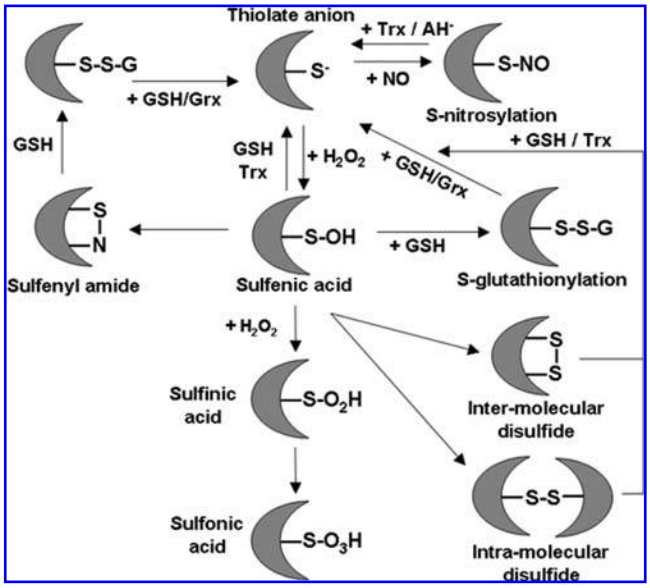

FIG. 1. Protein thiol modifications essential for redox signaling. Select cysteine thiolate anions in certain proteins are subject to posttranslational *S*-oxidation, *S*-nitrosation, and/or *S*-glutathiolynation that alter protein function and underlie redox signaling in cells. Certain redox modifications are reversible through the action of cellular reductants, including glutathione (GSH), thioredoxin (Trx), glutaredoxin (Grx), or ascorbate (AH^-).

tions of cysteines alter protein function with those mediated by H_2O_2 of particular relevance to cell signaling. Although it is a mild oxidant and hence relatively inert with most biomolecules, H_2O_2 oxidizes cysteine residues in proteins to produce disulfides (R-S-S-R) *via* formation of a sulfenic acid (R-SOH) intermediate in what is a biologically reversible reaction (91, 111). Disulfides occur between adjacent cysteines within a protein (intraprotein disulfide), between two proteins (interprotein disulfide), or a mixed-disulfide formed between a protein thiol and glutathione, termed *S*-glutathionylation. Protein thiols can be further oxidized by H_2O_2 to higher oxidation states to form sulfinic ($R\text{-}SO_2H$) and sulfonic ($R\text{-}SO_3H$) acids. In addition to oxidation, thiols are subject to *S*-nitrosation/*S*-nitrosylation, which describes the covalent binding of NO to cysteine thiols to form protein-*S*-nitrosothiols (R-S-NO) (217). Several enzymatic and nonenzymatic systems are responsible for the biologic reduction of oxidized or nitrosated cysteine thiols. For example, thioredoxin (Trx) efficiently reduces sulfenic acids and protein disulfides, whereas glutaredoxin (Grx) catalyzes the reduction of *S*-glutathionylated protein cysteines (226). To date, only the enzyme sulfiredoxin has been shown to reduce sulfinic acids (48), whereas formation of sulfonic acid is, for now, considered to represent a biologically irreversible reaction. Reduction of *S*-nitrosothiols can be achieved by Trx or Grx (226). Reversible posttranslational modification of specific cysteines in proteins *via* *S*-oxidation, *S*-nitrosylation, or *S*-glutathionylation is increasingly considered to represent

a ubiquitous, physiologic signaling modality controlling diverse cellular processes, analogous to protein *O*-phosphorylation (Fig. 1).

Although most proteins contain multiple cysteines, specific residues exhibit a predilection for redox modification. Thus, H_2O_2 reacts more readily with cysteine thiolate anions (Cys-S^-) than with protonated residues (Cys-SH). Most protein cysteines are protonated at physiologic pH (pK_a ~8.5). However, under physiologic conditions, selected proteins contain cysteine thiolate anions that are formed through the formation of salt bridges with surrounding positively charged amino acid residues and exhibit pK_a values of ~5.0. Thus, an important aspect underlying the signaling properties of H_2O_2 is its ability to target proteins containing oxidation-susceptible deprotonated cysteines critical for protein function. With respect to *S*-nitrosylation, a consensus sequence has been proposed in which a target cysteine resides within a sequential amino acid motif containing a polar amino acid, an acidic or basic amino acid, cysteine, and an acidic amino acid, an environment that facilitates the acid–base chemistry necessary for trans-nitrosation reactions (452). A more recent study using a high-throughput proteomics coupled to a bioinformatics approach failed to identify a linear Cys-flanking motif that predicts stable trans-nitrosation of cysteines in different proteins (201). Instead, specificity of *S*-nitrosylation appears dependent on the tertiary structure of proteins and how it affects the pK_a and local hydrogen bonding and electrostatic interactions. Overall, *S*-nitrosylation appears to be favored for reactive cysteines that reside in a low-pK_a and hydrophobic environment, in close proximity to aromatic amino acid residues (201). Of note, the chemical reactions responsible for *S*-nitrosylation are complex and do not represent a simple reaction between NO and thiol. Instead, multiple redox reactions are responsible for *S*-nitrosylation *in vivo*, and the chemical reaction favored appears to be a function of the target protein thiol (217).

Several classes of signaling proteins capable of conveying a broad spectrum of cellular signals and that contain conserved redox-sensitive cysteines have been identified. These include phosphatases, protein kinases, transcription factors, ion channels, structural proteins, and metabolic and antioxidant enzymes (Table 1).

1. Phosphatases. Phosphatases are a family of enzymes that remove phosphate groups from phosphorylated amino acid residues on proteins to counteract the signaling actions of protein kinases. Redox control of protein tyrosine phosphatase (PTP) activity has received considerable recent attention. PTPs are characterized by the presence of a catalytically critical cysteine residue (pK_a = 4.7–5.4) that, at neutral pH, resides as a thiolate anion in a signature active-site motif, *His-Cys-X-X-Gly-X-X-Arg-Ser/Thr* (where *X* is any amino acid) (487). This low-pK_a environment underlies the cysteine thiol function as a nucleophile important for phosphatase activity but also renders the amino acid susceptible to oxidation. Oxidation of the essential cysteine by H_2O_2 inactivates phosphatase activity (111). Reversible oxidative inactivation of several phosphatases by endogenous H_2O_2 has been identified as a key mechanism leading to the activation of receptor tyrosine kinase activation and initiation of downstream signaling pathways, in response to binding of various ligands to their specific receptors. Meng *et al*. (350) used a novel redox-sensitive, in-gel PTP assay to observe reversible oxidative inactivation of SHP-2 necessary for the activation of the PDGF-receptor tyrosine kinase activity in vascular smooth muscle cells stimulated with PDGF. A requirement for inactivation of the PTP by intracellular H_2O_2 was the recruitment and binding of SHP-2 to the PDGF receptor (350). Similarly, in endothelial cells, H_2O_2-induced activation of platelet endothelial adhesion molecule (PECAM)-1/CD-31 involves the recruitment and transient inactivation of SHP-2 to the membrane-bound glycoprotein that controls endothelial–leukocyte interactions (247). In addition to PTPs, other phosphatase family members are sensitive to redox control. Oxidation of the active-site Cys-124 of the lipid phosphatase and tumor-suppressor PTEN has been documented in different cell types stimulated with growth factors or insulin (283). H_2O_2 also inhibits MAP kinase phosphatases, allowing sustained activation of JNK signaling in cells treated with TNF-α (256).

Essential for oxidative inactivation of phosphatases to act as a physiologic signaling modality is that the process be chemically reversible *via* reduction of the oxidized cysteine by cellular reductants (see later). Oxidation of thiolate anions in phosphatases by H_2O_2 results in the initial formation of sulfenic acid. Rather than further oxidation and irreversible formation of sulfinic or sulfonic acid derivatives, the highly unstable sulfenic acid of PTP-1B forms a cyclic sulfenyl amide species (427, 501) (Fig. 1). Alternatively, PTP-1B is protected from irreversible oxidation through *S*-glutathionylation (36). For other phosphatases (*i.e.*, LMW-PTP, PTEN), formation of an intra-molecular disulfide with a nearby cysteine protects from irreversible oxidation (88, 429). Reduction of the cyclic sulfenyl amide or disulfides restores the catalytic activity of the PTP that acts to terminate the redox signal (Fig. 1).

2. Protein kinases. Certain protein kinases contain reactive cysteines, important for their function, that render them sensitive to redox control. For example, the nonreceptor tyrosine kinase Src has received recent attention. Integrin ligation stimulates the production of endogenous H_2O_2, which oxidizes Src kinase at Cys-245 in the SH2 domain and Cys-487 in the kinase domain, resulting in activation of the enzyme (175). H_2O_2 treatment or cyclic strain–induced ROS production activates Src in endothelial cells, leading to activation of MAP kinase signaling (87). Whereas oxidative inactivation of PTPs is considered to represent an initial event leading to receptor tyrosine kinase trans-activation (*i.e.*, ligand-independent stimulation of receptor tyrosine kinase activity) by oxidants, recent data also indicate a role for Src kinase. Thus, in H_2O_2-treated endothelial cells, activation of Src kinase is necessary for the trans-activation of the EGF receptor and resultant stimulation of the JNK (83) or PI3-K/Akt/eNOS (479) pathways that act to promote or protect against oxidative stress–induced endothelial cell death, respectively. Activation of Src is also crucial for H_2O_2 to induce nuclear export of telomerase reverse transcriptase (TERT) into the cytosol, resulting in endothelial cell senescence (196). Serine/threonine protein kinases are also susceptible to control through direct redox modification of cysteines. For example, the N-terminal regulatory domain of protein kinase C (PKC) contains zinc-binding, cysteine-rich motif that is susceptible to oxidation, leading to activation of the enzyme (184). Conversely, modification of reactive

cysteines in the C-terminal catalytic domain can inhibit PKC activity (184). Oxidative activation of PKC-α by NADPH oxidase–derived ROS in endothelial cells is essential for VCAM-1-dependent transendothelial migration of lymphocytes (1). Recent work indicates that H_2O_2 directly activates cyclic GMP– (PKG) and c-AMP–dependent (PKA) protein kinases in a manner independent of the cyclic nucleotides but dependent on oxidation of critical cysteines and resultant formation of an interprotein disulfide (54, 61). Oxidant activation of PKG represents one mechanism through which H_2O_2 can induce the relaxation of arteries (61). In addition to cysteine oxidation, several protein kinases, including Akt (558) and JNK (392), are subject to *S*-nitrosylation, resulting in inhibition of their kinase activity. Small GTPase enzymes are also subject to redox control, as exemplified by Ras that can undergo *S*-nitrosylation (286) or *S*-glutathionylation (6) at Cys-118, leading to activation of the enzyme and stimulation of downstream signaling, including the MAP kinase and PI3-K/Akt pathways.

3. Transcription factors. An important mechanism through which ROS and RNS alter vascular cell function is through redox-dependent alteration of transcriptional output. In mammalian cells, numerous transcription factors are considered to be subject to redox control, including NF-κB, Nrf2, AP-1, HIF-1, and P53 (Table 1) (82, 319). Of these, NF-κB, which plays a central role in the control of inflammatory and immune-response genes, is considered the prototypical transcription factor subject to redox control. In endothelial cells, H_2O_2 activates NF-κB (101), which is important for the oxidants' ability to promote tubular morphogenesis of human microvascular endothelial cells (443). Redox-sensitive activation of NF-κB is also necessary for VEGF to promote endothelial gene expression of the mitochondrial isoform of SOD (Mn-SOD) (2) or proinflammatory stimuli to induce the expression of leukocyte adhesion molecules (VCAM-1, ICAM-1) and resultant increased endothelium–leukocyte interactions (85, 334). Endothelial levels of the p65 subunit of NF-κB are elevated in arteries from aged versus young humans, and this is correlated with the extent of increase in endothelial 3-nitrotyrosine levels and impairment of endothelium-dependent relaxation in aged subjects (120). Enhanced endothelial activation of NF-κB in aged rat arteries is also linked to enhanced mitochondrial dysfunction, resulting in an increased production of H_2O_2 by the organelle (494).

The nonactivated NF-κB p50-p65 heterodimer resides in the cytoplasm bound to its inhibitory protein, I-κB. Activation of NF-κB occurs through phosphorylation and ubiquitination of I-κB (46), leading to translocation of NF-κB to the nucleus and phosphorylation of Ser-276 on the p65 subunit (174). Redox control of NF-κB is complex. An oxidizing stimulus in the cytoplasm is associated with activation of inhibitory κB kinase (IKK), leading to enhanced I-κB degradation and nuclear translocation (314). DNA binding of activated NF-κB requires a reducing environment within the nucleus to ensure that Cys-62 in the DNA-binding region of the p50 subunit is reduced (342). Maintenance of a reducing environment in the nucleus for DNA binding of active NF-κB is mediated by thioredoxin (223) and redox factor-1 (Ref-1) (547). Despite these factors, it is increasingly apparent that the extent to which NF-κB activation is subject to redox control and the mechanisms involved is stimulus and cell-type specific (388). Whereas H_2O_2 activates NF-κB in endothelial cells (101), the oxidant inhibits NF-κB activation in epithelial cells (415). Negative control of NF-κB by H_2O_2 involves inactivation of IKK through oxidation and resultant *S*-glutathionylation of Cys-179 of the β-subunit of the kinase (415). Similarly, IKK activity and hence NF-κB activation is inhibited by *S*-nitrosylation of Cys-179 (414). NO can also inhibit NF-κB p50-p65 heterodimer DNA binding through *S*-nitrosylation of cysteines in both p50 and p65 subunits (263, 332).

Recent studies have identified the Nrf2-Keap1 system, which coordinately regulates cytoprotective gene expression *via* the antioxidant responsive element (ARE), as a redox-sensitive transcription factor system that exhibits similarities to the control of NF-κB (178). Under basal conditions, the transcription factor Nrf2 is anchored within the cytoplasm by Keap1 targeting Nrf2 for ubiquitination and proteasome degradation. This represses the ability of Nrf2 to induce genes possessing AREs in their promoters. When cells are exposed to oxidative stress, distinct cysteines on Keap1 are oxidized, leading to the dissociation of the NrF2/Keap1 complex and the nuclear translocation of Nrf2, where it forms heterodimers with other transcription factors that bind to AREs present within phase II and antioxidant enzyme gene promoters, increasing their transcription, including glutathione-*S*-transferase and heme oxygenase-1 (178, 514, 568). Recent data show that activation of Nrf2 has implications for endothelial homeostasis. Exposure of human aortic endothelial cells to vasoprotective laminar flow maintains Nrf2 activity, whereas proatherogenic flow inhibits the binding capacity of Nrf2 to AREs (104, 229). Activation of Nrf2 in response to atheroprotective shear stress is dependent on the production of endogenous ROS and activation of the PI3-kinase/Akt pathway, and these events result in the upregulation of the expression of various antioxidant and cryoprotective genes that act to maintain the redox homeostasis of endothelial cells (104, 522). Finally, zinc supplements protect endothelial cells from H_2O_2-induced cytotoxicity *via* Nrf2-dependent stimulation of the biosynthesis of glutathione (95).

Other transcription factors of relevance to vascular homeostasis and disease are known to be subject to redox control (Table 1), and the control of these factors has been reviewed elsewhere (82, 319). Of note, a recent study highlights that redox control of p53 influences the expression of genes that govern the cellular redox status. Sablina *et al*. (424) reported that under basal conditions, p53 governs the upregulation of several genes with antioxidant capacity, and this is associated with the maintenance of intracellular ROS at low, nontoxic levels. However, downregulation of basal p53 levels results in increased cellular oxidative stress, leading to oxidation of DNA, increased mutation rate, and karyotype instability, deleterious events prevented by antioxidant supplements (424).

In addition to phosphatases, kinases, and transcription factors, direct redox modification of critical cysteines also represents a means of controlling ion channels and an array of other enzymes (Table 1). Examples of the redox control of these other proteins can be found throughout the remainder of this article.

B. Spatial control of redox signaling

In light of the cytotoxic potential of uncontrolled ROS and RNS production, if these species are to mediate cell signaling *via* redox-dependent, posttranslational modification of proteins, it is important that their production be focused at the intracellular site critical for signaling. Support for such spatial control of redox signaling has emerged in migrating endothelial cells. Site-specific ROS production linked to directed endothelial cell migration in response to VEGF appears to involve the targeting of active NADPH oxidase 2 (Nox2) and the VEGF receptor-2 from plasma membrane caveolae/lipid rafts to focal complexes/adhesions in the lamellipodia and membrane ruffles at the leading edge of the migrating cell (240, 496). Similar to VEGF, treatment of coronary artery endothelial cells with Fas ligand promotes lipid-raft clustering and recruitment of Nox2 and its subunits $p47^{phox}$ and Rac1 to these plasma-membrane microdomains, resulting in enhanced localized ROS production (564). This suggests that, in response to certain stimuli, subcellular microdomains, such as caveolae and lipid rafts, act as signaling platforms to convey redox signaling in endothelial cells. The redox-sensitive proteins enriched in these cellular microenvironments remain to be fully characterized.

Traditionally, H_2O_2 is thought to diffuse freely across lipid membranes. It has been suggested that H_2O_2 can traverse several cell diameters before reaction with its target or it is consumed. However, recent studies suggest that certain membranes are poorly permeable to H_2O_2 and instead regulate transport of the oxidant through changes in lipid composition or by aquaporins, a group of diffusion-facilitated channel proteins responsible for the transport of noncharged solutes (*e.g.*, water) (47). If true, then aquaporins or membrane lipid composition or both would represent important determinants of redox signaling and add a further level of control to H_2O_2 signaling. More work on subcellular H_2O_2 diffusion and the control mechanisms involved is warranted, as it will likely provide novel insights into the spatial control of redox signaling.

C. Antioxidant networks and redox signaling

In addition to spatial constraints, redox signaling is subject to temporal control. To mediate reversible signaling, H_2O_2 must initially evade antioxidant defenses such that its local concentration increases rapidly above a threshold necessary to oxidize target proteins. On mediating its signaling function, H_2O_2 must be removed in a timely manner, and the oxidized cysteine residues chemically repaired in targeted signaling proteins. The local intracellular concentration of H_2O_2 in mammalian cells is primarily governed by the antioxidant enzymes catalase, glutathione peroxidase (Gpx), and peroxiredoxins (Prx). Although catalase contains a catalytic heme prosthetic group, the catalytic reduction of H_2O_2 by Gpx and Prx involves conserved active-site selenocysteine and cysteine residues, respectively (Fig. 2). These antioxidant proteins, including members within the same family, are encoded by distinct genes and are targeted to different subcellular sites that may represent a means to confine H_2O_2 production to those cell compartments in which the oxidant is required for signaling reactions. Whereas catalase is exclusively localized to peroxisomes, Gpx1 is located primarily in the cytosol but also in the mitochondria. With

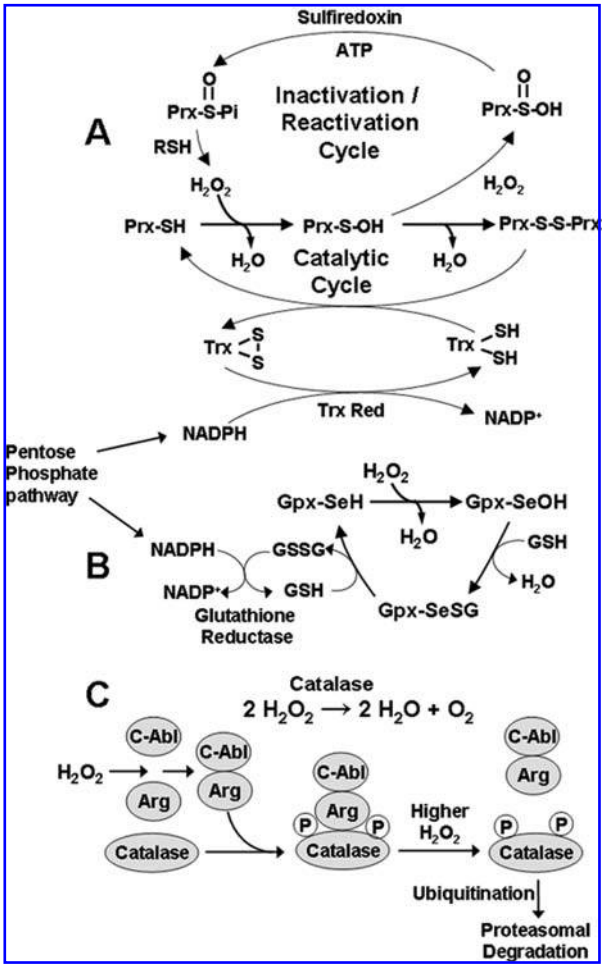

FIG. 2. Antioxidant networks important for redox signaling. The local concentration of H_2O_2 available for redox signaling is subject to control by peroxiredoxin (Prx)/thioredoxin (Trx) network, glutathione peroxidase (Gpx)/glutathione reductase network, and catalase. (**A**) The catalytic cycle of 2-Cys Prx subfamily involves the oxidation of a conserved cysteine (Cys^{51}) by H_2O_2 to a sulfenic acid (Prx-S-OH) that subsequently reacts with a conserved cysteine (Cys^{172}) of the other subunit to form an intermolecular disulfide (Prx-S-S-Prx). The disulfide-linked homodimer is selectively reduced by Trx, which receives reducing equivalents from NADPH (derived from the Pentose Phosphate Pathway) via the action of Trx reductase. When local H_2O_2 concentrations increase above a certain threshold, the Prx-S-OH intermediate formed during the catalytic cycle is subject to further oxidation to a catalytically inactive sulfinic acid (Prx-SO_2H). This transient inactivation of the peroxidase activity allows H_2O_2 to react with the cysteines of proteins to mediate redox signaling. Reactivation of Prx involves the reduction of the sulfinic acid in a reaction catalyzed by sulfiredoxin, which involves the hydrolysis of ATP and reducing equivalents donated by cellular reductants (RSH; GSH or Trx). (**B**) The catalytic action of Gpx requires the oxidation of a conserved selenocysteine (SeH) by H_2O_2. Recycling of the oxidized peroxidase is performed by reduced glutathione (GSH), the intracellular levels of which are maintained by glutathione reductase and NADPH. (**C**) The activity of catalase and Gpx (not shown) are enhanced through tyrosine phosphorylation mediated by the redox-sensitive tyrosine kinases c-Abl and Arg. Higher H_2O_2 concentrations cause dissociation of the catalase/kinase protein complex and the phosphorylated catalase is ubiquitinated and targeted for degradation by the proteasome.

respect to Prx, Prx 1 and 2 reside in the cytosol, Prx 3, in the mitochondria, and Prx 4, in the endoplasmic reticulum and extra-cellular space. Different Prx are subdivided into classes based on the mechanisms and number of cysteines involved during the catalysis; typical 2-Cys, atypical 2-Cys, or 1-Cys Prx. For example, typical 2-Cys Prx contain two conserved cysteine residues that both participate in peroxide reduction. Although the antioxidant enzymes act in concert to protect cells from the cytotoxic actions of high H_2O_2 concentrations, recent findings highlight that they are also active participants in the spatial and temporal control of redox signaling. Notably, these antioxidant enzymes are subject to posttranslational modifications that temporally control their H_2O_2-degrading activity to represent a mechanism to govern transient changes in local H_2O_2 levels, important for redox signaling in response to specific stimuli or conditions.

1. Peroxiredoxins. A redox-signaling role for antioxidant enzymes is exemplified by Prxs. Prxs show high reactivity for H_2O_2 when compared with other thiol oxidants and exhibit a rate constant (1.3×10^7 M/sec) sufficiently high to outcompete catalase and Gpx for H_2O_2 (396). During the catalytic reduction of H_2O_2, the active-site cysteine of Prxs occasionally interacts with two molecules of H_2O_2, resulting in hyperoxidation or sulfinylation ($-SO_2$) and transient inactivation of Prxs, thereby enabling H_2O_2 to target cysteines of local signaling proteins (544) (Fig. 2A). Reduction of sulfinylated Prx is catalyzed by a novel ATP-dependent enzyme termed sulfiredoxin (48). A recent study by Choi and colleagues (89) showed that Prx2 negatively regulates PDGF signaling by transiently associating with the PDGF receptor and suppressing the oxidative inactivation of PTPs by endogenous H_2O_2. Therefore, the transient recruitment of Prx2 to a growth-factor receptor provides both temporal and spatial control of local H_2O_2 concentrations at the focal point of signaling. A role of mitochondrial Prx3 for spatial control of local H_2O_2 concentration is highlighted by a study showing that depletion of Prx3 enhanced mitochondrial H_2O_2 production and apoptosis in cells stimulated with TNF-α (78). A screen of the *S*-nitrosoproteome in cultured endothelial cells identified Prx1 as an *S*-nitrosylated protein, raising the possibility that, in addition to control through cysteine oxidation, Prx1 activity is also subject to redox control by NO (556). In a further layer of control, Prxs are subject to phosphorylation at Thr-90 by cyclin B-dependant kinase. In cells, Prx1 phosphorylation is observed primarily in cells during mitosis, implicating a role for a temporal increase in H_2O_2 in this phase of the cell cycle (79).

2. Catalase and glutathione peroxidase. Recent data suggest that catalase and Gpx are subject to phosphorylation that allows these antioxidant enzymes to respond to changes in cellular H_2O_2 concentrations to control the signaling properties of the oxidant (Fig. 2). In response to low concentrations of H_2O_2, catalase and Gpx are subject to phosphorylation by the tyrosine kinases c-Abl and Arg, resulting in enhanced enzyme activity (69, 70). Accordingly, cells deficient in either c-Abl or Arg produce increased levels of H_2O_2 (69, 70). Higher levels of H_2O_2 promote dissociation of the tyrosine kinases from catalase, leading to ubiquitination and degradation of the antioxidant enzyme, resulting in reduced removal of H_2O_2 and increased cell death (70) (Fig. 2). The importance of Gpx for endothelial function has been recently highlighted. Thus, increasing Gpx expression and activity protects endothelial cells from H_2O_2-induced toxicity (575) or angiotensin II–induced impairment of EDNO bioactivity (90). Conversely, vessels from mice with heterozygous or homozygous deficiency of Gpx show impaired EDNO bioactivity and hence a dysfunctional endothelium (90, 151).

3. Thioredoxin. Reversion of redox signaling requires reductive repair of reactive cysteines in proteins. An antioxidant enzyme responsible for the repair of oxidized protein cysteines is the 12-kDa oxidoreductase Trx (226). Two types of Trx exist: Trx1 in the cytosol or Trx2 in the mitochondria, maintained in the reduced state *via* electrons donated by Trx1 or Trx2 reductase enzymes, respectively. The Trx family of proteins represents important regulators of cellular redox signaling by using Cys-32 and Cys-35 in the conserved active-site motif (Cys-Gly-Pro-Cys) to reduce intra- or intermolecular disulfides or sulfenic acids present on proteins. For example, Trx specifically maintains the conserved active-site cysteines of Prx in the reduced and hence catalytically active form (Fig. 2A). Also important to the signaling properties of Trx is the ability to bind selected proteins and modulate their function. For example, Trx1 forms a complex with apoptosis signal–related kinase-1 (ASK1), which is involved in the activation of c-Jun N-terminal kinase (JNK) and p38 kinase, two stress-activated components of the MAP kinase pathway that can induce apoptosis. Trx1 complex formation inactivates ASK1 (425), and this process is reversed by ROS produced in response to TNF-α (186). Association of Trx with ASK1 through a single cysteine induces ASK1 ubiquitination/degradation and reduced apoptosis. Also important for Trx1 control of ASK-1 is the stress-responsive thioredoxin-interacting protein (TXNIP). Binding of TXNIP to Trx attenuates the Trx–ASK1 interaction. In endothelial cells, fluid shear stress reduces TXNIP expression, leading to increased Trx1 binding to ASK1 and inhibition of TNF-α activation of JNK/p38 and VCAM1 expression (552). Trx2 also binds ASK1 in the mitochondria, and this inhibits apoptosis without interference of ASK-1–dependent activation of JNK, indicating that ASK1 in the cytosol and mitochondria promotes TNF-α–mediated apoptosis *via* distinct signaling pathways (570). Cytosolic Trx1 and mitochondrial Trx2 can differentially control redox signaling; whereas Trx1 stimulates Akt and HIF-1α trans-activation, Trx2 is inhibitory (577). In the nucleus, Trx associates with Ref-1, which reduces critical cysteines within various redox-sensitive transcription factors to promote DNA binding, including AP-1, NF-κB, HIF-1, and p53 (319). The N-terminal region of Ref-1 contains a redox regulatory domain characterized by two critical cysteines, Cys-65 and Cys-93, important in the redox-dependent modification of transcription factors and maintained in the reduced state by nuclear-translocated Trx (548).

In keeping with a central role of Trx as a regulator of redox signaling governing cell phenotype and viability, recent studies indicate that Trx itself is subject to posttranslational control of specific cysteine residues. Trx in endothelial cells maintains the cellular content of *S*-nitrosylated proteins, which is critical for sustained endothelial viability (197). Trx itself is subject to *S*-nitrosylation at Cys-69 from NO liberated from eNOS, and this preserves the redox regulatory activity of Trx and its ability to scavenge ROS to protect en-

dothelial cells from apoptosis (197). Atheroprotective stimuli or agents such as shear stress or statins augment *S*-nitrosylation of Trx (198, 224). In addition to *S*-nitrosylation, Trx is subject to redox control at the level of its expression. Trx protein levels in endothelial cells are subject to differential control by H_2O_2; low concentrations (10–50 μM) enhance Trx expression, whereas high concentrations (100–500 μM) promote Trx degradation in a cathepsin D–dependent process (199, 200). The antiapoptotic action of Trx in response to low H_2O_2 requires its nuclear transport, where it facilitates transcription factor binding to AREs and enhances expression of the phase 2 detoxifying enzyme, glutathione-*S*-transferase (435).

4. Glutaredoxin. As noted earlier, *S*-glutathionylation is an important redox-sensitive posttranslational modification that not only protects protein cysteines from irreversible oxidation but also alters protein function to control cell signaling. Grxs, members of the thioredoxin protein family, are glutathione-dependent oxidoreductase enzymes primarily responsible for reduction of *S*-glutathionylated proteins (226). Two different glutaredoxins exist: Grx-1 in the cytosol and Grx-2 in the mitochondria. Grx-2, but not Grx-1, is an iron–sulfur protein (226). The 2Fe-2S cluster bridges two molecules of Grx-2 to form an inactive dimer. Cluster destruction results in enzyme activation. As the cluster is destroyed by ROS or RNS, the iron–sulfur cluster is considered a redox sensor that ensures Grx-2 activation during oxidative stress (203). In contrast, Grx-1 is inactivated by *S*-nitrosylation of critical cysteines (203). Recent work is beginning to address the roles of Grx in endothelial cells. For example, flow-induced activation of eNOS depends on the thiol-transferase activity of Grx-1 (517). Upregulation of Grx-1 activity in response to TNF-α participated in the cytotoxic action of the cytokine toward endothelial cells (387). These studies demonstrate that Grx activity in endothelial cells can have beneficial or deleterious actions.

This highlights how ROS and RNS can control cell signaling. A key point is that although reversible redox modification of protein thiols represents a mechanism of physiologic signaling, malfunction in signaling pathways involving *S*-oxidation, *S*-nitrosylation, or *S*-glutathionylation are likely active participants in cellular dysfunction. Disease may relate to aberrations in the posttranslational redox modification of selected proteins, leading to altered cellular function. This aberration may reflect overproduction or impaired processing and removal of a particular ROS or RNS. Therefore, a better understanding of the enzymatic and nonenzymatic mechanisms that control ROS/RNS formation, processing, and degradation and defects that can occur in these processes may be important for the development of treatments of vascular disease states associated with aberrant redox signaling.

III. Oxidative Stress and Endothelial Dysfunction

A. Vascular homeostasis and endothelium-derived nitric oxide

The endothelium is critical for the maintenance of cardiovascular homeostasis. Fundamental for this is EDNO, synthesized by eNOS, which controls vascular tone (303), arterial pressure (412), and inhibits platelet aggregation (22) and SMC growth (166). With respect to vascular tone, the extent to which EDNO mediates local vasodilatation differs among blood vessels of differing size [*i.e.*, although under physiologic conditions, EDNO is a primary vasorelaxant in mid- to large-sized conduit and resistance arteries, its contribution to vasodilatation in smaller vascular beds (*e.g.*, coronary arterioles) is comparatively less (299)]. The importance of EDNO in vascular homeostasis is highlighted by observations with eNOS gene knockout mice that exhibit spontaneous hypertension, defective vascular remodeling plus enhanced vascular thrombosis, and leukocyte interactions (155, 234, 302, 421). Moreover, deficiency of eNOS results in accelerated arterial lesion formation in atherosclerosis-prone mice (270, 280). However, EDNO is not always beneficial. A recent study showed that eNOS gene deficiency protects mice against anaphylactic shock, highlighting that eNOS-derived NO is a principal vasodilator in the acute hypotensive response (74).

Critical to the paracrine signaling action or bioactivity of EDNO in the vasculature is its binding to heme of α/β heterodimer of soluble guanylate cyclase (sGC) in target cells (*e.g.*, platelets, smooth muscle cells) (364) (Fig. 3). The heme moiety of sGC resides in the N-terminal domain, whereas the catalytic functions reside in the C-terminal domain. Binding of NO to the ferrous (Fe^{II}) heme of sGC results in disruption of the axial histidine ligand, leading to formation of penta-coordinate iron that is shifted out of the plane of the porphyrin ring (142). This conformational change results in allosteric activation of sGC and resultant conversion of GTP to cyclic-3′,5′-guanosine monophosphate (cGMP). The primary target of cGMP in SMCs is the cGMP-dependent protein kinase (PKG), which in turn targets myosin light-chain phosphatase. The cGMP-dependent dephosphorylation of smooth muscle myosin signals for vasodilatation. In platelets, NO-mediated increases in intracellular cGMP signals for a reduction in intraplatelet calcium, resulting in the inhibition of platelet activation and aggregation (364). As outlined earlier, NO also mediates its cell-signaling actions through *S*-nitrosylation of critical cysteines in target proteins (217). Studies in endothelial cells indicate that cellular *S*-nitrosylation is concentrated at the primary site of active eNOS (188, 244). This supports that, in addition to mediating changes in cGMP, EDNO bioactivity also relates to *S*-nitrosylation reactions on selected proteins. For example, stimulus-coupled induction of EDNO synthesis results in *S*-nitrosylation and activation of plasma membrane transient-receptor-potential ion channels (TRP) and potentiated Ca^{2+} entry that may be important for sustained eNOS activity (560). It is, however, important to note that aberrant *S*-nitrosylation has pathogenic potential. For example, mice with targeted deficiency of *S*-nitrosoglutathione reductase, an enzyme responsible for removal of *S*-nitrosothiols, are hypotensive and exhibit significant increases in cellular *S*-nitrosylation, tissue damage, and mortality when subjected to a model of endotoxic shock (321).

B. Regulation of endothelium-derived nitric oxide production

In the endothelium, EDNO is produced constitutively by eNOS, a 135-kDa protein that structurally consists of a C-terminal reductase domain (which binds NADPH, flavin adenine dinucleotide, and flavin mononucleotide) linked by a

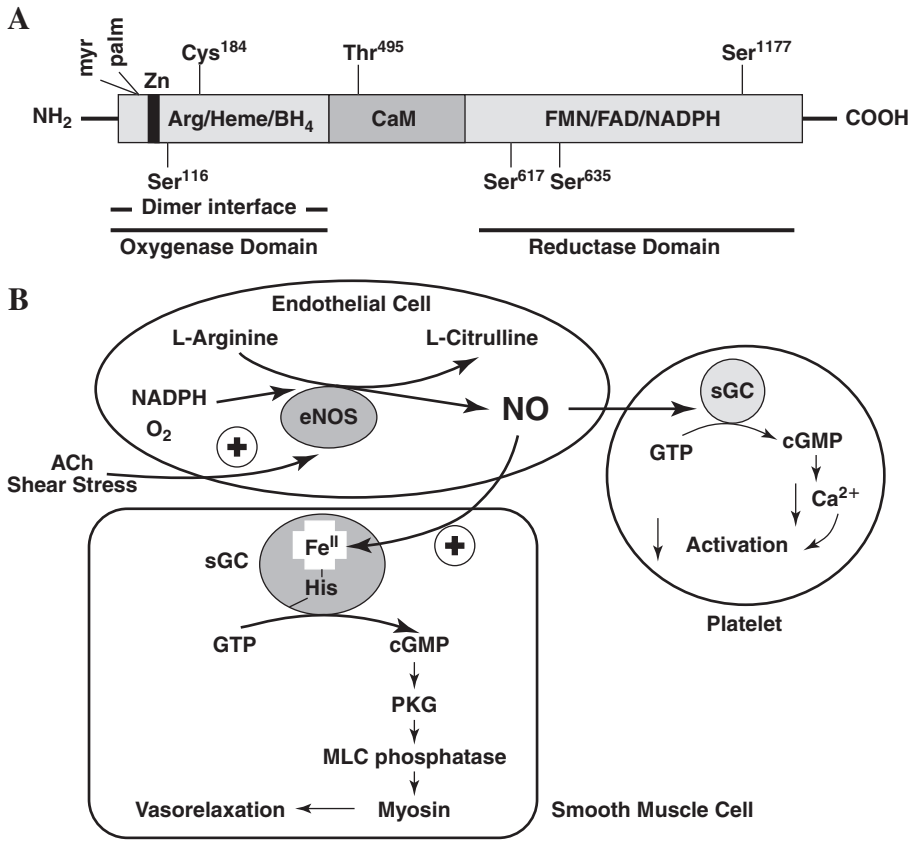

FIG. 3. Synthesis and bioactivity of EDNO. (**A**) Linear structure of eNOS. Areas involved in L-arginine (ARG), heme, tetrahydrobiopterin (BH_4), calmodulin (CaM), flavins (FMN, FAD), and NADPH binding sites are indicated. Also noted are sites of phosphorylation, zinc incorporation, palmitoylation (palm), myristolation (myr), and cysteine heme coordination (Cys^{184}). (**B**) In response to vasoactive stimuli, such as shear stress and acetylcholine (ACh), endothelial cells produce EDNO by activating eNOS. Central to the bioactivity of EDNO is its binding to the ferrous-heme group of soluble guanylate cyclase (sGC) in target cells. Activation of sGC by EDNO increases in the intracellular concentration of cGMP, which signals for smooth muscle relaxation or inhibition of platelet activation and aggregation.

regulatory calmodulin-binding site to an N-terminal oxygenase domain (Fig. 3). The oxygenase domain contains the heme prosthetic group and binds (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), molecular oxygen, and the substrate L-arginine. The catalytic action of eNOS involves the flavin-mediated transport of electrons from NADPH bound at the C-terminal reductase domain to the N-terminal heme, where molecular O_2 is reduced and incorporated into the guanidino group of L-arginine, resulting in the production of hydroxy-L-arginine. In a second catalytic step, the guanidino nitrogen of hydroxy-L-arginine is further oxidized to form L-citrulline, and NO is liberated.

NOS enzymes are unique in that they are the only heme proteins known to contain BH_4 as an essential cofactor. The pterin ensures the coupled transport of electrons through eNOS by exhibiting allosteric and redox activities (460). Thus, BH_4 stabilizes formation of the eNOS dimer, increases NOS affinity for L-arginine, and undergoes distinct cooperative redox transitions with the heme group that activate molecular O_2 for the two-step oxidation of L-arginine. Specifically, BH_4 donates a second electron to the ferrous-dioxygen complex in the oxygenase domain necessary for the initial hydroxylation of L-arginine. Electron donation by BH_4 results in formation of $BH_3{}^{\bullet}H^+$ (trihydropterin cation radical), which is subsequently reduced to BH_4 by electrons donated by eNOS flavins (460).

Active eNOS requires formation of a homodimer through a linkage between the N-terminal oxygenase domains. Dimer formation appears to be influenced by the binding of calmodulin, heme, L-arginine, and BH_4. Stabilization of the eNOS homodimer and binding of BH_4 also appear to depend on integrity of a zinc-thiolate cluster formed by a Zn^{2+} ion coordinated in a tetrahedral conformation with pairs of CysXXXXCys motifs at the N-terminal oxygenase domain and dimer interface (84, 216, 411).

Vascular eNOS is subject to strict controls that under physiologic conditions ensure appropriate tonic regulation of NO output. In the endothelium, eNOS is subject to transcriptional control [reviewed in (309)] and various forms of co- and posttranslational regulation that include substrate and cofactor availability, reversible enzyme acylation and subcellular localization, protein–protein interactions (Table 2), phosphorylation (Table 3), and *S*-nitrosylation [reviewed in detail in (127, 128, 187, 361)].

Posttranslational control ensures that eNOS activity is subject to rapid and tonic regulation in response to vasostimuli. Agonist stimulation of endothelial cells results in rapid enzyme activation through increases in the intracellular Ca^{2+} concentration that induces the displacement of the inhibitory protein caveolin-1 (351) with calmodulin and the release of an adjacent 45–amino acid autoinhibitory loop (426) that permits electron flow through the enzyme. Further criteria for the optimal activation of eNOS are the binding of heat shock protein 90 (Hsp90) (163), coordinated changes in the phosphorylation status of critical Ser and Thr residues (361), and the *S*-nitrosylation status of cysteines within the zinc-thiolate cluster, a modification that inhibits enzyme activity (127, 139). For example, transient activation of eNOS by VEGF requires the rapid and reversible denitrosylation that is inversely related to Akt-dependent eNOS phosphorylation at Ser-1177 (human) or Ser-1179 (bovine) (139). Also essential for optimal activity is the subcellular targeting of eNOS to

Table 2. Kinases and Phosphatases That Modulate the Phosphorylation Status of eNOS

Enzyme	*Phosphorylation site(s)*	*eNOS activity*
Kinases		
Akt	Ser-1177, Ser-617	Stimulatory
5′-AMP-activated kinase	Ser-1177	Stimulatory
	Thr-495	Inhibitory
Protein kinase C	Thr-495, Ser-116	Inhibitory
Protein kinase A	Ser-1177, Ser-617, Ser-635	Stimulatory
Calmodulin kinase II	Ser-1177	Stimulatory
MAP kinase	N.D.	Inhibitory
Phosphatases		
Protein phosphatase I	Thr-495	Stimulatory
Protein phosphatase 2A	Ser-1177	Inhibitory
Calcineurin	Thr-495, Ser-116	Stimulatory

For references, see recent review articles (127, 128, 187, 361) and citations therein.
N.D., Not determined.

caveolae and Golgi membranes, governed by dual acylation at the N-terminal domain of the enzyme [*i.e.*, myristoylation at Gly-2 and palmytoylation at Cys-15 and Cys-26 (320, 417)]. Accordingly, mistargeted, acylation-deficient eNOS mutants are resistant to Ser-1177 phosphorylation or *S*-nitrosylation and exhibit reduced enzyme activity (140, 160). Recent data show that Src-dependant caveolae-mediated endocytosis of eNOS is important for caveolin-1 dissociation and Akt-dependant phosphorylation of the enzyme (331).

Studies in intact blood vessels have shown that Akt-dependent phosphorylation of eNOS at Ser-1177 is essential for acetylcholine-induced endothelium-dependent relaxation (327), indicating that phosphorylation at this amino acid site is a primary regulator of EDNO release in intact blood vessels. Consistent with this, endothelium-specific overexpression of Akt attenuates neointima formation after carotid artery ligation in a manner that is sensitive to inhibition of eNOS (363). Also, transgenic mice expressing only a phosphomimetic (Bovine S1179D) form of eNOS exhibit enhanced vascular reactivity, develop fewer severe strokes, and have improved cerebral blood flow in a middle cerebral artery occlusion model compared with mice expressing a form of eNOS that is resistant to phosphorylation at Ser-1179 (20). In addition to Akt, a recent *in vivo* study reported that PKCα is important for the maintenance of eNOS phosphorylation at Ser-1177, NO production, and control of blood flow (394). Mechanistically, eNOS phosphorylation at Ser-1177 removes the influence of an autoinhibitory control sequence to facilitate calmodulin binding and promote electron flow through the reductase domain (294, 344). In addition to Ser-1177, human eNOS is subject to phosphorylation at Ser-617 and Ser-633, thought to activate the enzyme, whereas phosphorylation of Ser-116 and Thr-495 is considered inhibitory (361). Various protein kinases and phosphatase enzymes have been reported to modulate the coordinated phosphorylation of eNOS at these amino acid residues in endothelial cells (Table 3). The potential for these other phosphorylation sites to modulate vascular tone remains to be established, although mimicking phosphorylation of Ser-116 increases eNOS association with caveolin-1 and reduces the vascular reactivity of intact aortic rings (307).

Hsp90 represents an important activator of eNOS through allosteric modulation of eNOS activity (163), coupling of NADPH-derived electrons to L-arginine oxidation (402), enhancing the calmodulin-induced displacement of the caveolin inhibitory clamp from eNOS (189), and acting as a docking site to facilitate Akt-dependent phosphorylation of eNOS at Ser-1177 (58). Hsp90 itself is subject to posttranslational modification that influence its ability to control eNOS. Binding of Hsp90 to eNOS in response to VEGF requires Src-mediated phosphorylation of the chaperone protein at Tyr-300 (134). Hsp90 is also subject to *S*-nitrosylation of cysteines in

Table 3. 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 to eNOS *via* the receptors′ intracellular domain to inhibit enzyme activity
NOSIP and NOSTRIN	Bind eNOS to control the redistribution of the enzyme from the plasma membrane to intracellular compartments, resulting in the inhibition of enzyme activity
Calmodulin	Binds eNOS in the calmodulin-binding domain to activate enzyme activity
HSP90	Binds to the amino terminus of eNOS and activates enzyme activity
Dynamin-2	Activates eNOS
SIRT-1	Binds and deacetylates eNOS at lysines 496 and 506 in the calmodulin-binding domain to activate the enzyme (341).
Porin	Activates eNOS

For references, see recent review articles (127, 128, 187) and the references therein.

its C-terminal domain responsible for interaction with eNOS. *S*-nitrosylation inhibits the ATPase activity of Hsp90 and its positive effects on eNOS activity (333). Therefore, *S*-nitrosylation of eNOS (139) and Hsp90 (333) provides a feedback mechanism to limit EDNO production. In addition to Hsp90, various other proteins bind eNOS to influence the enzymes activity (Table 3).

C. Oxidative stress, superoxide anion radical, and endothelial dysfunction

Endothelial dysfunction is an imprecise term that refers to a loss of normal homeostatic functions of the endothelium (*e.g.*, vasodilatation, inhibition of platelet aggregation and leukocyte adhesion) that is often apparent early in the course of vascular diseases such as atherosclerosis, diabetes, and hypertension. Coronary arteries of humans with cardiovascular risk factors, but lacking gross atherosclerotic disease, vasoconstrict in response to acetylcholine (326, 512). This is in marked contrast to endothelium-dependent vasodilatory responses to acetylcholine exhibited by coronary arteries from humans free of risk factors (326, 512). One important manifestation of endothelial dysfunction is a decrease in EDNO bioactivity. Numerous prospective studies recognize that the reduction of EDNO bioactivity is an independent predictor of increased risk of cardiovascular events in CAD patients (180, 212, 431, 466). In light of the clinical implications of compromised EDNO bioactivity, it is important to define the mechanisms involved. Considerable evidence indicates that endothelial dysfunction is caused by enhanced vascular oxidative stress (64, 480). As such, currently significant interest exists in identifying the oxidative reactions responsible. We subsequently consider the various ways in which different oxidative reactions can affect EDNO bioactivity and hence endothelial function.

Several initial findings suggested that impaired EDNO bioactivity is not the result of attenuated production of NO but is due, in part, to the inactivation of EDNO before reaching its molecular target. Thus, diseased blood vessels from hypercholesterolemic rabbits produce substantial amounts of nitrogen oxides (NO oxidation products) despite the impairment in NO-dependent vascular relaxation (354). Also, eNOS protein levels are paradoxically increased rather than reduced in diseased blood vessels (221, 300). Subsequent studies have established that oxidative inactivation of EDNO frequently involves $O_2^{\bullet -}$. For example, hypertension, hypercholesterolemia, diabetes, and atherosclerosis are all associated with an increase in the steady-state flux of $O_2^{\bullet -}$ in the vascular wall (64), and $O_2^{\bullet -}$ reacts with NO at near diffusion-controlled rates ($k = 1.9 \times 10^{10}$ M/sec) to produce the potent oxidant peroxynitrite ($ONOO^-$; reaction 2) (267) (Fig. 4).

$$O_2^{\bullet -} + {}^{\bullet}NO \rightarrow ONOO^- \qquad [2]$$

This rate constant for peroxynitrite formation exceeds both NO autoxidation ($k = 2 \times 10^6$ M/sec) and spontaneous $O_2^{\bullet -}$ dismutation ($k = 5 \times 10^5$ M/sec). Moreover, the reaction of NO with $O_2^{\bullet -}$ is more rapid than either its reaction with enzyme-bound heme ($k = 10^2$–10^6 M/sec) or the reaction of $O_2^{\bullet -}$ with SOD ($k = 2 \times 10^9$ M/sec) (405). Thus, peroxynitrite formation is kinetically favored over other NO reactions and likely occurs whenever both NO and $O_2^{\bullet -}$ are present.

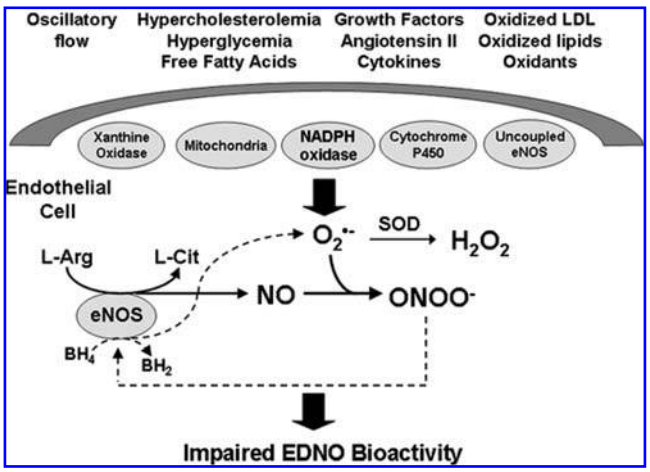

FIG. 4. Endothelial sources of superoxide anion radical important for the impairment of EDNO bioactivity during vascular disease. Numerous pathogenic stimuli promote the formation of superoxide anion radical ($O_2^{\bullet -}$) from various enzymatic sources in the vascular endothelium. The increased $O_2^{\bullet -}$ reacts rapidly with NO synthesized by eNOS, resulting in the formation of the oxidant peroxynitrite ($ONOO^-$), a reaction that out-competes the dismutation of $O_2^{\bullet -}$ catalyzed by SOD. The peroxynitrite formed can oxidize tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2), resulting in uncoupling of the eNOS and formation of $O_2^{\bullet -}$ (*dashed line*). These oxidative reactions impair EDNO bioactivity leading to endothelial dysfunction apparent in patients with vascular diseases.

Because peroxynitrite inefficiently activates the soluble isoform of guanylate cyclase (473), its formation effectively decreases EDNO bioactivity in the vascular wall.

Numerous studies indicate that direct inactivation of NO by $O_2^{\bullet -}$ represents a key event responsible for impaired EDNO bioactivity. For example, the addition of $O_2^{\bullet -}$ to vascular bioassay systems impairs NO-dependent vessel relaxation (192). Exogenous SOD improves the vascular relaxation response to EDNO under both basal and acetylcholine-stimulated conditions (192). Increasing vascular SOD activity affords a significant improvement in EDNO-mediated arterial relaxation in atherosclerotic animals (362). Also, blood vessels from mice deficient in Cu,Zn-SOD activity exhibit enhanced vascular $O_2^{\bullet -}$ production and impaired NO-mediated arterial relaxation (116, 328). Similarly, resistance vessels from hypertensive mice lacking EC-SOD (182, 251) or carotid arteries from $ApoE^{-/-}$ mice heterozygous for Mn-SOD exhibit enhanced $O_2^{\bullet -}$ and impaired EDNO bioactivity (382). Endothelium-bound EC-SOD is markedly decreased in CAD patients compared with healthy subjects, and the extent of reduction closely relates to the degree of impairment of endothelium-dependent vasodilatation exhibited by these patients (292). Finally, acute intraarterial infusion of ascorbate at concentrations that effectively prevent $O_2^{\bullet -}$ interaction with NO (245) improves endothelium-dependent relaxation in CAD patients (212). Patients that demonstrate the greatest improvement in EDNO 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 impor-

tant (212). Collectively, the evidence supports that increased vascular $O_2^{\bullet -}$ is intrinsically involved in the impairment of EDNO bioactivity. We next consider the major sources of $O_2^{\bullet -}$ in the vascular endothelium and their role in endothelial dysfunction and vascular disease. To date, several significant endothelial sources have been identified, including NADPH oxidase, mitochondria, xanthine oxidase, cytochrome P_{450}, and uncoupled eNOS, which, in response to various stimuli, produce increased $O_2^{\bullet -}$ capable of impairing EDNO bioactivity (Fig. 4).

IV. Major Sources of Superoxide Anion Radical in the Vascular Endothelium

A. NADPH oxidase

The NADPH oxidases are a family of enzyme complexes that catalyze the transfer of electrons from NADPH to molecular oxygen *via* their catalytic Nox or Duox subunit, to generate $O_2^{\bullet -}$ and, in some instances, H_2O_2 (43, 284, 383). Unlike other so-called oxidases, which produce ROS as a byproduct of their normal catalytic function (*e.g.*, enzymes of the mitochondrial electron transport chain, cytochrome P_{450}) or *via* a dysfunctional variant of the enzymes (eNOS, xanthine oxidases), NADPH oxidases appear to be unique in that their only known function is to generate ROS. This function of NADPH oxidases underlies their microbicidal role in phagocytic cells of the innate immune system, where they are acutely activated to generate a burst of ROS that recent data support play a signaling role leading to the removal of invading pathogens (413). NADPH oxidases are therefore strong candidates for the enzyme(s) responsible for deliberate ROS production required for redox-signaling purposes, especially those isoforms that generate moderate, noncytotoxic amounts of ROS in a controlled manner.

To date, a total of seven NADPH oxidase isoforms have been identified in different cells and tissues from mammalian species, characterized primarily by the catalytic subunit that they use. These include the Nox1-, Nox2- (formerly $gp91^{phox}$-), Nox3-, Nox4-, and Nox5-containing oxidases, as well Duox1- and Duox2-containing enzymes. Regarding endothelial cells, four of these isoforms—Nox1, Nox2, Nox4, and Nox5—have been identified under different states of physiology and pathophysiology, and hence we focus on these isoforms in this review. Of note, other cells of the vascular wall (i.e., vascular smooth muscle cells and adventitial fibroblasts) also express NADPH oxidase isoforms, as do macrophages, neutrophils, and platelets, all of which may reside in or on the vascular wall, especially in disease states. Hence, the influence of these cells must be taken into account when considering the roles of NADPH oxidases in vascular disease.

The various Nox-containing NADPH oxidase isoforms expressed in endothelial cells rely to varying extents on different sets of regulatory subunits for full activity. For example, Nox1-, Nox2-, and Nox4-dependent NADPH oxidase activity appears to require expression of a small, integral membrane protein, $p22^{phox}$, which likely acts to stabilize the catalytic subunit in different subcellular membranes. Although association with $p22^{phox}$ appears to be sufficient for full Nox4-dependent NADPH oxidase activity, Nox1- and Nox2-oxidase activity require the additional association of the small, isoprenylated, GTPase, Rac1, as well as at least two cytosolic regulatory subunits including an "organizer" protein, which forms a cytosolic complex with and chaperones an "activator" protein, to the membrane-bound Nox-$p22^{phox}$ complex (383). For Nox2, $p47^{phox}$ and $p67^{phox}$ appear to serve the function of organizer and activator, respectively. A third cytosolic subunit, termed $p40^{phox}$, may also associate with the Nox2-oxidase complex; however, its precise role in regulating NADPH oxidase function is currently unclear (343). Recently, homologues of $p47^{phox}$ and $p67^{phox}$, termed Noxo1 and Noxa1, respectively, were identified and demonstrated to support Nox1-dependent NADPH oxidase activity in colon epithelial cells [*i.e.*, the prototypical Nox1-expressing cell type (34, 471)]. Although one report demonstrated that Noxo1 and Noxa1 are expressed in endothelial cells from cerebral arteries (8), no studies have directly examined their role in regulating endothelial Nox1-dependent NADPH oxidase activity. Hence, it remains to be determined whether $p47^{phox}$ and $p67^{phox}$, Noxo1 and Noxa1, or combinations thereof [*i.e.*, as is the case in vascular smooth muscle cells (14)] act as the organizer and activator proteins for the Nox1-oxidase in endothelial cells. The only Nox that appears to have the ability to function as a ROS-producing enzyme in the absence of other "phox" subunits or Rac is Nox5. Furthermore, unlike Nox1, Nox2, and Nox4, Nox5 contains an amino-terminal calmodulin-like domain with four binding sites for Ca^{2+} (EF hands) (35), making it the only Nox expressed in endothelial cells whose activity is modulated by Ca^{2+}. Figure 5 provides a schematic diagram depicting the putative structures of the four Nox-containing oxidases expressed in endothelial cells.

1. Expression and subcellular localization of NADPH oxidase subunits in endothelial cells

a. Nox2. From the seminal observation by Griendling *et al*. (190) that nonphagocytic cells–specifically vascular smooth muscle cells–express certain subunits of the phagocytic NADPH oxidase, Jones *et al*. (249) demonstrated that human umbilical vein endothelial cells in culture express virtually all of the components of the traditional leukocytic NADPH oxidase [*i.e.*, Nox2, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$–at least at an mRNA level (249)]. These authors also provided immunohistochemical data indicating that HUVECs express $p47^{phox}$ and $p67^{phox}$ at the protein level but questioned whether they also express Nox2 and $p22^{phox}$ protein, on the basis that heme-spectroscopy failed to detect the presence of the low-potential cytochrome *b558* in the plasma membrane fractions (249). As discussed later, the reason for this finding probably relates to the fact that, in contrast to phagocytic cells, the majority of the Nox2-containing NADPH oxidase complex in endothelial cells is localized to the nuclear and endoplasmic reticulum membranes, which were disregarded in the assays by Jones *et al*. (249). Hence, as reliable antibodies became available, protein expression of $p22^{phox}$ and Nox2 in endothelial cells, both in culture and *in situ*, was confirmed by numerous investigators (40, 41, 185, 311). Western blot analyses in many of these studies revealed that $p22^{phox}$ invariably ran as a single band at ~22 kDa, which is identical to its predicted molecular mass based on its amino acid sequence. By contrast, Nox2 often ran as two distinct bands, one at ~65 kDa, corresponding to its predicted mo-

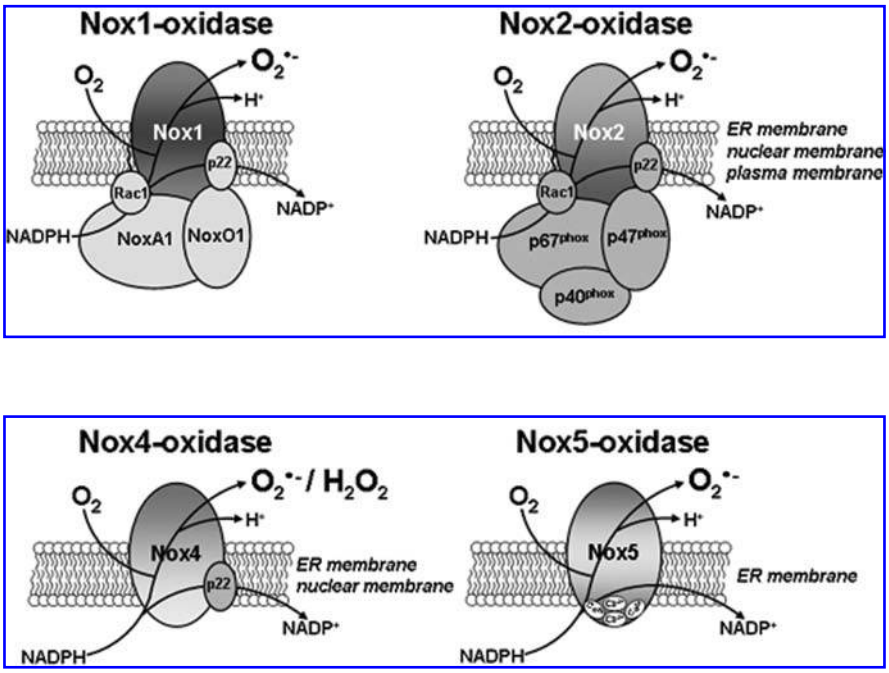

FIG. 5. Subunit composition of the four NADPH oxidase isoforms expressed in endothelial cells. Each NADPH oxidase isoform is comprised of a membrane-bound catalytic 'Nox' domain that contains all elements required for the transfer of electrons from NADPH to molecular oxygen, including an NADPH binding domain, FAD, and two heme groups. Additionally, Nox1, 2, and 4 require the association of up to four additional subunits for activity, including $p22^{phox}$, which serves to stabilize the Nox subunit in the membrane, a cytosolic organizer protein ($p47^{phox}$ or NoxO1), a cytosolic activator protein ($p67^{phox}$ or NoxA1), and a small G-protein, Rac1. In endothelial cells, Nox2, 4, and 5 appear to be predominantly expressed in the membranes of the ER and nucleus. A small proportion of Nox2 may also be present in the plasma membrane. To date, no studies have examined the subcellular distribution of Nox1 in endothelial cells.

lecular size, and another at 91 kDa. This high-molecular-weight variant has the same electrophoretic mobility as the fully *N*-glycosylated protein in neutrophils, suggesting that this posttranslational modification of Nox2 also occurs in endothelial cells. However, the role *N*-glycosylation plays in Nox2 expression and activity in endothelial cells, and indeed other cell types, remains to be explored.

Unlike the phagocytic Nox2-containing oxidase, which is unassembled and virtually inactive under basal conditions, and generates an acute burst of ROS only in response to pathogenic stimuli, a large portion of the endothelial Nox2-containing oxidase pool appears to be constitutively assembled to generate ROS in a tonic fashion (312). Furthermore, whereas the cytochrome *b558* complex is localized in the plasma membrane of phagocytic cells, in endothelial cells, much of the Nox2 protein (*i.e.*, ~90%) appears to colocalize with $p22^{phox}$, $p67^{phox}$, and $p47^{phox}$ in a perinuclear location–probably on the membranes of the nucleus and endoplasmic reticulum–with only a small proportion of the protein being expressed at the plasma membrane (40, 312).

The differential distribution pattern of Nox2-oxidases in phagocytic *versus* endothelial cells, together with their markedly different biochemistries, highlights the fact that the enzymes serve entirely different functions in the two cell types. In phagocytic cells, the Nox2-oxidase is responsible for producing bursts of ROS in the phagosome and extracellular space to participate in the destruction of pathogens. By contrast, Nox2 in endothelial cells does not display appreciable microbicidal activity, and hence, it has been suggested that Nox2-oxidases in these cells are more likely to act as intermediates in intracellular redox-dependent signaling pathways. One such signaling role for Nox2-dependent NADPH oxidase activity is that involved in endothelial cell proliferation and migration, essential processes in wound repair and angiogenesis. Substantial evidence indicates that Nox2-dependent NADPH oxidase activity in endothelial cells is critical for angiogenesis. For example, *in vivo* angiogenic responses induced by VEGF (497) or hindlimb ischemia (486) were markedly reduced in $Nox2^{-/-}$ *versus* wild-type mice. Likewise, overexpression of dominant-negative Rac1 or transfection with Nox2 antisense inhibited the proliferation and migration of cultured endothelial cells after stimulation with VEGF (497). Recently, further evidence for the participation of Nox2-dependent NADPH oxidase activity in angiogenesis surfaced—somewhat surprisingly—from a line of investigation into the antiangiogenic effects of Dicer suppression in endothelial cells. Dicer is the key enzyme involved in micro-RNA synthesis, and it is well established that downregulation of this enzyme suppresses angiogenesis (277, 555). Shilo *et al*. (439) further demonstrated that siRNA knockdown of Dicer in human endothelial cells is associated with an increase in expression of the suppressor transcription factor, HBP1, and a concominant reduction in $p47^{phox}$ expression and VEGF-induced ROS production. Importantly, supplementation with exogenous H_2O_2 restored angiogenic responses in Dicer-depleted cells (439).

We propose that a perinuclear location of Nox2 under physiologic conditions may be additionally important in endothelial cells for compartmentalizing of redox signaling to ensure that $O_2^{\bullet -}$ is generated away from NO, which is predominantly generated near the plasma and Golgi membranes by eNOS. Such compartmentalization would limit the potential for the near-diffusion limited reaction between $O_2^{\bullet -}$ and NO, which not only reduces the bioavailability of each species, but also gives rise to the powerful oxidizing species, peroxynitrite.

b. Nox4. Nox4 was identified independently by two separate laboratories as a protein with similar homology (~40%) and overall structure (*i.e.*, conserved transmembrane domains and motifs for NADPH, FAD, and heme binding) to Nox2 that was critical to the NADPH oxidase activity of kidney cells from mice and humans (169, 441). Soon after, Nox4 mRNA was shown to be expressed in endothelial cells at

markedly higher levels than Nox2 (20- to 5,000-fold) and Nox1 (>300-fold) and, based on siRNA and dominant-negative gene expression studies, to be a primary contributor to intracellular ROS production in these cells (9, 106, 446, 498).

Western blot studies suggest that, in endothelial cells, Nox4 is likely to be expressed as a 65-kDa protein. This apparent molecular mass is predicted based on the amino acid composition of Nox4 and assuming that the protein does not undergo significant posttranslational modifications. Indeed, sequence alignment of Nox4 with Nox2 reveals that it lacks at least two (Asn148 and Asn239) of the three asparagine residues required for *N*-glycosylation and the slower electrophoretic mobility of the latter homologue (86, 515). Nevertheless, similar to Nox2, Nox4 appears to be expressed predominantly in a perinuclear location in endothelial cells (282, 498). Co-immunostaining studies with specific endoplasmic reticulum markers (397, 498), plus cell fractionation and confocal microscopy studies focusing on the nucleus (282), reveal that Nox4 localizes primarily to the membranes of these organelles.

Studies indicate a role for Nox4 in endothelial proliferation and migration. First, Nox4 protein expression is markedly upregulated in proliferating *versus* quiescent endothelial cells. For example, TGF-β–induced increases in ROS production and cytoskeletal arrangement (filopodia formation and F-actin organization) in HUVECs was blocked by overexpression of dominant-negative Nox4 (232). Likewise, both basal and VEGF-stimulated endothelial cell migration and proliferation are inhibited by both Nox4 siRNA and a dominant-negative construct (106). By contrast, overexpression of wild-type Nox4 increased these measures (106). Accordingly, tube formation in Matrigel and wound healing in an *in vitro* scrape model were inhibited and potentiated, respectively, by overexpression of dominant-negative and wild-type Nox4 (106).

c. Nox1. Relatively few studies have investigated a role for Nox1 in endothelial cell biology and pathobiology. Compared with Nox2 and Nox4, which appear to be ubiquitously expressed in all endothelial cell types and preparations (*i.e.*, cultured or *in situ*), Nox1 often goes undetected or is expressed at very low levels. For example, human cultured coronary artery endothelial cells expressed mRNA for Nox1, although at much lower levels than Nox4 (0.3%) or Nox2 (7%) (446), as did rat aortic endothelial cells (9), whereas cultured HUVECs do not appear to express this homologue (498). Nox1 protein expression was detected in endothelial cells of the rat basilar artery (8) and the placental vasculature from normotensive pregnant women (103). Interestingly, Nox1 expression appeared to be elevated in placental endothelial cells from women with severe preeclampsia (103) or in endothelial cells in response to oscillatory shear stress (447). To date, no studies have characterized the subcellular distribution of Nox1 within endothelial cells.

d. Nox5. A most recent report identified the expression of Nox5 in primary cultures of HUVECs and in an immortalized human microdermal cell line (HMEC) (44). RT-PCR revealed that these cells actually express two mRNA splice variants of Nox5, termed Nox5β and Nox5δ, which differ from one another in the sequences of their Ca^{2+}-binding domains. Furthermore, Western blotting revealed specific bands of 75 kDa and 60 kDa, the former size corresponding to the predicted molecular mass of the two mRNA splice variants described earlier, and the latter to a truncated protein variant of Nox5 (Nox5S), which lacks a Ca^{2+}-binding domain. When overexpressed in HMEC-1, both protein variants caused a similar increase in basal ROS levels; however, only the activity of full-length Nox5 could be further enhanced by a Ca^{2+} ionophore. Both proteins were associated with $p22^{phox}$, although this association was not necessary for activity, and both proteins were colocalized with calreticulin in an intracellular compartment surrounding the nucleus, suggesting that, like Nox2 and Nox4, they reside in the endoplasmic reticulum.

2. NADPH oxidase regulation by proatherogenic stimuli and roles in endothelial dysfunction and vascular disease. *In vivo* animal models generally support the concept that vascular proinflammatory/proatherogenic states, such as hypertension, diabetes, and hypercholesterolemia, are associated with elevated expression and activity of Nox2 (and possibly Nox1)-containing oxidases within the vessel wall, and with either no change or even downregulation of Nox4 expression (45, 357, 389, 532). Furthermore, in human arteries from coronary artery disease patients, Nox2 appears to be upregulated in endothelial cells and macrophages, whereas expression of Nox4 is either unchanged or diminished across the vessel wall (194, 446). Studies in "Nox" and "phox" -knockout mice suggest that upregulation of Nox1 and Nox2 is not merely a symptom but is an active participant of vascular disease. For example, infusion of angiotensin II into wild-type mice caused increases in systolic blood pressure and aortic medial thickening, which were associated with marked elevations in aortic $O_2^{\bullet -}$ generation and 3-nitrotyrosine staining in both the endothelium and adventitia (516). Although angiotensin II caused a similar increase in blood pressure in $Nox2^{-/-}$ mice, it failed to increase $O_2^{\bullet -}$ and 3-nitrotyrosine levels in these animals or to induce medial thickening (516). Likewise, the impairment in endothelium-dependent vasorelaxation responses that is normally associated with renovascular hypertension induced by renal artery clipping in wild-type mice was not evident in $Nox2^{-/-}$ mice, despite the surgery having a similar effect on blood pressure in both strains (252). Hence, although Nox2 may not play a significant role in modulating blood pressure—at least in response to elevated plasma levels of angiotensin II—it does appear to be an important mediator of the associated vascular pathology.

Three separate studies, examining the effect of global deletion of Nox1 on vascular function and hemodynamic responses after angiotensin II infusion in mice, similarly suggest an important pathophysiologic role for this isoform in the setting of hypertension. Deletion of Nox1 significantly blunted the pressor response to angiotensin II in mice (167, 340). Nox1 deletion also prevented the angiotensin II–induced increase in ROS production in the aorta, the impairment in endothelium-dependent vasorelaxation responses to acetylcholine, and the incidence of aortic dissection (167, 168, 340). However, Nox1 deletion had little or no effect on smooth muscle cell proliferation and medial hypertrophy after angiotensin II infusion (167, 340). This is somewhat surprising, given that targeted overexpression of human Nox1 in smooth muscle cells of mice on its own led to a thickened

medial layer and also potentiated the vascular hypertrophic responses to angiotensin infusion (119). Hence, it is possible that the effects of Nox1 deletion in the knockout studies described earlier were due to its absence in the endothelial cells rather than in the vascular smooth muscle.

The potential role of NADPH oxidases in hypercholesterolemia-induced atherogenesis has also been examined by using apolipoprotein-deficient ($ApoE^{-/-}$) mice crossbred with mice lacking $p47^{phox}$ ($p47^{phox-/-}$). Although no significant difference was found between the extent of atherosclerosis in the ascending aortae of $ApoE^{-/-}$ *versus* $ApoE^{-/-}/p47^{phox-/-}$ mice, lesion burden in the descending aorta was markedly reduced in the double-knockout animals (37, 230). Interestingly, similar findings have been reported in $ApoE^{-/-}$ mice treated with antioxidant supplements (481). Mice lacking $p47^{phox}$ would be expected to have markedly reduced Nox2- (and possibly Nox1-) dependent NADPH oxidase activity, but normal Nox4- and Nox5-dependent NADPH oxidase activity. Nevertheless, only one study has examined the impact of Nox2 deletion on atherogenesis in $ApoE^{-/-}$ mice. As predicted from the aforementioned studies using $p47^{phox}$-deficient animals, lesion cross-section area in the aortic sinus was similar between $ApoE^{-/-}$ and $ApoE^{-/-}/Nox2^{-/-}$ mice (266). However, these authors focused only on lesion burden at this site, and hence it remains to be determined if Nox2 deletion, like $p47^{phox}$ deletion, affects atherogenesis in the descending aorta, or indeed in any other regions of the vascular tree.

In all of the whole-animal studies described, it is difficult to ascertain the relative contribution of individual cell types (*i.e.*, endothelial *vs.* phagocytic, vascular smooth muscle, and adventitial fibroblasts) to the overall changes in Nox-expression profiles across the blood vessel wall. However, as discussed later, studies investigating the effects of known proatherogenic stimuli on Nox-expression profiles in cell-culture models suggest that many of these changes do indeed occur at the level of the endothelium.

Mechanical forces. Within the vasculature, branch points are most susceptible to the development of atherosclerotic lesions, and it is thought that the turbulent flow profiles, resulting in disturbed shear forces on the endothelium, may be an early trigger for atherogenesis in these regions. A number of studies have examined the effects of *in vitro* models of disturbed (*i.e.*, oscillatory) *versus* laminar or pulsatile flow on Nox-expression profiles in endothelial cells. Short-term exposure to oscillatory flow (*i.e.*, <6 h) appears to result in upregulation of Nox2 and Nox4 alike (236). However, this is not the case for more physiologically relevant longer-term exposure to disturbed shear forces (*i.e.*, >18 h), which is likely to have opposing effects on the expression of Nox1 and Nox2 *versus* Nox4. For example, in mouse aortic endothelial cells (MAECs), oscillatory shear stress increased the expression of both Nox1 and Nox2, while reducing that of Nox4 (447). Shear-induced elevations in ROS production and monocyte binding to the endothelial cell monolayer were markedly attenuated after gene silencing of Nox1 with specific siRNA (447) or in endothelial cells isolated from $p47^{phox-/-}$ mice (239), suggesting a proinflammatory role for Nox1- and Nox2-dependent oxidases, respectively, in this model. In a separate study on bovine aortic endothelial cells (BAECs), oscillatory shear stress similarly resulted in upregulation of Nox2 expression. However, additionally, Nox4 levels were increased in these studies, which potentially confounds the conclusions drawn (236). These changes in Nox-expression profiles were accompanied by increases in ROS production, as measured by dihydroethidium fluorescence, as well as an increase in the ability of the cells to oxidize native LDL, which was included in the incubating medium throughout the experiment (236). This latter finding is important, as it highlights a major difference between this study in BAECs and the previously mentioned studies in MAECs and may provide an explanation for the contrasting findings with regard to Nox4 expression. One way of rationalizing these independent findings is that exposure to oscillatory shear stress initially caused an increase only in Nox2 expression and that the associated increase in ROS production and LDL oxidation was ultimately responsible for upregulating the expression of Nox4. Indeed, as discussed later, treatment of endothelial cells with certain components of modified LDL has been shown to cause upregulation of Nox4 expression. To our knowledge, no studies examined the effects of various flow profiles on expression of Nox5.

Proatherogenic lipids. Elevated levels of circulating lipids are recognized as a major risk factor for atherosclerosis. In cultured human umbilical vein and coronary artery endothelial cells, treatment with oxidized LDL or remnant lipoprotein particles (RLPs) isolated from the plasma of hyperlipidemic patients increased both ROS production and expression of Nox2 (105, 440). Although the expression of other Nox isoforms was not examined specifically in these studies, the oxidized phospholipids oxidized-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC), a biologically active component of minimally modified LDL, was shown to increase expression of Nox4 in BAECs (419). No studies examined the effects of atherogenic lipid particles on endothelial expression of Nox1 or 5.

Proinflammatory cytokines, hormones, and infectious agents. Much of this discussion focused on expressional regulation of NADPH oxidase activity. However, some evidence suggests that certain proatherogenic/proinflammatory stimuli may, in addition to affecting Nox-isoform expression, directly modulate the activity of these enzymes. For example, prolonged treatment of HUVECs with angiotensin II for up to 10 h caused an increase in Nox2 mRNA and protein expression and a corresponding three- to fourfold increase in DPI-inhibitable ROS production over the same time course (423). Shorter-term incubations with angiotensin II (5–60 min) also increase ROS production in endothelial cells, albeit to a far lesser extent (*i.e.*, ~1.5-fold) (313). Such short-term effects of angiotensin II are unlikely to be associated with significant changes in protein expression of any NADPH oxidase subunits. Rather, over this period, angiotensin II appears to act by increasing serine phosphorylation of $p47^{phox}$ and its association with $p22^{phox}$, leading to increased NADPH oxidase activity (313). TNF-α also has been shown to increase endothelial ROS production by inducing the association of PKC-ζ and TNF-α receptor–associated factor, TRAF4, with $p47^{phox}$ (157, 310). These interactions facilitate phosphorylation of the NADPH oxidase organizer protein and its targeting to the Nox2-$p22^{phox}$ heterodimer (310), possibly in lipid-raft domains of the plasma membrane (553). Finally, a growing body of evidence suggests that bacterial infection is a novel risk factor for endothelial dysfunction and atherosclerosis. Recently, Park *et al.* (391) demonstrated that, in human aortic endothelial cells, Nox4 colocalizes *via* the COOH-terminal domain (amino acids 451–530) with TLR4 (391), a known receptor for the LPS

of gram-negative bacteria, which include pathogens associated with coronary artery disease in patients, such as *Chlamydia pneumoniae* and *Helicobacter pylori*. Downregulation of Nox4 with siRNA or overexpression of the Nox4 COOH-terminus (*i.e.*, to act as a decoy) prevented LPS-induced increases in ROS production, inflammatory markers (NF-κB, ICAM-1, MCP-1, and IL-8) and monocyte adhesion (391).

B. Xanthine oxidase

The molybdoenzyme, flavoprotein xanthine oxidase, is derived from the posttranslational oxidation or proteolytic conversion or both of xanthine dehydrogenase. The oxidative pathway involves reversible oxidation of critical cysteine sulfhydryls (372). Xanthine oxidase catalyzes the metabolism of NADH, O_2, and hypoxanthine/xanthine to produce $O_2^{\bullet-}$ and H_2O_2. Studies with human coronary arteries from CAD patients indicate that xanthine oxidase, but not xanthine dehydrogenase, expression and activity are elevated, and this contributes to increased levels of $O_2^{\bullet-}$ produced by diseased human vessels (194, 449). Perhaps more important, several clinical studies have reported that pharmacologic inhibition of xanthine oxidase partially improves endothelial function in patients with type 2 diabetes (62), CAD (29, 71, 171, 293), and chronic heart failure (143). A mechanism potentially responsible for these observations is derived from studies with rabbits fed a high-cholesterol diet that exhibit increased circulating levels of xanthine oxidase that bind to heparan sulfate-glycosaminoglycans expressed on the endothelial cell surface and mediate $O_2^{\bullet-}$ production and endothelial dysfunction (536). Indeed, endothelium-bound xanthine oxidase is significantly increased in CAD patients (449). Studies with cultured endothelial cells support that increased H_2O_2 derived from NADPH oxidase in response to angiotensin II or oscillatory shear stress may also contribute to the elevation of xanthine oxidase in CAD patients (293, 348). Consistent with a role of angiotensin II in promoting xanthine oxidase–mediated endothelial dysfunction, endothelium-bound xanthine oxidase activity was reduced by the AT1-receptor antagonist losartan, and xanthine oxidase inhibition with oxypurinol improved endothelium-dependent vasodilatation before, but not after, losartan therapy (293).

C. Mitochondria

During normal respiration, partial reduction of molecular O_2 by a reduced component of complex I (NADH dehydrogenase) or semiquinone radical produced by complex III (ubiquinone-cytochrome *bc1*) of the electron-transport chain results in production of $O_2^{\bullet-}$ (Fig. 6). Estimates predict that

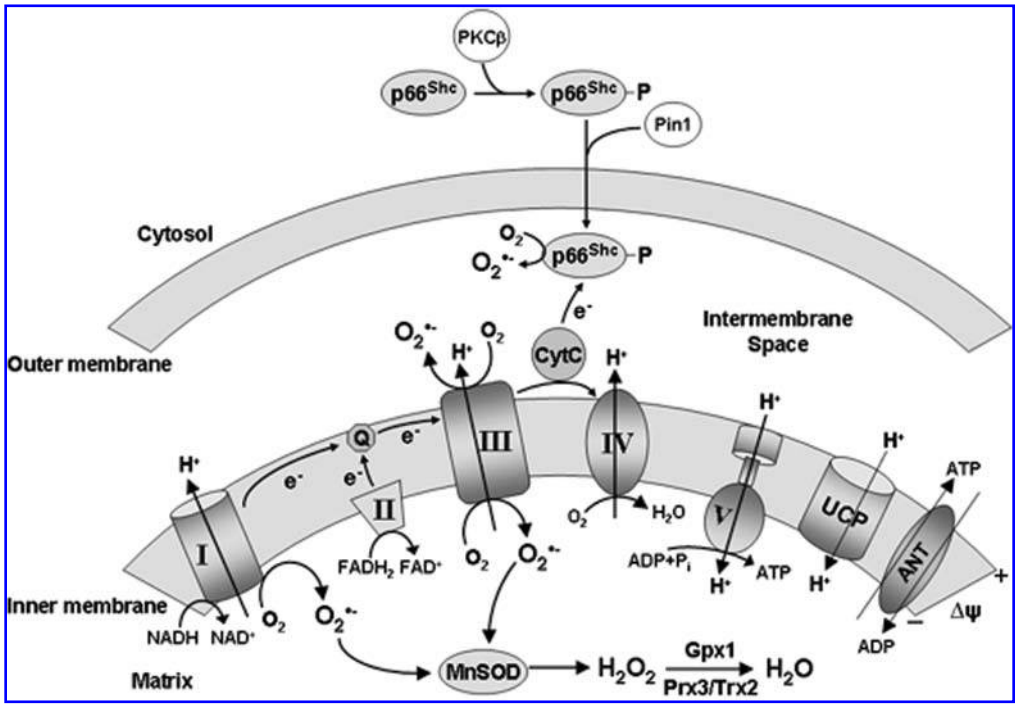

FIG. 6. Mitochondria and ROS production. Respiring mitochondria transfer electrons (e^-) from NADH or $FADH_2$ through complexes I or II, respectively, then complex III via reduced coenzyme Q_{10} (Q). The transfer of electrons from complex III and IV is mediated by cytochrome c, resulting in the reduction of O_2 to H_2O. Concomitant with the transfer of electrons is the transport of protons (H^+) across the inner mitochondrial membrane at complexes I, III, and IV to establish an electrochemical gradient (Ψ). Re-entry of protons into the mitochondrial matrix via complex V provides the protomotive force for ATP generation and ANT-mediated ATP-ADP exchange. Leakage of electrons at complex I and III leads to the reduction of molecular oxygen (O_2) and resultant formation of $O_2^{\bullet-}$. Superoxide formed at complex I is directed to the matrix, while $O_2^{\bullet-}$ formed at complex III can be directed to both the matrix and intermembrane space. Mitochondrial formation of $O_2^{\bullet-}$ can also be achieved by $p66^{Shc}$, which, upon phosphorylation by PKCβ and Pin1-dependent recruitment to the organelle, subtracts electrons from cytochrome c in the intermembrane space. Mitochondrial uncoupling proteins (UCP) reduce $O_2^{\bullet-}$ formation by facilitating the re-entry of protons into the matrix. Superoxide formed in the mitochondrial matrix is dismutated to H_2O_2 by MnSOD. The H_2O_2 produced in the mitochondria is reduced to H_2O by glutathione peroxidase (Gpx1) or peroxiredoxin 3 (Prx3)/thioredoxin 2 (Trx2) antioxidant systems.

1–2% of the total O_2 consumed is converted to ROS (76). Therefore, mitochondrial generation of $O_2^{\bullet -}$ represents a major intracellular source of ROS under physiologic conditions. The steady-state levels of mitochondrial $O_2^{\bullet -}$ are influenced by the local O_2 concentration and efficiency of mitochondrial electron transport or electrochemical gradient ($\Delta\Psi$); the higher the $\Delta\Psi$, the greater the mitochondrial ROS production (445). The rationale for this is that at high $\Delta\Psi$, protons are not pumped out of the matrix against the electrochemical gradient, meaning that the electron-transport chain slows, and this increases the half-life of reduced intermediates and the chance that electrons escape to reduce molecular O_2. Mitochondria levels of $O_2^{\bullet -}$ are also governed by the activities of MnSOD, located in the matrix of the organelle, and uncoupling proteins (UCPs), inner mitochondrial membrane anion transporters that, when activated, facilitate the transport of protons back into the mitochondrial matrix, resulting in decreased Ψ and capacity to produce $O_2^{\bullet -}$. Dismutation of $O_2^{\bullet -}$ by MnSOD results in the formation of the H_2O_2 available to mediate redox signaling that is subject to control by mitochondrial Trx-2, Grx-2, Prx-3, or a combination of these. In endothelial cells, VEGF upregulates the gene expression of MnSOD by inducing H_2O_2 production that signals for activation of NF-κB (2, 3).

Although mitochondria are responsible for steady-state cellular ROS production, it is becoming increasingly apparent that ROS production by the organelle can also be enhanced in response to numerous stimuli or alterations in the cellular environment, resulting in physiologic or pathologic consequences. Various metabolites and ligands can stimulate mitochondrial ROS generation, resulting in activation of redox-signaling cascades that act to relate mitochondrial status to cell function and phenotype (227). For example, mitochondrial metabolism of pyruvate stimulates the production of H_2O_2 by the organelle required for the activation of JNK in the cytosol that acts to feedback inhibit metabolic enzymes, glycogen synthase kinase 3β, and glycogen synthase (369). In endothelial cells, angiotensin II–mediated activation of NF-κB and VCAM-1 expression (404) or TNF-α–induced cytotoxicity (113, 183) involves enhanced generation of mitochondrial ROS. Treatment of bovine aortic endothelial cells with the adipokine leptin stimulates mitochondria $O_2^{\bullet -}$ production by increasing FFA oxidation *via* activation of PKA (550). In aortic endothelial cells, angiotensin II (121) or CD40 (108) promotes mitochondrial $O_2^{\bullet -}$ production, and this decreases NO bioactivity in these cells. Integrin engagement can also stimulate mitochondrial ROS production that mediates changes in gene expression that signals for changes in cellular shape (534).

Oxidative and metabolic stresses also promote mitochondrial ROS production in endothelial cells. For example, hypoxia-induced ROS generation and decreased AP-1 transcriptional activity are inhibited by the mitochondrial complex III inhibitor myxothiazol, confirming a signaling role of mitochondria-derived ROS during hypoxia (77). Mitochondrial ROS also signal for stabilization of the redox-sensitive transcription factor HIF-1α in hypoxic endothelial cells (195) and cell death of detached endothelial cells *via* anoikis (306). Exposure of endothelial cells to cyclic strain (11), H_2O_2 (114), oxLDL (578), lysoPC (527), or electrophilic lipid oxidation products (285) promotes mitochondria-dependent ROS production. H_2O_2 appears to act by enhancing the transport of redox-active iron into the mitochondria (114), the organelles responsible for biosynthesis of heme and iron–sulfur centers. Similarly, prolonged exposure of endothelial cells to high concentrations of NO increases liberation of redox-active iron from iron–sulfur proteins present in the mitochondria (409).

With respect to endothelial dysfunction, complications of diabetes are increasingly linked to an enhanced mitochondrial ROS flux. Insulin resistance during diabetes is characterized by hyperglycemia and elevated circulating concentrations of FFAs. Exposure of endothelial cells to diabetic glucose or FFA concentrations promotes mitochondria $O_2^{\bullet -}$ production (371). Overproduction of $O_2^{\bullet -}$ by the mitochondrial electron-transport chain is linked to a number of pathologic changes in endothelial cells characteristic of diabetes, such as formation of advanced glycation end products (AGEs), activation of PKC or NF-κB, plus the inactivation of the glycolytic enzyme GAPDH, eNOS, and prostacyclin synthase (124, 125, 371). Interruption of mitochondrial $O_2^{\bullet -}$, through overexpression of MnSOD or UCPs ameliorates many of the deleterious consequences of hyperglycemia or elevated FFAs (126, 301, 371). A role for mitochondrial dysfunction in diabetes is consistent with a relation between increased genetic alterations in mitochondrial energy metabolism and a predisposition to glucose intolerance and diabetes (335, 444).

Recent evidence shows that changes in the mitochondrial redox status and ROS production have important implications for endothelial dysfunction during atherosclerosis. For example, MnSOD deficiency enhances endothelial dysfunction noted in atherosclerotic, ApoE$^{-/-}$ mice (382). Endothelial cell-specific transgenesis of the mitochondrial Trx2 gene in mice attenuates endothelial production of ROS, preserves EDNO bioactivity, and inhibits atherosclerosis in ApoE$^{-/-}$ mice (569).

Organic nitrates represent effective antiischemic drugs for treating patients with stable angina, acute myocardial infarction, and heart failure. However, long-term therapy with these agents leads to the rapid development of nitrate tolerance associated with adverse consequences in human patients, including impaired endothelium-dependent responses (436). Recent data suggested that reduced EDNO bioactivity afforded by prolonged nitrate treatment is linked to the enhanced production of mitochondrial ROS and subsequent inactivation of mitochondrial complex I (141) and aldehyde dehydrogenase (468), a mitochondrial enzyme responsible for nitrate reduction and biotransformation *in vivo*.

Recent insights have been gained into the potential mechanisms controlling pathologic mitochondrial ROS production. These studies highlight a role of the 66-kDa isoform of growth-factor adaptor Shc (p66^{Shc}), which controls cellular responses to oxidative stress; mice lacking this protein exhibit increased resistance to oxidative stress and a prolonged life span (352). Other studies show that p66^{Shc} translates oxidative damage into apoptosis by promoting the production of ROS within the mitochondria (177). In response to oxidative stress, activated PKC-β phosphorylates p66^{Shc}, which triggers its recruitment to the mitochondria after it is recognized by the prolyl isomerase, Pin1 (398). Mitochondrial p66^{Shc} then uses reducing equivalents derived from the mitochondrial electron-transfer chain through oxidation of cytochrome *c* to generate ROS (177, 368) (Fig. 6). Recent evidence

indicates PKC-β and $p66^{Shc}$ have important implications for vascular complications. A selective PKC-β inhibitor alleviates impaired endothelium-dependent vasodilatation in healthy humans exposed to hyperglycemia (42). Deficiency of the $p66^{Shc}$ gene protects against atherosclerosis in mice fed a high-fat diet (366) and ROS-induced impairment of EDNO bioactivity and endothelial dysfunction associated with aging (152, 551) and hyperglycemia (68). In addition to promoting endothelial oxidative stress, $p66^{Shc}$ may also promote endothelial dysfunction by inhibiting eNOS activity through attenuating Ser-1177 phosphorylation of the enzyme (551). Together, these studies support that targeting of $p66^{Shc}$ represents a potential strategy to reduce mitochondria ROS production to restore endothelial function.

Although it is clear that mitochondrial ROS production can affect on EDNO bioactivity, several studies indicate that NO controls mitochondrial content and ROS production, particularly in endothelial cells. For example, eNOS-derived NO signals for mitochondrial biogenesis in response to calorie restriction, which may be important for extension of the mammalian life span (373, 374). Paxinou and colleagues (395) demonstrated that the protective action of eNOS-derived NO for endothelial cells exposed to oxidative stress is dependent on functional mitochondria (395). Therefore, it is likely that dynamic control of mitochondrial respiration by NO represents an important line of defense against oxidative stress. In endothelial cells, mitochondria represent a subcellular focal point of protein *S*-nitrosylation and tyrosine nitration reactions (272, 556). Elimination of functional mitochondria or inhibitors of mitochondrial electron transport reduce intracellular *S*-nitrosothiol content, indicating that functioning mitochondria are required for optimal *S*-nitrosoprotein formation in cultured endothelial cells (556). These various studies support an intimate relation between mitochondrial function and NO signaling. Consistent with this, several independent studies report that NO is produced within the organelle by a putative mitochondrial isoform of NOS (172). Also, in endothelial cells, eNOS can associate with the outer membrane of the mitochondria (162), and inhibitors of the mitochondria respiratory chain attenuate NO production in BAECs (109). Generation of NO within mitochondria can inhibit the electron-transport chain at multiple sites to promote $O_2^{\bullet -}$ and hence to increase H_2O_2 production (410). Physiologic NO modulates mitochondrial respiration by competing with O_2 and reversibly inhibiting the activity of heme-containing cytochrome *c* oxidase, the terminal enzyme in the electron-transport chain. Such transient inhibition is thought to respond to changing cellular energy and oxygen requirements. Transient modulation of mitochondrial H_2O_2 production by NO may therefore underlie the redox-signaling activity of the organelle (53). Dysfunction of this signaling pathway can result in the mitochondria exhibiting characteristics of hypoxia that favor the formation of peroxynitrite, leading in turn to the irreversible modification and dysfunction of mitochondrial proteins (442).

Another factor influencing the redox control of mitochondrial respiratory chain proteins is *S*-glutathionylation (474). For example, *S*-glutathionylation of complex I increases $O_2^{\bullet -}$ production by the complex that is primarily converted into H_2O_2 (474). Such reversible control of $O_2^{\bullet -}$ production by complex I may represent a further mechanism underlying the redox-signaling role of this organelle. It is becoming increasingly apparent that a variety of redox-sensitive signaling proteins can be recruited to the mitochondria in response to stimuli to mediate redox signaling (227). This is exemplified by a study in TNF-α–stimulated endothelial cells, showing that the anchorage protein Dok-4 recruits Src to the mitochondrion, which in turn stimulates mitochondrial ROS production and resultant activation of NF-κB (243).

In addition to acting as a significant cellular source of ROS, recent data indicate that mitochondria can sense and transduce the redox signal. Chen and colleagues (81) showed that functioning mitochondria are a proximal event in cell signaling induced by H_2O_2 exposure. Thus, H_2O_2-induced trans-activation of growth-factor receptors (*i.e.*, EGF, PDGF, and VEGF receptors) and resultant stimulation of distal signaling events, including JNK, p53, and Akt, were attenuated in cells deficient in respiring mitochondria or exposed to pharmacologic inhibitors of the mitochondrial respiratory chain (81). In contrast to H_2O_2 as a stimulus, inhibition of mitochondria did not affect growth factor–receptor activation in response to the relevant ligand or to UV light (81). The mechanism through which mitochondria sense and transduce oxidative signaling remains unknown, although it appears to involve redox reactions, as mitochondria-targeted antioxidants abrogated H_2O_2-induced cell signaling in a manner similar to inhibition of mitochondrial function.

Oxidative damage to mitochondrial proteins, lipids, and nucleic acid, leading to mitochondrial dysfunction, are increasingly recognized as important events in cardiovascular diseases. Mitochondrial DNA (mtDNA), which encodes for the majority of protein products that are essential components of the mitochondrial respiratory chain, is particularly sensitive to oxidative damage. Exposure of endothelial cells to H_2O_2 or peroxynitrite results in preferential damage to mtDNA over nuclear DNA, resulting in decreased mitochondrial protein synthesis and mitochondrial dysfunction (33). Sensitivity to oxidative damage relates to the location of mtDNA in the inner mitochondrial membrane in close proximity to ROS production and absence of protective histones and repair enzymes that are afforded to the nuclear genome. Damage to mtDNA and the resulting mutation of respiratory-chain proteins can lead to aberrant ROS production (31). Considering that the repair of mitochondrial proteins is dependent on *de novo* protein synthesis and eNOS-derived NO signals for mitochondrial biogenesis, a further consequence of impaired EDNO bioactivity during vascular disease may be the buildup of dysfunctional mitochondria in the endothelium. Consistent with this, atherosclerotic vessels display increased mtDNA damage when compared with nondiseased vessels (32, 269), and MnSOD deficiency correlates with increased mtDNA damage and accelerated atherosclerosis in ApoE-deficient mice (32).

D. Uncoupled eNOS

In the presence of sufficient substrate and cofactors, eNOS efficiently transports the 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, studies with purified enzyme demonstrate that eNOS, under conditions of limiting L-arginine or BH_4 concentrations or both, can exhibit NADPH oxidase activity to produce $O_2^{\bullet -}$, a pro-

cess known as "uncoupling" (503) (Fig. 7). In this process, O_2 acts as the terminal electron acceptor (rather than L-arginine), resulting in the production of $O_2^{\bullet -}$. Initial studies indicated that BH_4-depleted eNOS generates $O_2^{\bullet -}$ in a Ca^{2+}/calmodulin-dependent manner by the oxygenase domain *via* dissociation of the ferrous–dioxygen complex (503). Therefore, the intracellular BH_4 concentration represents an important determinant of the NO to $O_2^{\bullet -}$ ratio generated by eNOS (433). Other studies conclude that eNOS interaction with Hsp90 protects against eNOS uncoupling (385, 401, 402, 461), although others have challenged this conclusion (118, 537). The phosphorylation status of eNOS at Thr-495 may also represent a determinant of the extent of eNOS uncoupling; a Thr495Ala variant of eNOS produced increased $O_2^{\bullet -}$ compared with the wild-type enzyme, suggesting that dephosphorylation at Thr495 may promote eNOS uncoupling (318).

Numerous reports support that eNOS uncoupling is an important mechanism of pathologic $O_2^{\bullet -}$ production in the vascular endothelium. Evidence for eNOS-derived $O_2^{\bullet -}$ production has been documented in blood vessels derived from experimental animals with atherosclerosis (300), hypertension (98, 289), diabetes (221), myocardial ischemia (131), and angiotensin II treatment (357). Notably, evidence for eNOS uncoupling has also been recognized in humans with endothelial dysfunction in the pathologic settings of hypercholesterolemia (459), diabetes (211), hypertension (219), and smoking (209). As such considerable interest exists in defining the stimuli and biochemical mechanism(s) responsible for eNOS uncoupling *in vivo*.

A number of pathophysiologically relevant stimuli have been implicated in promoting eNOS uncoupling in cultured endothelial cells, including native LDL (403), oxidized LDL (506), angiostatin (273), high glucose (580), ceramide (308), homocysteine (488), $ONOO^-$ (580), and HOCl (456, 549). A recent *in vivo* study identified angiotensin II as an import mediator of eNOS uncoupling in diabetic mice (378).

It is increasingly apparent that limitation of BH_4 availability for eNOS represents the primary mechanism leading to uncoupling of the enzyme during vascular disease. Thus, BH_4 levels are reduced in diseased *versus* healthy vessels in animal models of diabetes, hypertension, or atherosclerosis (13). A recent study by d'Uscio and colleagues (132) reported that although BH_4 in healthy aortas from wild-type mice was largely localized to the endothelium, *ex vivo* denudation of the endothelium from aortas isolated from $ApoE^{-/-}$ mice had little impact on vascular BH_4 levels, indicating lesion cells other than endothelial cells (*e.g.*, immune-stimulated macrophages or SMCs) contained the BH_4 (132). These findings support that endothelial BH_4 levels are selectively depleted during atherogenesis. Consistent with this contention, impaired endothelium-dependent relaxation exhibited by diseased vessels can be reversed on restoration of vascular BH_4 levels through supplementation (209, 329). Similarly, endothelium-targeted, transgenic overexpression of GTP cyclohydrolase I (GTPCH-1; the initial and rate-limiting enzyme of the *de novo* BH_4 biosynthesis pathway) in $ApoE^{-/-}$ or streptozotocin-treated diabetic mice results in increased aortic BH_4 levels, reduced eNOS uncoupling and preserved endothelium-dependent vasorelaxation compared with controls (12, 13). The successful restoration of endothelial function after provision of BH_4 supplements to patients with hypercholesterolemia, diabetes, hypertension, and cigarette smoking (209, 211, 219, 459) highlights that eNOS uncoupling is also likely a feature of human vascular disease. This indicates that maintenance of the endothelial intracellular BH_4 concentration represents a key determinant of the NO-to-$O_2^{\bullet -}$ ratio generated by eNOS (96–98).

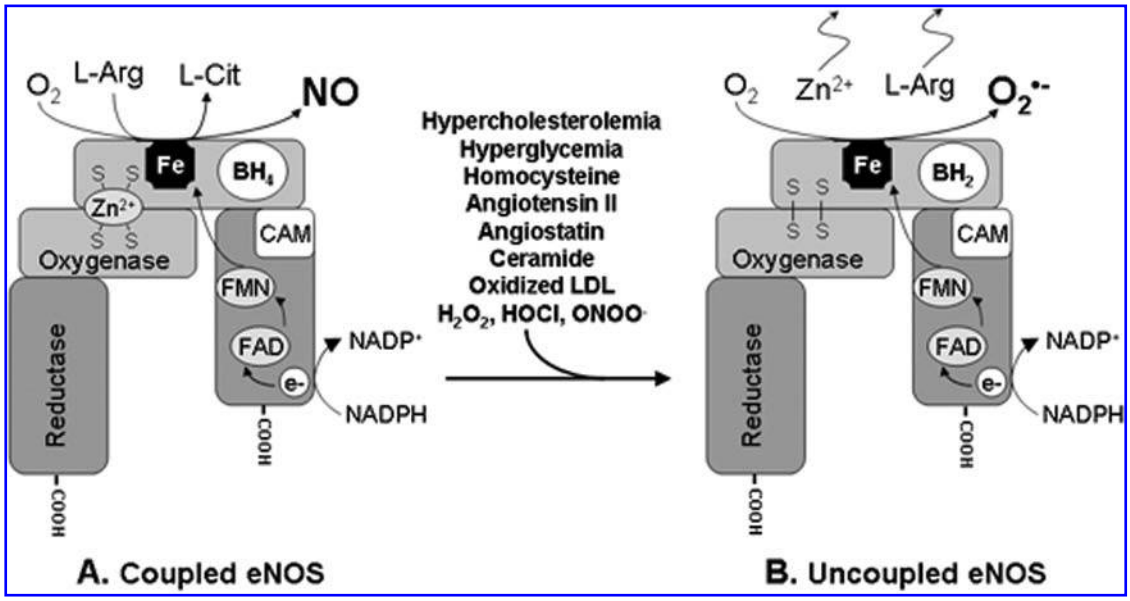

FIG. 7. Uncoupling of eNOS. (**A**) In the presence of adequate amounts of substrate and co-factors, eNOS efficiently channels the transport of electrons from NADPH bound at the C-terminal reductase to the N-terminal heme for O_2 reduction and incorporation into the guanidine group of L-arginine to produce NO and L-citrulline. (**B**) Under conditions of limiting concentrations of L-arginine or BH_4 and the oxidation of BH_4 to BH_2 and/or the Zn^{2+}-thiolate cluster, eNOS exhibits NADPH oxidase activity to produce $O_2^{\bullet -}$.

Endothelial levels of BH_4 likely reflect the level of *de novo* synthesis and recycling *versus* the degree of oxidative degradation. In endothelial cells, biosynthesis of BH_4 is governed by the expression level and activity of GTPCH-1. Physiologic laminar shear stress increases and maintains the enzymatic activity of GTP cyclohydrolase (GTPCH)-1 in endothelial cells (538). However, some evidence indicates that decreased vascular BH_4 levels are related to reduced expression of GTPCH-1 in animal models of hypertension (355, 576). Several recent studies also point to oxidation of BH_4 as a primary reason for limiting the levels of the biopterin in diseased blood vessels. Thus, Laursen *et al*. (300) provided evidence that peroxynitrite-mediated oxidation of endothelial BH_4 is responsible for eNOS uncoupling and impaired endothelium-dependent relaxations of mouse atherosclerotic vessels. The two-electron oxidation of BH_4 yields BH_2, which is not a cofactor for eNOS and can compete with BH_4 for binding to the oxygenase domain of the enzyme. However, BH_2 can be reduced to BH_4 *via* DHFR. Recent work indicates that selective inhibition of DHFR in endothelial cells with RNA interference results in a marked reduction in intracellular BH_4 levels and eNOS uncoupling (75). Also, angiotensin II treatment downregulates endothelial expression of DHFR and BH_4 levels, resulting in eNOS uncoupling, changes prevented by overexpression of DHFR (75). These various studies support that prevention of BH_4 oxidation and sustained recycling of BH_2 to BH_4 *via* DHFR represent suitable strategies to reduce eNOS uncoupling during vascular disease.

A recent study by Zou and colleagues (580) led to the proposal that eNOS uncoupling can be achieved by oxidative reactions, independent of BH_4 oxidation (580). They reported that peroxynitrite-mediated oxidative destruction of the Zn^{2+}-thiolate cluster resulted in Zn^{2+} ion release and formation of a disulfide-linked eNOS dimer, and this led to uncoupling of the enzyme. As peroxynitrite concentrations required to disrupt the Zn^{2+}-thiolate complex in eNOS were at least 10-fold lower than those required to oxidize BH_4, the authors concluded that oxidation of the Zn^{2+}–thiolate cluster was primarily responsible for enzyme uncoupling (580). However, caution is warranted with such a conclusion. This is because Cys-99 present in the thiolate cluster is also essential for the correct binding of BH_4 (84). Oxidation of this cysteine is also likely to result in release of BH_4 from the enzyme that is expected to contribute to eNOS uncoupling (Fig. 7).

Studies into the molecular signals leading to eNOS uncoupling indicate a central role for PKC. Thus, pharmacologic inhibition of PKC in vessels from diabetic or angiotensin II–treated animals showed reduced eNOS uncoupling (193, 221, 357). The same *in vivo* studies provided support that an important molecular target of PKC leading to eNOS uncoupling is NADPH oxidase (193, 221, 357). In support of this, in hypertensive mice, a deficiency of the NADPH oxidase subunit $p47^{phox}$ attenuates eNOS uncoupling (288). Also, the ability of angiotensin II to downregulate the expression of DHFR, resulting in reduced BH_4 levels and eNOS uncoupling, is reversed by a pharmacologic inhibitor of the Nox subunit, Rac1 (75). Together, these findings support that PKC-mediated NADPH oxidase activation represents a critical initial event leading to eNOS uncoupling in certain vascular disorders.

Recent studies support that eNOS uncoupling can ultimately lead to increased atherosclerosis. Thus, an initial study reported that transgenic overexpression of eNOS in $ApoE^{-/-}$ mice promoted atherosclerosis, and this correlated with enhanced endothelium- and eNOS-dependent $O_2^{\bullet-}$ production (386). Augmenting BH_4 levels in the endothelium through dietary supplements or overexpression of GTP-cyclohydrolase I reduced the rate of lesion formation in $ApoE^{-/-}$/eNOS transgenic mice, and this was associated with decreased vascular $O_2^{\bullet-}$ generation from uncoupled eNOS (386, 469).

E. Cytochrome P_{450}

Arachidonic acid metabolism by cytochrome P_{450} (CYP) enzymes expressed within the cardiovascular system is increasingly recognized as playing an important role in governing vascular homeostasis and tone (147). Epoxyeicostrienoic acids (EETs) produced by CYP2 epoxygenases expressed in endothelial cells account for the NO- and prostacyclin-independent vasodilatation in several vascular beds, including resistance and coronary arteries (146). As such, EETs represent an important endothelium-derived hyperpolarizing factor (EDHF). More recently, CYP 2C9 expressed by coronary artery endothelial cells has been identified as a significant source of ROS responsible for impaired EDNO bioactivity and redox signaling, leading to the activation of NF-κB (148). In support of this, a CYP 2C9 inhibitor, sulfaphenazole, improves endothelium-dependent vasodilatation in CAD patients (148).

F. Interaction of oxidative pathways in endothelial cells

The preceding data indicate that increased endothelial $O_2^{\bullet-}$ can be derived from various enzymatic sources. As such, it is likely that the precise sources of pathologic $O_2^{\bullet-}$ production may depend on the nature of the vascular disease, the type of blood vessel in question, and the stage of disease progression. Increasing evidence supports cross-communication between different oxidative enzymes in endothelial cells, where an initial minor increase in ROS production from one enzyme can initiate a feed-forward, self-propagating pathway of amplified ROS production from other enzymes that has important implications for endothelial dysfunction. For example, NADPH oxidase–derived H_2O_2 signals for the enhanced expression and activity of xanthine oxidase in endothelial cells exposed to oscillatory shear stress (348, 349) or angiotensin II (293). NADPH oxidase–initiated reactions can also underscore enhanced mitochondrial ROS production (121) or the reduction of cellular BH_4 levels in endothelial cells, resulting in an amplification of ROS production by uncoupled eNOS (75, 289). Also, H_2O_2 or mitochondria-derived ROS activate NADPH oxidase in endothelial cells, which may lead to a self-perpetuating cycle of enzyme activation (315, 432). Finally, increased H_2O_2 enhances extracellular iron uptake or liberation of intracellular labile iron to promote iron-dependent oxidative reactions in endothelial cells (339, 472, 483). The extent to which the different oxidative enzymes or events act in concert to promote endothelial cell oxidative reactions during vascular disease requires further attention. Recent studies, however, support that overstimulation of local endothelial ROS production from NADPH oxidase acts to initiate and expand the uncontrolled production of pathogenic ROS from dysfunctional cellular sources, including uncoupled eNOS in hypertensive

mice (289) or xanthine oxidase in CAD patients (293), leading to endothelial dysfunction.

V. Endothelial Dysfunction and Other Forms of Oxidative Stress

Although it is clear that EDNO bioactivity is dependent on the local vascular concentration of $O_2^{\bullet -}$, the full extent of endothelial dysfunction associated with vascular disease is only partially explained by this ROS. For example, increasing vascular SOD activity only partially improves EDNO bioactivity (362), and the effect of SOD is dependent on the stage of vascular disease. Reducing the $O_2^{\bullet -}$ flux with SOD treatment improves EDNO bioactivity more effectively in the early (362) compared with advanced stages of atherogenesis (254). Subsequently, we consider in more detail how different forms of oxidative stress can affect on EDNO bioactivity to influence endothelial dysfunction during vascular disease.

A. Hydrogen peroxide

Diseased vessels produce increased levels of H_2O_2 (356), and this oxidant is also produced in significant concentrations by activated leukocytes (322). Numerous studies report that H_2O_2 represents an important vasoactive substance capable of modulating vascular tone, although its mode of action is complex. Thus, H_2O_2 can elicit contractile or relaxant responses, depending on the animal species, vascular bed, contractile state, and disease status of the vessel (18). A recent study using mice overexpressing a catalase transgene targeted to blood vessels reported that these mice exhibited significantly reduced blood pressure compared with wild-type controls, consistent with an overall contractile *in vivo* action of H_2O_2 in mice (465). The various complex mechanisms by which H_2O_2 can directly elicit smooth muscle contraction were recently the subject of an excellent review (18) and are not discussed here. We focus on the implications of elevated endothelial H_2O_2 for eNOS activity and EDNO bioactivity.

Depending on blood vessel size and physiologic or pathologic status of the vessel, H_2O_2 is capable of inducing endothelium-dependent and -independent vasorelaxation (Fig. 8). Endothelium-dependent relaxations can be EDNO dependent or independent. Exposure of large-sized arteries to H_2O_2 induces their relaxation in an endothelium-dependent manner that involves activation of eNOS and increased EDNO production (479, 563). For example, H_2O_2-induced vasorelaxation of preconstricted rabbit aorta is inhibited by removal of the endothelium or inhibition of eNOS activity with L-NAME (479, 563). Accordingly, H_2O_2 treatment acutely enhances eNOS activity and NO production in cultured aortic endothelial cells (65, 479). Acute activation of eNOS by H_2O_2 requires the induction of a redox-signaling pathway involving Src kinase–dependent trans-activation of the EGF receptor and resultant stimulation of PI3-kinase signaling, resulting in the phosphorylation of the enzyme at Ser-1177 and dephosphorylation at Thr-495 (83, 479, 483) (Fig. 8). H_2O_2-induced eNOS phosphorylation at Ser-1177 is dependent on Akt, whereas the PI3-kinase–dependent events leading to dephosphorylation at Thr-495 remain to be defined (479). A more recent study indicates that, in addition to PI3-K/Akt signaling, maximal activation of eNOS by H_2O_2 also requires stimulation of the MAP kinase, ERK1/2 (65). Activation of eNOS with high H_2O_2 concentrations can also involve Src kinase–dependent phosphorylation of the enzyme at Tyr-83 (159). Endogenous H_2O_2 produced by NADPH oxidase also activates eNOS in cultured endothelial cells stimulated with angiotensin II (66), likely through phosphorylation at Ser-1177 (467, 559). Together, these studies support that endogenous H_2O_2 represents an important signal for eNOS activation induced by certain agonists in endothelial cells.

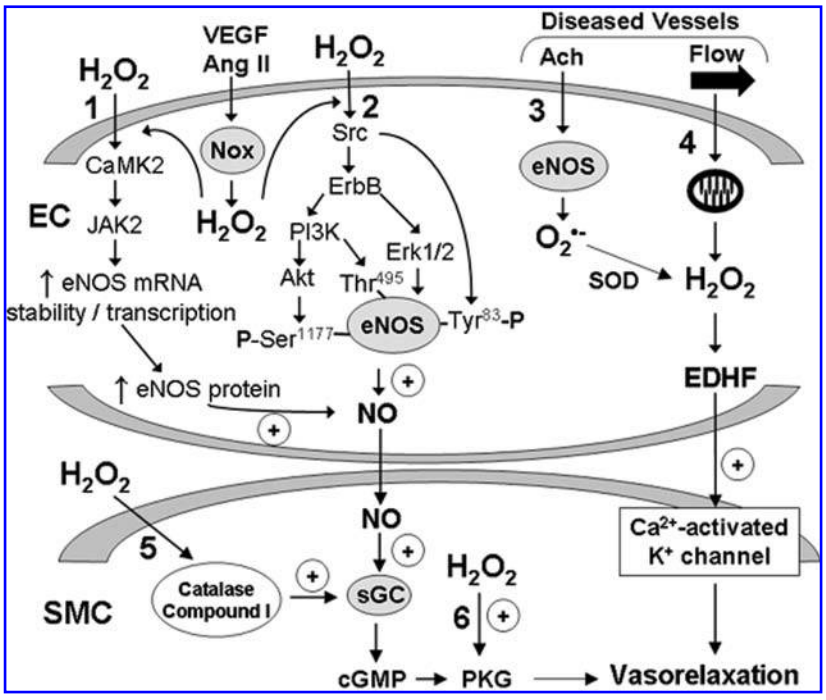

FIG. 8. Vasorelaxant mechanisms of H_2O_2. H_2O_2 can mediate vasorelaxation of vessels by several potential mechanisms depending on the vessel type and disease status. H_2O_2 activates redox-sensitive signaling pathways that result in increased eNOS activity and EDNO production through (**1**) long-term upregulation of eNOS expression at the transcriptional level and (**2**) acute activation of the enzyme through increased phosphorylation of eNOS at Ser^{1177} and Tyr^{83} and de-phosphorylation at Thr^{495}. (**3**) Hypertensive mouse aorta exposed to Ach or (**4**) human coronary arterioles subject to shear stress produce increased H_2O_2 derived from uncoupled eNOS or mitochondria, respectively, which mediates vasorelaxation likely by exhibiting endothelial-derived hyperpolarizing (EDHF) activity and resultant activation of Ca^{2+}-activated K^+ channels (BK_{Ca}) expressed on SMC. (**5**) H_2O_2 directly promotes SMC relaxation by a cGMP-dependent mechanism requiring the formation of Compound I of catalase that stimulates sGC activity. (**6**) H_2O_2 induces cGMP-independent SMC relaxation by directly activating PKG.

In addition to acute stimulation of enzyme activity, H_2O_2 upregulates endothelial expression of eNOS *via* stimulation of mRNA transcription and enhancing mRNA stability (123) (Fig. 8). These effects of H_2O_2 on eNOS expression have been linked to activation of Ca^{2+}/calmodulin kinase II and Janus kinase 2 signaling pathways (63). Oxidant-induced eNOS upregulation is consistent with observations that the eNOS promoter contains an ARE and consensus sequences for the redox-sensitive transcription factors (*i.e.*, AP-1, NF-κB, and SP-1). The ability of H_2O_2 to upregulate eNOS expression may, in part, explain the consistent finding that the expression levels of eNOS protein are paradoxically increased during atherosclerosis, hypertension, and diabetes, conditions associated with increased vascular oxidative stress (221, 300). Also, exercise training of mice (297) or therapeutic agents such as cyclosporin A and doxorubicin elevate eNOS expression in an H_2O_2-dependent manner (255). The ability of H_2O_2 to induce redox-signaling pathways that enhance eNOS activity and expression may represent a compensatory and beneficial response of endothelial cells to maintain EDNO production in the face of oxidative stress. Consistent with this proposal are studies demonstrating that eNOS-derived NO protects endothelial cells against H_2O_2-induced toxicity (395, 478). The protective action of NO in endothelial cells relates to control of mitochondrial respiration (395) or enhanced proteasomal activity necessary for the degradation of nitrated transferrin receptor and subsequent abrogation of cellular uptake of redox-active, labile iron (275, 478) (Fig. 8).

H_2O_2 and EDHF. In addition to EDNO and PGI_2, the endothelium mediates vasodilatation *via* an additional mechanism characterized as the hyperpolarization-mediated relaxation of smooth muscle that remains after the inhibition of the synthesis of NO and prostaglandins. This mechanism is due to the action of EDHF and is dependent on the release of diffusible factor(s). Whereas the role of EDNO is dominant in larger vessels, the importance of EDHF appears to increase with decreasing vessel size. The chemical nature and mechanism of action of EDHF is a contentious issue, although it may vary depending on the stimuli, vascular beds, species, and gender, plus during development, aging, and disease (137, 491).

Several recent studies reported that in certain vessels, and in response to selected stimuli, H_2O_2 can act as an EDHF (Fig. 8) [see, however (137, 491), that question the extent to which H_2O_2 represents an EDHF of physiologic relevance]. For example, Matoba and colleagues (338) first reported that H_2O_2 derived from uncoupled eNOS mediates the relaxation of mouse small mesenteric arteries stimulated with acetylcholine. Consistent with this initial report, a subsequent study using Cu,Zn-SOD–deficient mice provided support for the contention that SOD isoforms can acts as an EDHF synthase by catalyzing the dismutation of $O_2^{\bullet -}$ into H_2O_2 (360). EDHF activity of H_2O_2 has also been reported in human mesenteric arteries (337) and coronary arterioles (356). Exposure of isolated coronary arterioles from human CAD patients to shear stress stimulates flow-induced dilatation that is dependent on the EDHF activity of H_2O_2 derived from respiring mitochondria (356). Recent data indicate that the EDHF activity of H_2O_2 relates to the oxidants' ability to directly activate PKG-α and the resultant phosphorylation and activation of Ca^{2+}-activated K^+ channels (BK_{Ca}) in vascular smooth muscle cells (61, 323). Activation of PKG-α by H_2O_2 involves oxidation of critical cysteines in the kinase, resulting in enzyme dimerization (61). Wolin and Burke (543) provided evidence that H_2O_2 also promotes smooth muscle cell relaxation *via* a cGMP-dependent mechanism dependent on the formation of catalase compound I that stimulates guanylyl cyclase to increase intracellular cGMP (543) (reactions 3 and 4) (Fig. 8).

$$\text{Catalase-Fe}^{3+} + H_2O_2 \rightarrow$$

$$H_2O + \text{Compound I} \rightarrow \uparrow \text{cGMP} \quad [3]$$

$$\text{Compound I} + H_2O_2 \rightarrow {}^1/_2O_2 + H_2O + \text{Catalase-Fe}^{3+} \quad [4]$$

The ability of H_2O_2 to mediate vasorelaxation may partially explain the redundancy of endothelium-dependent relaxation caused by EDHF and EDNO (258) and the inhibitory action of EDNO on EDHF activity (358). Therefore, under certain conditions, SOD isoforms may act to preserve endothelium-dependent relaxation, not only by increasing the half-life of EDNO through removal of $O_2^{\bullet -}$, but also by converting $O_2^{\bullet -}$ into H_2O_2 that can exhibit EDHF activity (360). The precise contributions of H_2O_2 *versus* NO to vasodilation are not clear. However, under pathologic conditions, when BH_4 levels are limited, leading to eNOS uncoupling, the endothelium can switch from EDNO to H_2O_2 as a vasodilator (97). This paradigm is exemplified in a study using mice that display a 90% reduction in GTPCH activity and hence significantly reduced tissue BH_4 levels than do wild-type controls. In these mice, arterial relaxation in response to acetylcholine is mediated by H_2O_2 derived from uncoupled eNOS (96). Similarly, deoxycorticosterone acetate-salt (DOCA-salt) hypertensive mice showed equivalent endothelium-dependent relaxation responses to acetylcholine compared with control mice in a manner that is mediated by H_2O_2 produced by uncoupled eNOS (289). Importantly, however, whereas *ex vivo* endothelium-dependent relaxations remain essentially intact in the hypertensive mice, this study provided evidence that eNOS uncoupling and enhanced endothelial production of H_2O_2 participates in the long-term elevation in blood pressure noted in DOCA-salt mice. In further support of this contention, mice overexpressing catalase in the vascular wall exhibit decreased blood pressure indicative of a vasoconstrictive role of H_2O_2 (465). Therefore, although H_2O_2 may mediate endothelium-dependent relaxations *in vivo* in the short term, long-term overproduction of the oxidant can elicit pathogenic consequences, either through its ability to catalyze oxidative reactions (see later) or by inducing aberrant cell signaling that alters vascular cell function and phenotype (*e.g.*, induction of SMC hypertrophy and vascular remodeling important for the progression of hypertension) (18).

H_2O_2 and impaired EDNO bioactivity. Growing evidence indicates that elevated endothelial H_2O_2 has detrimental consequences for overall EDNO bioactivity and endothelial function (Fig. 9). Thus, although H_2O_2 does not appreciably react with NO, and treatment of endothelial cells with the oxidant promotes eNOS activity (479), several studies have reported that pretreatment of cultured endothelial cells or isolated arteries with H_2O_2 inhibits agonist-stimulated EDNO bioactivity and endothelium-dependent relaxation (246, 483). Recent studies report that exposure of isolated vessels or cultured arterial endothelial cells to H_2O_2 impairs

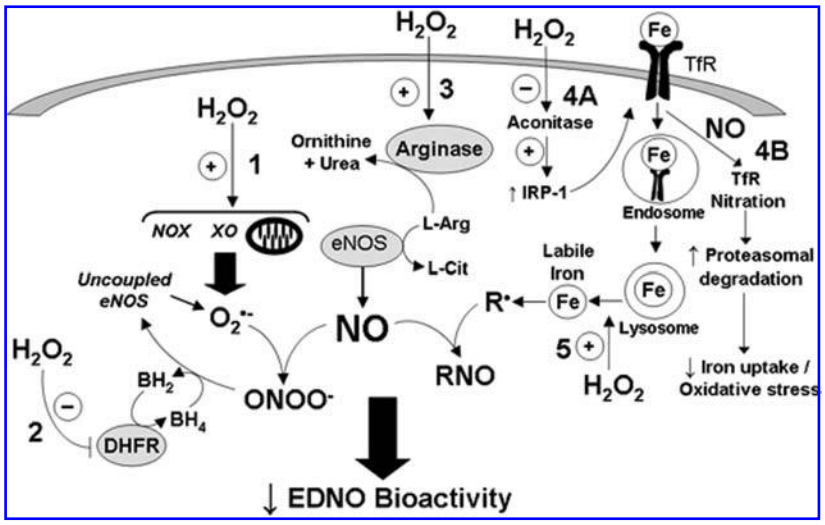

FIG. 9. Potential mechanisms through which elevated vascular H_2O_2 concentrations can impair EDNO bioactivity. Elevated endothelial H_2O_2 impairs EDNO bioactivity via several mechanisms. (**1**) H_2O_2 stimulates the production of $O_2^{\bullet-}$ from several sources, including NADPH oxidase (Nox), xanthine oxidase (XO), mitochondria, and uncoupled eNOS. The $O_2^{\bullet-}$ produced is available to scavenge NO to form peroxynitrite ($ONOO^-$). (**2**) H_2O_2 can downregulate the expression of dihydrofolate reductase (DHFR), resulting in the inhibition of recycling of BH_2 back to BH_4 and enhanced $O_2^{\bullet-}$ production by uncoupled eNOS. (**3**) H_2O_2 upregulates arginase expression, leading to reduced L-Arginine substrate for eNOS and hence reduced NO production. (**4A**) H_2O_2 activates iron-regulatory protein-1 (IRP1), which signals for increased expression of the transferrin receptor (TfR) and enhanced endothelial iron uptake. (**4B**) NO inhibits the ability of H_2O_2 to promote iron uptake by mediating tyrosine nitration of TfR, which signals for the increased proteasomal removal of the receptor. (**5**) H_2O_2 induces the release of iron likely from lysosomal stores and this labile iron catalyses the formation of free radicals ($R^{\bullet}$) capable of scavenging NO.

EDNO bioactivity and that SOD mimetics or cell-permeable forms of SOD reverse H_2O_2-induced impairment of EDNO bioactivity and endothelium-dependant relaxation, implying a role for $O_2^{\bullet-}$ (483, 531, 542). Consistent with this, H_2O_2 treatment of endothelial cells increases $O_2^{\bullet-}$ production (99, 542). Various cellular sources of $O_2^{\bullet-}$ have been proposed, including NADPH oxidase, mitochondria, xanthine oxidase, and uncoupled eNOS (52, 99, 542). Treatment of endothelial cells with H_2O_2 can activate NADPH oxidase or xanthine oxidase activities (99, 349) and promote mitochondrial $O_2^{\bullet-}$ production (115). With respect to uncoupled eNOS, whereas H_2O_2 itself is not a potent oxidant of BH_4, *repeated* H_2O_2 treatment of endothelial cells can coactivate eNOS and NADPH oxidase, resulting in the simultaneous production of NO and $O_2^{\bullet-}$ necessary for the formation of peroxynitrite that readily oxidizes BH_4, leading to eNOS uncoupling (52).

Recent evidence indicates that, in addition to $O_2^{\bullet-}$, alterations in endothelial iron homeostasis in response to H_2O_2 have important implications for EDNO bioactivity (Fig. 9). Thus, increased endothelial oxidative stress in response to H_2O_2 treatment is accompanied by an acute increase in intracellular levels of labile iron (483). Chelation of intracellular iron in cultured endothelial cells with desferrioxamine not only prevents intracellular oxidative reactions induced by H_2O_2 but also significantly reverses the impairment of EDNO bioactivity afforded by the oxidant (483). Similarly, iron chelation with desferrioxamine prevents the H_2O_2-induced impairment of vasodilation of porcine coronary arterioles in response to adenosine (476). The subcellular source of acutely released labile iron in H_2O_2-treated endothelial cells is currently unknown, although recent studies in T cells support reversible and dynamic release from endosomes or lysosomes (475). Studies by Kalyanaraman and colleagues (472) also noted that H_2O_2 has important implications for intracellular iron status in endothelial cells; treatment of endothelial cells with H_2O_2 in the presence of serum enhances the transferrin-mediated uptake of iron that is important for the oxidant to promote intracellular oxidative stress and endothelial apoptosis (472). Enhanced endothelial iron uptake, oxidative stress, and apoptosis in response to H_2O_2 are all inhibited by mitochondria-targeted antioxidants, implying a role for ROS production by the organelle (114, 115). This has led to a proposal that H_2O_2-mediated upregulation of endothelial iron uptake involves oxidative inactivation of cytosolic and mitochondrial aconitase activity, and activation of iron-regulatory protein-1 (IRP-1), leading to increased mRNA binding to iron-responsive elements (IREs) present in the transferrin receptor promoter that signals for its increased expression (274) (Fig. 9). Interestingly, NO inhibits H_2O_2-induced iron signaling and oxidative stress in endothelial cells by promoting proteasomal degradation of tyrosine nitrated transferrin receptor, thereby reducing the intracellular flux of labile iron (275).

The studies outlined using cultured endothelial cells or isolated arteries are consistent with observations that vascular iron is important for endothelial dysfunction in humans. Thus, infusion of desferrioxamine improves endothelium-dependent vasodilation in CAD (129) and diabetic (375) patients. These clinical data support a recent study using noninvasive, electron paramagnetic resonance spectroscopy, which reported a significantly increased content of low-molecular-weight labile iron complexes in human atherosclerotic arteries (451). In light of these findings, an increase in vascular H_2O_2 content during atherosclerosis may, in part, be responsible for increases in labile iron present in diseased vessels, which in turn promotes oxidative reactions with implications for EDNO bioactivity and hence endothelial dysfunction. Intracellular iron signaling also appears important for the increased endothelial expression of leukocyte adhesion molecules in response to proinflammatory cytokines (573).

Additional mechanisms through which H_2O_2 may impair EDNO bioactivity may relate to the oxidants' ability to activate poly(ADP-ribose) polymerase (336, 406) or upregulate arginase activity, leading to a decreased levels of L-arginine available for eNOS (476). Although oxidant-induced activa-

tion of poly(ADP-ribose) polymerase depletes endothelial energy resources through cellular loss of NAD^+ and ATP, inhibition of the enzyme maintains these energy reserves and activates a cell-survival pathway involving VEGF receptor 2, Akt, and Bad (336). Elevation of endothelial ADP-ribose levels is also linked to the oxidants' ability to promote endothelial barrier dysfunction. Thus, a recent study reports that H_2O_2-induced increases in ADP(ribose) can activate Ca^{2+} entry *via* transient receptor potential melastatin (TRPM)2 ion channels, resulting in increased endothelial permeability (208). The extent to which H_2O_2 mediates the impairment of EDNO bioactivity during different vascular pathologies and the mechanisms involved require further attention.

A deleterious role of H_2O_2 on EDNO bioactivity is consistent with a study reporting that treatment with polyethylene glycolated-catalase or transgenic overexpression of Gpx protects mice against angiotensin II–induced impairment of endothelium-dependent relaxation (90). Accordingly, mice with heterozygous or homozygous Gpx deficiency exhibit impaired EDNO bioactivity compared with control mice (90, 151). In addition to removing H_2O_2, Gpx may also improve EDNO bioactivity by detoxifying lipid hydroperoxides or catalyzing the decomposition of endogenous S-nitroso-glutathione or both, thereby liberating bioavailable NO (154).

Together these studies indicate that elevated H_2O_2 has important implications for endothelial dysfunction in arteries. Although H_2O_2 has the ability to promote cell-signaling pathways that modulate eNOS phosphorylation, enzyme activation, and increased EDNO production, elevated levels of the oxidant can also promote oxidative reactions and aberrant signaling events in endothelial cells capable of impairing EDNO bioavailability. The propensity for H_2O_2 to catalyze oxidative reactions in endothelial cells may increase in pathologic settings such as atherosclerosis, in which vascular concentrations of small-molecular-weight iron complexes increase (451). Therefore, inhibition of deleterious H_2O_2-catalyzed oxidative and signaling reactions in endothelial cells represents a potential intervention strategy aimed at preserving EDNO bioactivity and hence endothelial function during cardiovascular disease.

H_2O_2 and atherosclerosis. Recent animal studies indicate that increased vascular levels of H_2O_2 have important implications for the atherosclerosis; overexpression of catalase in apolipoprotein E gene knockout mice attenuates the extent of atherosclerosis, indicative of a proatherogenic role for the oxidant (554). Because of the pleiotropic oxidative and signaling actions of H_2O_2, the proatherogenic mechanisms through which the oxidant act are potentially numerous. As alluded to earlier, increased vascular production of H_2O_2 is capable of amplifying ROS production from other sources in diseased blood vessels. In particular, H_2O_2 derived from NADPH oxidase appears to have significant pathogenic consequences, in that it can activate endothelial xanthine oxidase (349), uncouple eNOS (75, 288), and promote iron-dependent oxidative reactions (472, 483).

B. Oxidized lipoproteins

The oxidative-modification hypothesis of atherogenesis states that oxidation of LDL is an important early event in the pathogenesis of the disease (454, 457). As a consequence, many studies have examined the effects of oxidized LDL on eNOS activity and EDNO bioactivity. Exposure of vascular rings to oxidized LDL impairs endothelium-dependent relaxation (261, 278). Prolonged exposure of endothelial cells to copper-oxidized LDL impairs EDNO bioactivity through various mechanisms, including increased endothelial $O_2^{\bullet -}$ production (149, 506), modulation of eNOS expression and phosphorylation status (149, 179, 222, 316), promotion of eNOS uncoupling (149), inhibition of Akt-dependent eNOS activation (80), displacement of eNOS from plasmalemmal caveolae or Golgi to intracellular membranes (493), and reduction in eNOS substrate availability (506). Antiatherogenic, 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 (493). HDL binding to the scavenger receptor B1 induces Src/PI3K/Akt and MAP kinase signaling pathways that lead to eNOS activation and endothelium-dependent vasorelaxation (353, 561). The beneficial actions of HDL may relate, in part, to lipid factors that associate with the lipoprotein, including sphingosine 1-phosphate (376) and estrogen (181), which activate eNOS in endothelial cells.

Although the precise oxidants responsible for LDL modification in early atherogenesis are not known with certainty, increasing evidence implicates hypochlorous acid (HOCl) in this process (205, 206). Notably, 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 (377), cellular sites where eNOS is optimally activated (160).

C. Lipid peroxidation

Because of its small molecular radius and uncharged nature, $^{\bullet}NO$ readily diffuses through the hydrophilic surface to accumulate preferentially within the hydrophobic core of lipid membranes. Vascular diseases such as atherosclerosis are characterized by increased levels of oxidized lipids in the vessel wall (457), and the process of lipid peroxidation has potential consequences for EDNO bioactivity. Similar to its reaction with $O_2^{\bullet -}$, NO undergoes rapid radical–radical combination reactions with lipid alkoxyl ($L^{\bullet}$) or lipid peroxyl radicals ($LOO^{\bullet}$) ($k = 2 \times 10^9$ M/sec), resulting in the formation of nitrated lipid derivatives (379). The endothelium expresses several enzymes that catalyze the oxidation of unsaturated lipids, predominantly arachidonic acid, to generate lipid hydroperoxides and other lipid signaling molecules. These include constitutive and inducible isoforms of prostaglandin H synthase (PGHS), 15-lipoxygenase (LOX), and cytochrome P_{450}. A feature of these enzymes is the transient formation of lipid radicals during their catalytic cycle. Several studies indicate that generation of lipid radicals by these enzymes can result in catalytic consumption of NO, resulting in impaired bioactivity (93, 380, 381). In the context of vascular disease, lipoxygenase appears to have important implications for EDNO bioactivity; 15/12-lipoxygenase gene–deficient mice are resistant to angiotensin II–mediated impairment of EDNO bioactivity and hypertension (15). Interestingly, inhibition of cyclooxygenase activity with aspirin improves NO bioactivity in platelets from healthy blood

donors, but the NSAID beneficial effects on NO bioactivity may be compromised in hypercholesterolemic patients (539).

Although scavenging of EDNO by lipid radicals may be problematic during vascular disease, emerging evidence supports that nitrated lipid derivatives formed under physiologic conditions serve as cell-signaling molecules (317). For example, the nitroalkene derivative of linoleic acid (LNO_2) mediates cGMP-dependent vascular relaxation, induces endothelial expression of heme oxygenase-1, attenuates NF-κB–dependent VCAM-1 expression and monocyte adhesion in endothelial cells, and stimulates peroxisome proliferator–activated receptors (PPARs), a class of nuclear hormone receptors that modulate the expression of metabolic, cellular differentiation, and inflammation-related genes (24, 102, 317, 434, 545). The signaling properties of nitrated lipid derivatives is thought to involve the reversible, electrophilic nitroalkylation of cysteine residues in target proteins (38). Compared with other NO-derived species, such as nitrite, nitrosothiols (RSNO), and heme-nitrosyl complexes, LNO_2 has been proposed to represent a significant pool of bioactive oxides of nitrogen in the healthy vasculature (25).

Lipid peroxidation also has indirect implications for EDNO bioactivity. Oxidized lipids can induce cell signaling that can affect EDNO bioactivity through interruption of G protein–dependent signal transduction (278). Oxidized phospholipids, in particular, may have important implications for endothelial function. Atherosclerotic plaques contain oxidized phospholipids that are formed by nonenzymatic oxidation of esterified polyunsaturated fatty acids (529). Similar to that with H_2O_2 (479), exposure of endothelial cells to oxidized phospholipids promotes eNOS phosphorylation at Ser-1177 *via* a PI3K/Akt-dependent mechanism and dephosphorylation at Thr-495, events expected to enhance eNOS activity and production of NO (173). Despite this, oxidized phospholipids appear to uncouple eNOS, leading to enhanced $O_2^{\bullet -}$ production (173). In addition to effects on EDNO, the formation of oxidized lipids has important consequences for redox signaling in endothelial cells. For example, exposure of endothelial cells to oxidized phospholipids activates various signal-transduction pathways, leading to enhanced tissue factor (49) and IL-8 (50) expression, angiogenesis (50), and enhanced endothelial expression of adhesion molecules and monocyte binding to the endothelium (235, 529). Several signaling pathways have been implicated in the actions of oxidized phospholipids on endothelial function and phenotype, including elevation of intracellular cAMP and Ca^{2+}, activation of Src, PKA, PKC, PI3-K/Akt, and MAP kinases (Erk1/2, p38), which can induce various transcription factors, notably Egr-1, NFAT, CREB, and PPAR but not NF-κB (253). The extent and mechanisms by which oxidized lipids contribute to endothelial dysfunction during vascular disease remain largely unknown.

A consequence of insulin resistance during diabetes is the elevation of circulating free fatty acids (FFAs) because of the enhanced lipolytic activity of adipocytes. Numerous studies indicate that exposure of endothelial cells to diabetic FFA concentrations impairs endothelial function. Increased β-oxidation of FFAs in the mitochondria of human aortic endothelial cells promotes $O_2^{\bullet -}$ production by the organelles' electron-transport chain that results in the induction of proinflammatory signals, inhibition of eNOS activity, and inactivation of prostacyclin synthase (124). The latter enzyme is responsible for the production of prostacyclin, which acts in concert with EDNO to promote vasorelaxation and preserve vascular homeostasis; deficiency of the prostacyclin receptor promotes atherogenesis in $ApoE^{-/-}$ mice (271). Therefore, elevated plasma glucose and FFAs both represent important stimuli leading to the pathologic production of ROS by the mitochondria in endothelial cells that contribute to the vascular complications apparent in diabetic, insulin-resistant patients.

D. Peroxynitrite

Peroxynitrite, the reaction product of NO and $O_2^{\bullet -}$, is a strong oxidant capable of promoting oxidative damage and endothelial dysfunction. Peroxynitrite oxidizes BH_4 *via* a trihydrobiopterin radical to BH_2 with a rate-constant of $k = 6 \times 10^3$ M/sec, severalfold higher than reactions with ascorbate, glutathione, or protein thiols. Peroxynitrite-mediated oxidation of BH_4 promotes eNOS uncoupling and endothelial dysfunction in aortic segments from $apoE^{-/-}$ mice (300). Peroxynitrite has also been reported to promote eNOS uncoupling by inducing the oxidation of the Zn^{2+}-thiolate cluster present in the enzyme, resulting in Zn^{2+} release and destabilization of eNOS dimers (580). The production of $O_2^{\bullet -}$ by uncoupled eNOS may also be enhanced in the presence of peroxynitrite, as the oxidant promotes eNOS phosphorylation at Ser-1177 *via* stimulation of 5′-AMP-activated kinase (579), an event that stimulates electron flow through the enzyme (344). Therefore, by inducing oxidation of BH_4 and Zn^{2+}-thiolate cluster and promoting eNOS electron flow, peroxynitrite may act as a potent stimulus for eNOS-derived $O_2^{\bullet -}$ production. In addition to eNOS, prostacyclin synthase represents a sensitive protein target for peroxynitrite produced within endothelial cells, resulting in tyrosine nitration and inactivation of the enzyme (108). Inactivation of these two atheroprotective enzymes, eNOS and prostacyclin synthase, in endothelial cells represents a primary mechanism through which peroxynitrite can promote endothelial dysfunction.

Although peroxynitrite production within endothelial cells may have deleterious consequences, a recent study indicates that production of low, physiologic levels of the oxidant in vascular smooth muscle cells is important for mediating vasorelaxation. Adachi and colleagues (7) reported that intracellular production of peroxynitrite induces reversible *S*-glutathionylation of Cys-674 of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity, resulting in decreased intracellular Ca^{2+} levels and relaxation of vascular smooth muscle. Moreover, the same study provided evidence that irreversible oxidation of Cys-674 of SERCA to sulfonic acid may represent an important mechanism underlying impaired NO-dependent relaxation during atherosclerosis (7). In addition to oxidation of critical cysteines in SERCA, elevated smooth muscle concentrations of peroxynitrite can also impair NO-mediated vasorelaxation through direct inactivation of sGC. Stasch and colleagues (453) reported that endothelial dysfunction during vascular disease states involves the accumulation of oxidized and/or heme-free sGC in diseased blood vessels, refractory from activation by NO (453). Exposure of intact vessels to peroxynitrite produces a state of NO resistance in which both NO-depen-

dent cGMP accumulation and vasodilatation are impaired, and evidence indicates that the oxidant acts through oxidation of the heme in sGC to a ferric, NO-insensitive state (453). In addition to heme oxidation, a recent study indicates that *S*-nitrosylation of Cys-243 or Cys-122 present in the α and β subunits, respectively, desensitizes sGC to activation by NO (430). Peroxynitrite may also impair vascular smooth muscle relaxation through inhibition of the calcium-activated potassium channels important for the action of EDHF (324).

E. Myeloperoxidase

Myeloperoxidase (MPO) is a member of the heme peroxidase family that is abundantly expressed in phagocytes, including polymorphonuclear neutrophils, monocytes, and subpopulations of tissue macrophages. On leukocyte activation, MPO is secreted from the azurophilic granules into both the extracellular milieu and the phagolysosome, where it uses its co-substrate H_2O_2 and physiologic chloride to catalyze the formation of the potent, two-electron oxidant, HOCl. MPO is capable of generating several other oxidants capable of initiating lipid peroxidation and modification of amino acid residues in proteins, including halogenation, nitration, cross-linking, and carbamylation. In the presence of the NO-oxidation product nitrite (NO_2^-), the levels of which are elevated in inflammatory tissues, MPO catalyzes the formation of the free radical nitrogen dioxide ($^{\bullet}NO_2$) (136, 499). Similar to peroxynitrite, $^{\bullet}NO_2$ catalyzes the formation of 3-nitrotyrosine, and recent studies using MPO gene knockout mice indicate that the peroxidase significantly contributes to the increased levels of 3-nitrotyrosine measured in inflammatory tissues (57). Studies with MPO knockout mice also support a significant role of the enzyme for mediating *in vivo* lipid peroxidation during acute inflammation (572). More recent studies indicate that when exposed to physiologic chloride ion and thiocyanite concentrations (100 μM), MPO preferentially uses the latter as a substrate to generate hypothiocyanate, which appears to have potent signaling activities when exposed to endothelial cells, leading to enhanced expression of leukocyte adhesion molecules (518, 519). A recent study highlights that MPO in the presence of H_2O_2 converts thiocyanite to cyanate capable of reacting with lysine groups of proteins to form *e*-carbamyllysine in a process called protein carbamylation (521). MPO-catalyzed reactions appear to underlie the increase in carbamylated proteins apparent during atherosclerosis (521).

Human atherosclerotic lesions contain active MPO (107) and evidence of protein and lipid oxidation products characteristic of MPO (205, 206). Immunoreactive MPO and HOCl-modified epitopes in human atherosclerotic lesions are detected not only inside monocytes/macrophages but also associated with endothelial cells (28, 205, 330, 463). Myocardial tissue from human patients with acute myocardial infarction exhibit intense recruitment of MPO-positive leukocytes and deposits of free MPO in the endothelium of infarct-related vessels (28).

Although it is clear that MPO is present in diseased human vessels and that the enzyme contributes to the oxidative reactions apparent in such tissue, the situation in murine models of atherosclerosis is more complex. Thus, the LDL receptor (LDLr)/MPO double-gene knockout mouse unexpectedly revealed *increased* atherosclerosis relative to the LDLr knockout mouse (55). Atherosclerotic lesions from LDLr gene knockouts are devoid of MPO and markers of MPO-mediated oxidative reactions. It is thought that the difference between human and murine atherosclerotic lesions relates to the content of MPO in murine *versus* human leukocytes; murine neutrophils express 10–20% of the content of MPO expressed by human neutrophils. Consistent with this notion, a recent study reported that repopulation of irradiated LDLr knockout mice with bone marrow derived from a transgenic mouse overexpressing MPO resulted in increased development of atherosclerosis compared with irradiated LDLr knockout mice receiving wild-type bone marrow (347). This discussion therefore suggests that the commonly used mouse models of atherosclerosis are not suitable models to examine the role of MPO during atherosclerosis.

Clinical evidence indicates a pathologic role of MPO in cardiovascular disease and endothelial dysfunction in humans. Thus, circulating MPO levels are elevated in CAD patients (571) and represent a strong, independent predictor of cardiovascular clinical events (27, 56) and endothelial dysfunction (510). With respect to the latter, Vita and colleagues (510) reported a strong inverse correlation between serum MPO levels and flow-mediated, endothelium-dependent dilatation of the brachial artery. After adjustment for cardiovascular risk factors, medications, and prevalence of cardiovascular disease, patients with MPO levels in the fourth quartile were >6 times more likely to exhibit endothelial dysfunction when compared with subjects in the bottom quartile (510).

Studies with experimental animals also strongly support that MPO represents an important determinant of endothelial dysfunction during inflammatory vascular disease. Thus, recent studies indicate that MPO released by activated phagocytes interacts with and deposits into the vascular endothelium, where the enzyme is anatomically positioned to promote endothelial dysfunction (26, 135). Eiserich and colleagues (135), by using a mouse model of acute vascular inflammation, reported that endotoxin challenge of wild-type mice induced the accumulation of MPO into the aortic endothelium and significantly impaired endothelium-dependent relaxation in response to acetylcholine, whereas arterial rings from MPO gene–deficient mice exhibited normal vasorelaxation. Infusion of MPO into cannulated arteries results in deposition of enzyme into the vascular endothelium, and this affords a greater impairment of endothelium-dependent relaxation in response to elevation of vascular ROS production through angiotensin II treatment (566). Elevated vascular levels of MPO are also detected in the arteries of diabetic Zucker rats, in which the enzyme contributes to the impairment EDNO bioactivity apparent when these vessels are exposed to diabetic glucose concentrations *ex vivo* (567).

The cellular processes leading to endothelial internalization of MPO are currently being explored. Exposure of endothelial cells to MPO results in the rapid binding of the cationic enzyme to the anionic heparan sulfate (HS) glycosaminoglycans expressed on the endothelial cell surface (26) (Fig. 10). Such binding is required for the subsequent internalization and transcytosis of MPO through the endothelial cell, resulting in the deposition of the enzyme into the subendothelial space (26). In addition to HS, binding of MPO to other proteins facilitates endothelial transcytosis. Thus, Ma-

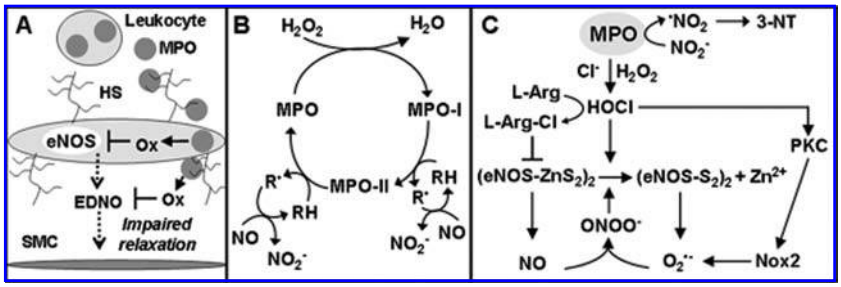

FIG. 10. Myeloperoxidase and endothelial dysfunction. (**A**) Myeloperoxidase (MPO) liberated by activated leukocytes can bind to heparan sulfates expressed on the endothelium, resulting in the enzymes transcytoses to the subendothelial space where the enzyme is positioned to catalyse oxidative reactions that can impair EDNO bioactivity. Several MPO reactions attenuate EDNO bioactivity: (**B**) MPO in the presence of H_2O_2 catalytically consumes NO through the production of substrate radicals ($R^•$) generated by compound I or II of the enzyme. (**C**) HOCl produced by active MPO chlorinates L-arginine and these chlorination products represent inhibitors of eNOS activity. HOCl can oxidize the zinc-sulfur cluster of eNOS leading to uncoupling of the enzyme and $O_2^{•-}$ production. HOCl may also increase endothelial $O_2^{•-}$ production by inducing the protein kinase C (PKC)-dependent activation of NADPH oxidase (Nox). The $O_2^{•-}$ produced reacts with NO to form peroxynitrite ($ONOO^-$) that itself can oxidize the zinc-sulfur cluster of eNOS and BH_4 leading to enzyme uncoupling.

lik and colleagues (485) reported that MPO binds serum albumin *via* positively charged residues 425 to 454 present in the MPO heavy chain, and this facilitates endothelial MPO binding and transcytosis *via* the caveolae. A recent study has identified cytokeratin 1 as an endothelium-expressed binding protein of MPO that facilitates the internalization of the oxidant enzyme (19). Complete endothelial transcytosis results in the deposition of MPO into the endothelial basement membrane, where the enzyme associates with the extracellular matrix protein fibronectin, which is a target for nitration by active MPO (26, 135).

Endothelium-localized MPO can promote the impairment of EDNO bioactivity and hence endothelial dysfunction in various ways (Fig. 10). First, active MPO can act as a catalytic sink to consume NO avidly through scavenging by compound I or II of the enzyme or by substrate radicals produced during the enzyme's catalytic cycle (4, 135). Physiologic substrate radicals include the ascorbyl radical and tyrosyl radical (135). Second, MPO produces HOCl, which itself can mediate endothelial dysfunction. Thus, HOCl treatment inhibits endothelium-dependent relaxation in isolated vessels or receptor-dependent activation of NO production in cultured endothelial cells (246, 456, 549, 565). Such inhibition may relate to reaction of HOCl with L-Arg to produce chlorinated products that inhibit eNOS activity (565) or the ability of the oxidant to oxidize eNOS cofactors such as NADPH (21) and enhance eNOS dimer instability and uncoupling, resulting in $O_2^{•-}$ production (456, 549). HOCl may also promote endothelial $O_2^{•-}$ production by stimulating PKC-mediated activation of NADPH oxidase, leading to formation of peroxynitrite, which itself impairs EDNO bioactivity (see earlier) (549). Moreover, HOCl may promote endothelial dysfunction through activation of poly(ADP-ribose) polymerase; inhibitors of poly(ADP-ribose) polymerase improve endothelial function in vessels exposed to HOCl (407). Together these findings point toward an important causal role of MPO-catalyzed oxidative reactions for the impairment of EDNO bioactivity noted during vascular inflammation.

MPO may also promote the inflammatory and thrombotic phenotype of the endothelium. Exposure of endothelial cells to low levels of HOCl enhances the expression and activity of tissue factor and promotes endothelial cell apoptosis (462). MPO may also promote vascular inflammation *via* binding to CD11b/CD18 integrins expressed on neutrophils, resulting in activation of these cells (296).

VI. Therapeutic Opportunities for Treatment of Endothelial Dysfunction

Enhanced vascular oxidative stress represents a primary mechanism underlying endothelial dysfunction. Therefore, it is not surprising that many researchers have attempted to normalize EDNO synthesis and bioactivity in the setting of vascular disease through the administration of various antioxidant strategies, including small-molecular-weight antioxidants or inhibitors of ROS-producing enzymes. Other strategies include treatment with eNOS substrate and cofactors to enhance EDNO production, novel drugs that target heme-free sGC or improving endothelial progenitor cell (EPC) number/function to aid in the repair of dysfunctional endothelium. It is also becoming increasingly apparent that pharmacologic agents known to improve clinical outcome in cardiovascular disease patients may act, in part, by ameliorating endothelial dysfunction. We next discuss in more detail the modes through which different strategies act to improve endothelial function that may provide therapeutic potential.

A. L-Arginine and tetrahydrobiopterin

Numerous studies have reported that dietary and intravenous administration of L-arginine increases EDNO bioactivity and endothelium-dependent relaxation in humans (100, 122). This is despite the fact that plasma (~100 μM) and endothelial cell (mM range) L-arginine concentrations exceed the K_m for eNOS (~2 μM). The mechanisms behind the beneficial activity of L-arginine are unknown. One 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 (345). This paradox may also relate to the upregulation of endothelial arginases that compete with eNOS for its substrate. Increased expression and activity of arginase is noted in animal models of vascular disease (418) or in endothelial cells exposed to H_2O_2 and high glucose (418, 476). Enhanced expression of the inducible isoform of NOS (iNOS, NOS2) promotes *S*-nitrosylation of arginase,

increasing the enzyme's K_m for L-arginine, and this contributes to age-related endothelial dysfunction in rats (428). The beneficial effects of L-arginine supplements may also indicate the presence of endogenous inhibitors of NOS. For example, vascular disease is associated with increased levels of the eNOS inhibitor asymmetric-dimethyl arginine (ADMA) (51), suggesting that increased L-arginine may effectively compete with ADMA for eNOS. It remains to be established, however, whether *in vivo* ADMA concentrations are sufficient to compete with L-arginine for eNOS. Finally, a recent study highlights that improved endothelial function elicited by L-arginine supplements may relate to the amino acid ability to bind membrane receptors in endothelial cells to stimulate eNOS activity and EDNO production (250).

As noted earlier, BH_4 supplements can successfully restore endothelial function in human patients with hypercholesterolemia, diabetes, hypertension, or cigarette smoking (209, 211, 219, 459). These studies support the proposal that eNOS uncoupling is a common feature of human vascular disease, that augmenting endothelial BH_4 levels in vascular disease patients has therapeutic potential, and that clinical studies on the effects of long-term BH_4 supplements are warranted.

B. Antioxidants

1. Water-soluble antioxidants. Ascorbate (vitamin C) is a most efficient aqueous extra- and intracellular antioxidant. Numerous studies have consistently reported a beneficial effect of short- and long-term ascorbate administration on the bioactivity of EDNO in human subjects. Both intraarterial infusion and oral supplementation of ascorbate improves endothelium-dependent vasodilation in human patients with atherosclerosis, diabetes, hypertension, and cigarette smoking [*e.g.*, (210, 305)]. It was initially proposed that ascorbate improves EDNO bioactivity by scavenging of $O_2^{\bullet -}$. However, the rate constant for the reaction of ascorbate with $O_2^{\bullet -}$ is $\sim 10^5$ M/sec, 10^4-fold slower than the reaction of NO with $O_2^{\bullet -}$ (267). As such, supraphysiologic concentrations of ascorbate are required to compete effectively with the bimolecular reaction of $O_2^{\bullet -}$ and NO. In support of this, *ex vivo* vessel studies determined that millimolar concentrations of ascorbate are required to prevent $O_2^{\bullet -}$-mediated impairment in EDNO bioactivity (245). Therefore, although direct scavenging of $O_2^{\bullet -}$ may explain the beneficial actions of intraarterial infusion of supraphysiologic concentrations of ascorbate (1–10 m*M*), other mechanisms explain the benefit afforded by physiologic doses of ascorbate (50–100 μM).

Mechanistic studies in cultured endothelial cells have provided insights into the beneficial actions of ascorbate on EDNO bioactivity. Cultured cells are typically scorbutic, as ascorbate added to culture media is frequently oxidized. Two independent studies have reported that incubation of cultured endothelial cells with physiologic ascorbate concentrations results in a time- and concentration-dependent accumulation of the vitamin and this enhanced EDNO bioactivity (214, 233). The mechanism underlying these observations relates to the ability of ascorbate to elevate endothelial levels of BH_4 (233) and maintain the eNOS cofactor in its reduced and hence active state (215). Similarly, an *in vivo* study reported that ascorbate supplements preserved vascular BH_4 levels and eNOS activity in $ApoE^{-/-}$ mice (133). Consistent with the notion that ascorbate acts *via* maintenance of endothelial BH_4 levels in humans, Heitzer and colleagues (209) reported that whereas administration of BH_4 or ascorbate alone to CAD patients improves EDNO bioactivity, the combination does not provide an additive effect. It is important to note that despite the clear benefits of ascorbate with respect to endothelial dysfunction, large-scale randomized clinical trials examining the potential benefits of ascorbate supplements on cardiovascular end points after several years of follow-up have been overall disappointing [*e.g.*, (191)]. The reasons for this may relate to the prescription of a suboptimal dose of the vitamin or initiation of intervention too late in the progression of the vascular disease to provide significant clinical benefits.

Glutathione, present in cells at millimolar concentrations, is a major determinant of the intracellular redox status. GSH levels are reduced in arteries from hypercholesterolemic rabbits, and supplementation with glutathione ester significantly improves EDNO bioactivity in these vessels (5). In CAD patients, treatment with L-oxo-4-thiazoldine carboxylate, an agent that selectively increases intracellular glutathione concentrations, improved EDNO bioactivity in the brachial artery (511). Similarly, intraarterial infusion of glutathione improves endothelium-dependent relaxation in response to acetylcholine (279). The clinical utility of prolonged augmentation of endothelial GSH levels remains unclear.

2. Lipid-soluble antioxidants. Vitamin E, the major biologic and chemical active form of which is α-tocopherol, is an important lipid-soluble antioxidant. Although vitamin E can scavenge $O_2^{\bullet -}$ ($k = 5 \times 10^3$ M/sec), it is unlikely to compete with NO for $O_2^{\bullet -}$ *in vivo*. Despite this, increasing vascular vitamin E levels can improve endothelium-dependent relaxation in experimental animal models of vascular disease (259, 260). This beneficial action appears independent of changes in lipid oxidation (260) and instead may relate to the vitamin's ability to inhibit PKC-dependent $O_2^{\bullet -}$ production (261). Although a consistent beneficial action of vitamin E supplements for endothelium-dependent relaxation is consistent in experimental animals, the situation in humans is contradictory; some studies report that vitamin E supplements improve endothelium-dependent vasodilatation in hypercholesterolemic or CAD patients (213, 370), but others report no effect of the vitamin (176, 346). The reasons for the contradictory findings in animals and humans are unknown but may reflect the stage of disease when intervention is applied. Intervention typically occurs in experimental animals early in the disease process, whereas human studies involve patients with established vascular disease. Similarly, large-scale clinical trials aimed at examining the ability of vitamin E supplements to prevent cardiovascular disease have been overall disappointing (276). Therefore, a clear therapeutic benefit of vitamin E supplements for endothelial dysfunction and cardiovascular disorders in humans is yet to be established.

Coenzyme Q_{10}, in its reduced and antioxidant form (ubiquinol-10, $CoQ_{10}H_2$), represents an effective lipid-soluble antioxidant defense against lipoprotein lipid peroxidation *ex vivo* and *in vivo* (455, 481, 482). Dietary supplementation with coenzyme Q_{10} increases plasma and artery levels of ubiquinol-10 and inhibits atherosclerosis in $ApoE^{-/-}$ mice (481, 540). Several studies have examined the effect of coenzyme Q_{10} supplements on endothelial dysfunction. Coen-

zyme Q_{10} supplements provided no apparent benefit in hypercholesterolemic young adults with endothelial dysfunction (408), but supplementation of type II diabetics improved endothelial function of the brachial artery, independent of changes in plasma F_2-isoprostanes levels, an index of *in vivo* lipid peroxidation (530). Consistent with the benefits to diabetic patients, a recent study reported that treatment of cultured endothelial cells with coenzyme Q_{10} protects against numerous abnormalities afforded by exposure to diabetic glucose concentrations (492).

Probucol is a lipid-soluble, lipid-lowering compound with antioxidant properties; it consistently inhibits the development of atherosclerosis in animal models (72, 541). Probucol also preserves EDNO bioactivity in animal models of vascular disease, independent of its lipid-lowering properties (262). The mechanisms underlying this protective activity of probucol may relate to a reduction in the vascular $O_2^{\bullet -}$ flux in cholesterol-fed rabbits (262) or an ability to promote reendothelialization (295). More recently, the beneficial actions of probucol have been ascribed to the drug's ability to upregulate the expression of heme oxygenase-1, which exhibits an array of beneficial vasoprotective properties (110, 546).

3. Iron chelators. Clinical studies have reported that infusion of the iron chelator desferrioxamine improves endothelium-dependent, but not endothelial-independent, vasodilatation in CAD (129) and diabetic (375) patients. These data suggest that iron availability represents an important determinant for EDNO bioactivity. The underlying mechanisms by which iron chelators improve endothelial function is unknown but may relate to recent *in vitro* studies indicating that elevated H_2O_2 promotes endothelial levels of labile iron capable of catalyzing oxidative reactions that impair EDNO bioactivity (472, 483).

4. Polyphenols. A growing interest exists in dietary polyphenolic agents as protective agents against cardiovascular disease (509). Polyphenols are enriched in various dietary sources, including oils, fruits, nuts, vegetables, wine, and tea. Several studies have reported that polyphenol-enriched beverages can improve EDNO activity and endothelial function in CAD patients. For example, Duffy and colleagues (130) reported that black tea polyphenols improved endothelium-dependent relaxation in CAD patients (130). Mechanistically, polyphenols may improve endothelial function *via* their action as water-soluble antioxidants. However, the beneficial actions of polyphenol consumption on endothelial function in patients do not correspond to a reduction in plasma concentrations of F_2-isoprostanes, markers of systemic lipid peroxidation, or 8-hydroxydeoxyguanosine, a marker of DNA oxidation, suggesting that actions other than antioxidant activity are involved (130). Instead, the beneficial activities of black tea polyphenols may relate to their ability to immediately activate eNOS. Activation of eNOS involved changes in the eNOS phosphorylation status at Ser-1177 and Thr-495 (17). Signaling studies identified p38 MAPK as an upstream component of PI3-kinase/Akt-mediated eNOS activation in response to polyphenols (17). Interestingly, polyphenol-mediated induction of MAPK in endothelial cells occurs *via* activation of the estrogen receptor (16, 268). Recent studies indicate that the green tea polyphenol, epigallocatechin gallate, or red wine polyphenols induce endothelium-dependent vasodilation of isolated arteries *via* redox-sensitive PI3-kinase/Akt-dependent phosphorylation of eNOS (265, 367). Also, the ability of resveratrol to alleviate cardiac dysfunction in diabetic rats related to the polyphenols' ability to stimulate Akt-dependent activation of eNOS (477). In addition to immediate effects, long-term exposure of endothelial cells to red wine polyphenols enhances eNOS expression (304). The extent to which these mechanisms are important for the beneficial activities of dietary polyphenols in patients with vascular complications is currently unknown.

C. Clinical drugs

Key pharmacologic agents that improve clinical outcome in high-risk patients are statins, ACE inhibitors, or angiotensin-receptor antagonists, and emerging data supports that these medications are effective in improving endothelial function (384).

1. HMG-CoA reductase inhibitors. Hypercholesterolemia is a major independent risk factor for CAD and produces endothelial dysfunction because of a decrease in EDNO bioactivity (73). Inhibitors of the enzyme 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, are 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, a body of evidence indicates that HMG-CoA reductase inhibitors have clinical benefit independent of the drug effects on circulating lipid levels (470). One such effect of HMG-CoA reductase inhibition is an improvement in EDNO bioactivity and endothelial function (490). Studies with endothelial cells indicate that HMG-CoA reductase inhibitors can increase eNOS activity by increasing the enzyme expression (298), decreasing the binding of inhibitory caveolin-1 to eNOS (144), and enhancing PI3-K/Akt-dependent phosphorylation of the enzyme (281) that requires the recruitment of Hsp90 to eNOS (59). Inhibition of HMG-CoA reductase may also improve EDNO bioactivity by decreasing vascular oxidative stress (287). These compounds decrease endothelial $O_2^{\bullet -}$ production by reducing PKC- and Rac-dependent NADPH oxidase activation (513, 526). Moreover, HMG-CoA reductase inhibitors increase the expression of GTPCH-1 and hence endothelial BH_4 levels (204), leading to inhibition of $O_2^{\bullet -}$ production from uncoupled eNOS. Also, statins promote *S*-nitrosylation and enhance the activity of Trx in endothelial cells, resulting in a reduction in intracellular ROS production (198). Withdrawal of HMG-CoA reductase inhibition is not without hazard, however, as it produces impaired EDNO bioactivity because of increased vascular production of $O_2^{\bullet -}$ by NADPH oxidase (505). The precise mechanisms by which HMG-CoA reductase inhibitors improve EDNO bioactivity in human cardiovascular disease patients remains a topic for further study.

2. Angiotensin-receptor antagonists and ACE inhibitors. Increased angiotensin II produced through the renin–angiotensin system represents a primary stimulus leading to enhanced $O_2^{\bullet -}$ production and endothelial dysfunction in

diseased blood vessels (523, 524). Accordingly, angiotensin-receptor antagonists and angiotensin-converting enzyme (ACE) inhibitors are increasingly recognized as effective therapeutics to ameliorate endothelial dysfunction. Indeed, anti-renin–angiotensin system agents improve endothelial function in patients with hypercholesterolemia and coronary artery disease, independent of changes in blood pressure (228, 293, 525). Hornig and colleagues (228) reported that angiotensin-receptor antagonists ramipril and losartan improve endothelial function in patients with CAD by increasing the bioavailability of NO through reduction of oxidative stress within the arterial wall, mediated in part by increased EC-SOD activity. More recent studies suggest that angiotensin type 1–receptor blockers have vascular protective effects beyond blood pressure reduction. Thus, losartan stimulates eNOS phosphorylation and suppresses TNF-α–induced endothelial apoptosis by activating the VEGFR2/PI3K/Akt pathway (528). These studies support the notion that interference with the renin–angiotensin system represents a means to reduce vascular oxidative stress and improve endothelial function in patients.

3. Peroxisome proliferator–activated receptors (PPARs). PPARs are ligand-activated transcription factors that have been shown to mediate antiinflammatory actions in vascular cells. Recent evidence suggests that PPAR agonists may beneficially influence endothelial function. For example, activators of PPARα (lipid-lowering fibrate derivatives) and PPARγ (antidiabetic thiazolidinediones) can inhibit NADPH oxidase activity by inhibiting the expression of Nox subunits (117, 237) and can promote the production of NO in endothelial cells (67, 399). Accordingly, PPAR agonists diminish ROS production in angiotensin II–infused rats and decrease $p22^{phox}$ expression and improve endothelial function in diabetic rats (117, 238).

D. Selective inhibition of superoxide-producing enzymes

1. NADPH oxidase. In light of evidence supporting Nox2 as a primary source of pathogenic ROS production during vascular disease, one might predict that drugs that selectively target Nox2 over Nox4 would be beneficial against vascular disease. Apocynin has such a profile, being a relatively potent inhibitor of Nox2 activity in isolated neutrophils (IC_{50}, ~10–20 μM) (458, 500), yet a poor inhibitor of both Nox4-dependent (IC_{50}, >200 μM) (138, 437) and Nox5-dependent (IC_{50}, >63 μM) (438) ROS production in other cell types. Moreover, numerous reports in the literature describe beneficial effects of apocynin in animal models of vascular disease, especially hypertension. Such actions include suppression of NADPH oxidase activity and markers of vascular oxidative stress, increased NO bioavailability and restoration of endothelial function, inhibition of vascular inflammatory markers, and reductions in blood pressure (39, 231, 248, 393, 507, 508, 574).

Given that Nox2 appears to play a critical role in endothelial cell signaling, particularly in the setting of angiogenesis, one could argue that pharmacologic inhibition of vascular Nox2-dependent NADPH oxidase activity under physiologic conditions might have deleterious effects on the vascular wall. However, this is unlikely to be an issue with apocynin, because the compound itself is unlikely to be a direct inhibitor of NADPH oxidase activity (218). Rather, apocynin is a prodrug requiring conversion into its active form–possibly a symmetric dimer–by the actions of MPO in concert with hydrogen peroxide (458). Given that MPO expression is normally low in the blood vessel wall but increases in proatherogenic settings (28, 463), one might predict that apocynin would be relatively inactive against endothelial Nox2 activity during normal physiology and would inhibit only the excessive Nox2-dependent NADPH oxidase activity associated with atherogenesis.

Another potential criticism of the use of Nox2-oxidase inhibitors as treatments for chronic disease is that they could induce a chronic granulomatous disease-like state in patients through systemic suppression of NADPH oxidase activity in the cells of the immune system. Again, this does not seem to be a problem with apocynin, as the drug appears to inhibit NADPH oxidase activity only at the plasma membrane of neutrophils and not within the phagolysosome (458), which is the site where microorganisms are killed within leucocytes. Holland *et al*. (225) demonstrated that hypercholesterolemic rabbits maintained on high doses of apocynin (~1–5 mM orally in the drinking water), even for extended periods of 3 months, demonstrated no overt signs of ill health. These authors reported that the rabbits gained weight in a fashion that was comparable to that in placebo-treated animals and did not display an increase in the incidence of infections, which would not be predicted if apocynin inhibited phagocytic NADPH oxidase activity indiscriminately. Importantly, atherogenesis in these animals was attenuated by apocynin (225).

No reports concern the use of apocynin in humans. However, in light of the previous discussion highlighting that apocynin is both safe and effective at reducing Nox2-dependent NADPH oxidase activity and vascular pathology associated with hypertension and hypercholesterolemia in experimental animals models, such clinical studies would certainly seem to be warranted.

As a postscript to this section on NADPH oxidase inhibitors, it is interesting to note that the two most common and effective cardiovascular therapies currently in clinical use–angiotensin-pathway modulators and statins–may be selective Nox1 and Nox2 inhibitors. Statins block Rac isoprenylation, which is required by Nox1 and Nox2 but not by Nox4 or Nox5. Likewise, Ang II, *via* its AT-1 receptor, appears to upregulate Nox1 and Nox2 preferentially over Nox4. Interestingly, Nox2 expression in mammary arteries from CAD patients after prolonged treatment with AT1-receptor blockers or statins was lower than that seen in patients taking other medications (359, 422, 423).

2. Xanthine oxidase. Several studies indicate that pharmacologic inhibition of xanthine oxidase partially improves endothelial function in patients with type-2 diabetes (62), CAD (29, 71, 171, 293), and chronic heart failure (143). In addition to direct inhibition of xanthine oxidase activity, AT_1-receptor blockade with losartan has been recently reported to reduce endothelium-bound XO activity in CAD patients (293). The clinical benefits of inhibitors of xanthine oxidase for cardiovascular disease patients remain unknown, and studies investigating the long-term effects of allopurinol on disease appear warranted.

3. Mitochondria. Although perhaps not as advanced as selective strategies to target NADPH oxidase and xanthine oxidase, recent promising advances have been noted in the development of selective mitochondria-targeted antioxidants aimed at attenuating deleterious oxidative reactions within the organelle (365). Design of these agents involves the conjugation of lipophilic cations with antioxidants (*e.g.*, coenzyme Q, vitamin E), resulting in synthesis of a membrane-permeable, positively charged agent with antioxidant potential capable of accumulating within the mitochondria up to several-hundred-fold, driven by the membrane potential (365). The therapeutic potential of these agents in the context of endothelial dysfunction is supported by recent studies showing that mitochondria-targeted coenzyme Q, vitamin E, or SOD mimetic protects endothelial cells against oxidative stress–induced cell death (81, 114, 115). Mitochondria-targeted coenzyme Q also ameliorates mitochondrial ROS production and tolerance to nitroglycerin in an *in vivo* rat model of nitrate tolerance (141). Promising treatments for endothelial dysfunction related to nitrate tolerance also include the organic nitrate pentaerithrityl tetranitrate (PETN), which, in contrast to other organic nitrates, does not promote mitochondrial ROS production and upregulates the antioxidant enzyme heme oxygenase-1, which aids in the prevention of tolerance (533).

E. Heparan sulfates

Heparan sulfates, the principal proteoglycans expressed on the endothelial surface and throughout the subendothelial matrix, exhibit key roles in inflammation and maintaining the integrity and function of the vascular endothelium (390). For example, heparan sulfates expressed on the endothelial luminal surface act as mechanosensors to mediate EDNO synthesis in response to shear stress (150) and are essential for leukocyte binding to the endothelial surface and subsequent extravasation into tissue sites of inflammation (520). In addition, endothelial heparan sulfates selectively bind various proteins, including xanthine oxidase and MPO, two enzymes implicated in mediating oxidative stress–induced endothelial dysfunction. Thus, therapeutic strategies aimed at removal of these oxidative stress enzymes from the endothelium represent a promising approach to alleviate endothelial dysfunction promoted by these enzymes. In support of this idea, heparin is able to inhibit the binding of MPO and xanthine oxidase to the endothelium or mobilize enzyme deposits from the endothelium (26, 30, 536). A recent clinical study reported that intravenous administration of heparin improved EDNO bioactivity in CAD patients, and this strongly correlated with the extent of mobilization of MPO from the vessel wall into the circulation (30). Also, compared with healthy controls, CAD patients exhibited a greater degree of liberation of MPO into the circulation in response to heparin (30). This study supports that heparan sulfate–sequestered MPO deposited into the vascular endothelium is causally linked to the compromised EDNO bioactivity occurring in CAD patients. Heparin as a therapy is, however, confounded by its side effects (*e.g.*, anticoagulant activity) and lack specificity; whereas heparin can mobilize MPO and xanthine oxidase from the endothelium, it also can liberate beneficial proteins such as EC-SOD (257, 292). The recent focus has turned to the development of small heparan sulfate mimetics that selectively bind specific proteins (390). This is possible, as heparan sulfates exhibit extraordinary structural diversity that allows different heparan sulfate molecules to interact with an array of functionally distinct proteins. Different proteins bind to unique structural motifs within the heparan sulfate chains, highlighting the potential for the design and synthesis of heparan sulfate mimetics capable of selectively preventing undesirable protein–heparan sulfate interactions *in vivo* (156). For example, the heparan sulfate mimetic phosphomannopentaose sulfate (PI-88) inhibits intimal thickening after arterial injury by binding FGF-2 to inhibit the growth factor's interaction with heparan sulfates expressed on smooth muscle cell (153). Novel heparan sulfate mimetics that selectively prevent the association of MPO and xanthine oxidase with the vascular endothelium may represent potential therapeutics to improve endothelial function in CAD patients.

F. Endothelial progenitor cells

Endothelial progenitor cells (EPCs) are bone marrow–derived, circulating cells that express certain markers characteristic of endothelial cells, including VEGFR2/KDR and eNOS, plus hematopoietic progenitor cell markers CD34 and CD133 (495). Compared with mature endothelial cells, EPCs possess a superior angiogenic potential *in vitro* and, on transplantation, enhanced capacity to improve the neovascularization of ischemic tissue *in vivo* (495). Interestingly, the improved function of EPCs with respect to re-endothelialization is thought to relate to the increased expression of antioxidant enzymes, notably MnSOD and Gpx-1 (112). Consistent with this, EPCs from Gpx-1–deficient mice exhibit reduced functional capacity compared with wild-type cells (161), whereas overexpression of MnSOD enhances the functional capacity of EPCs (158, 207). Similarly, extracellular SOD represents an essential factor for EPC function during reparative neovascularization induced by hindlimb ischemia (264).

Recent data indicate that insufficient numbers of circulating EPCs correlate with both endothelial dysfunction (220) and adverse clinical outcome (535). Also, EPCs isolated from diabetic, CAD, or hypertensive patients exhibit decreased capacity for *in vivo* re-endothelialization (495) or enhanced *in vitro* senescence compared with EPCs from healthy subjects (220, 242). These findings support the contention that endothelial injury and dysfunction represent a critical step for the initiation and progression of atherosclerosis. Therefore, improving EPC numbers and/or function may result in improved re-endothelialization and hence endothelial function in patients with vascular complications.

Similar to that in mature endothelial cells, EPC function and mobilization relies on competent eNOS activity and EDNO bioactivity (10). Recent studies have shown that oxidant stress and the resulting impairment of EDNO bioactivity holds detrimental consequences for the re-endothelialization capacity of EPCs. EPCs from diabetic patients produce increased levels of $O_2^{\bullet -}$ derived from NADPH oxidase, and treatment with a PPAR-γ agonist inhibited ROS production and improved the re-endothelialization capacity of these cells (448). Also, EPCs isolated from diabetics produced increased $O_2^{\bullet -}$ from uncoupled eNOS. Parallel *in vitro* studies showed that exposure of EPCs to diabetic glucose

concentrations promoted eNOS uncoupling in a manner dependent on the activation of PKC and reduction of BH_4 (484). Exposure of EPCs to pathophysiologic concentrations of C-reactive protein impairs antioxidant enzyme ability to promote oxidative stress and apoptosis (158). Therefore, treatments that inhibit EPC oxidative stress and enhance EDNO bioactivity in these cells may prove beneficial with respect to improving the regenerative potential of these cells and hence endothelial function in patients with vascular complications. Indeed, HMG-CoA reductase inhibitors improve EPC numbers and functionality in CAD patients *via* mechanisms involving Akt-dependent activation of eNOS and NO production (325, 450, 502). Similarly, the beneficial actions of statins on EPC mobilization resulting in the improvement of cardiac function and myocardial neovascularization are dependent on eNOS (290). The renin–angiotensin system also has implications for EPC function. Thus, angiotensin promotes EPC oxidative stress and senescence (241), and angiotensin-receptor antagonists improve EPC function in type-2 diabetics (23).

G. Activators of oxidized/heme-free sGC

Recent studies by Stasch and colleagues (453) highlight the therapeutic potential of novel drugs capable of activating NO-insensitive, oxidized, or heme-free sGC, the levels of which are increased in diseased vessels (453). These studies indicate that BAY 58-2667 has the ability to potently bind and activate oxidized and heme-free sGC to activate the enzyme selectively and thereby promote vasodilatation of diseased blood vessels (Fig. 11). These findings support the notion that accumulation of oxidized, heme-free, and NO-insensitive sGC contributes to endothelial dysfunction in diseased blood vessels and that novel compounds such as BAY 58-2667 have therapeutic potential in the treatment of endothelial dysfunction in human patients (142).

VII. Conclusions

From the evidence outlined in this review, it is clear that redox reactions represent important determinants of EDNO bioactivity and hence endothelial function and dysfunction in the setting of vascular homeostasis and disease. With respect to disease, considerable support now indicates that disturbances in intracellular redox potentials play a central role in the impairment of EDNO bioactivity that has prognostic implications for cardiovascular disease patients. Initial reports identified increased $O_2^{\bullet -}$ production and direct scavenging of EDNO as a primary mechanism for oxidative stress–induced endothelial dysfunction. However, more recent findings highlight the complexity with which oxidative reactions reduce the bioavailability of EDNO. Thus, an array of stimuli can activate multiple enzymes that reside in different intracellular compartments that contribute to endothelial $O_2^{\bullet -}$ production. These different enzymes can cross-talk with each other to potentiate endothelial $O_2^{\bullet -}$ generation. Moreover, $O_2^{\bullet -}$ production leads to the formation of a variety of ROS and RNS capable of affecting the bioactivity of EDNO *via* different redox reactions and involving a variety of protein targets. Adding to this complexity is that ROS and RNS represent important physiologic signaling molecules, and as such, neither global removal nor altering the overall cellular redox status appears appropriate. Such complexity therefore highlights the need for studies that accurately define the cellular sources and nature of the oxidative reactions primarily responsible for impaired EDNO bioactivity, which will likely vary with the type and stage of vascular disease. Critical for these studies will be the use of selective and sensitive methods to measure accurately the cellular sources, concentrations, and identity of ROS and RNS involved. A better understanding of the oxidative pathways involved should aid in the effective design of therapeutic strategies aimed at improving EDNO bioactivity and potentially reducing clinical cardiovascular events. Studies to date support that overstimulation of local endothelial ROS production from one cellular source (*e.g.*, NADPH oxidase) acts to initiate and expand the uncontrolled production of pathogenic ROS from other dysfunctional cellular sources (*e.g.*, xanthine oxidase, mitochondria, uncoupled eNOS). Multiple interventions may be required to combat endothelial dysfunction fully. Of note, recent support suggests that some of the beneficial actions of agents currently in clinical use relates to their ability to intervene with vascular ROS production, leading to improved endothelial function. A key goal of future research will be to determine the extent to which current and novel drugs aimed at restoring endothelial function in human patients with vascular complications provide benefits with respect to reducing clinical cardiovascular event rates.

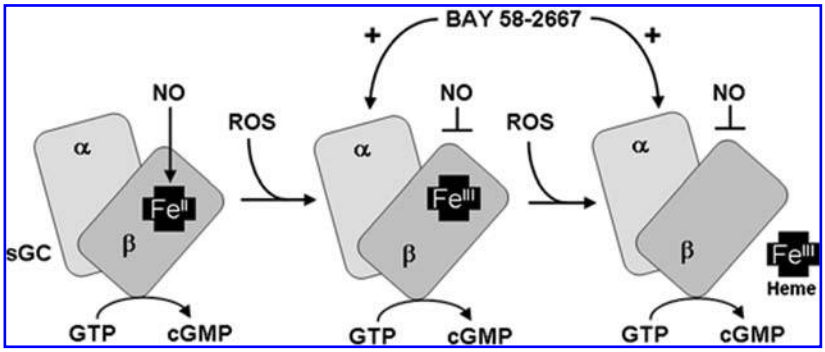

FIG. 11. BAY 58-2667 represents a novel agent capable of activating oxidized or heme-free sGC that is unresponsive to NO and present in diseased arteries. In healthy arteries, eNOS-derived NO diffuses from the endothelial cell into the adjacent smooth muscle and binds to the reduced/ferrous (Fe^{II}) heme prosthetic group of the β-subunit of soluble guanylate cyclase (sGC) heterodimer, activating the enzyme, which then converts GTP to cGMP leading to vasodilation. Endothelial dysfunction associated with oxidative stress involves the ROS-induced accumulation of oxidized/ferric (Fe^{III}) heme or heme-free sGC, an enzyme form that is unresponsive to NO. BAY 58-2667 represents a novel compound capable of binding to and activating oxidized and heme-free sGC to induce the formation of cGMP and hence restore vasodilation. Such agents may assist in the treatment of endothelial dysfunction in patients with vascular disease.

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Abbreviations

ACE, angiotensin-converting enzyme; ADMA, asymmetric-dimethyl arginine; ASK1, apoptosis signal-related kinase-1; ARE, antioxidant response element; BAEC, bovine aortic endothelial cell; BH_2, dihydrobiopterin; BH_4, (6R)-5,6,7,8-tetrahydrobiopterin; CAD, coronary artery disease; EC-SOD, extracellular superoxide dismutase; EDHF, endothelium-derived hyperpolarizing factor; EDNO, endothelium-derived nitric oxide; EGF, epidermal growth factor; EPC, endothelial progenitor cell; GTPCH, GTP cyclohydrolase; eNOS, endothelial isoform of nitric oxide synthase; Gpx, glutathione peroxidase; Grx, glutathione reductase; HDL, high-density lipoprotein; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; LNO_2, nitroalkene derivative of linoleic acid; MAEC, mouse aortic endothelial cell; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; NF-κB, nuclear factor κB; NO, nitric oxide; Nox, NADPH oxidase isoform; Nrf2, nuclear redox factor 2; $O_2^{\bullet -}$, superoxide anion radical; $ONOO^-$, peroxynitrite; PDGF, platelet-derived growth factor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PPAR, peroxisome proliferator–activated receptors; Prx, peroxiredoxin; PTP, protein tyrosine phosphatase; redox, reduction and oxidation; Ref-1, redox factor-1; ROS, reactive oxygen species; RNS, reactive nitrogen species; sGC, soluble guanylate cyclase; SMC, smooth muscle cell; SOD, superoxide dismutase; Trx, thioredoxin; VEGF, vascular endothelial growth factor.

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