Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology

Jian-Mei Li and Ajay M Shah
Department of Cardiology, Guy’s King’s and St Thomas’s School of Medicine, King’s College London, London SE5 9PJ, United Kingdom

Endothelial cells generate reactive oxygen species (ROS), including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), NO, peroxynitrite (ONOO$^{-}$), hydroxyl radicals ($\cdot$OH), and other radicals (29). The physiological functions of endothelial NO are well recognized, have been comprehensively reviewed (18, 138), and will not be considered in detail herein, except inasmuch as they are affected by reaction with other ROS (notably O$_2^-$). More recently, it has become clear that ROS such as O$_2^-$ and H$_2$O$_2$ also have several potentially important effects on endothelial function and phenotype and are implicated both in physiological regulation and disease pathophysiology. O$_2^-$ production usually involves a one-electron reduction of molecular O$_2$. The negatively charged O$_2^-$ radical is unstable in aqueous solution (half-life of a few seconds) and is rapidly dismutated to H$_2$O$_2$. It is poorly cell membrane permeable and is generally restricted to the cell compartment in which it is produced. It can undergo several chemical reactions depending on the amount generated and the localization and proximity to other radicals and enzymes. O$_2^-$ reacts rather
poorly with itself to produce H₂O₂ and O₂ (rate constant 8 × 10⁹ mol⁻¹·l⁻¹·s⁻¹), but this reaction is substantially accelerated (rate constant ∼2 × 10⁸ mol⁻¹·l⁻¹·s⁻¹) by superoxide dismutase (SOD). Thus, when O₂⁻ production is relatively low (picomolar range), most biological effects are likely to be secondary to H₂O₂ production. Indeed, H₂O₂ is more stable and diffusible than O₂⁻ and is more cell membrane permeable and may therefore be more relevant than O₂⁻ with respect to modulation of signal transduction pathways. O₂⁻ reacts with NO at a significantly faster rate than with SOD (rate constant ∼7 × 10⁹ mol⁻¹·l⁻¹·s⁻¹), so that when levels of NO are in the high nanomolar range, NO may outcompete SOD and react with O₂⁻ to generate ONOO⁻, this reaction also resulting in NO inactivation. When O₂⁻ levels are higher still, it can react with iron-sulfur centers in many proteins and can also release iron, which reacts with H₂O₂ to produce the highly reactive ·OH radical; these last reactions are often involved in oxidative stress-associated tissue injury. In summary, O₂⁻ may 1) serve as a precursor for other ROS such as H₂O₂ and thereby act as a regulatory mediator in signaling processes leading to altered gene transcription and protein and enzyme activities (so-called “redox signaling”); 2) rapidly inactivate NO, thereby causing endothelial dysfunction; 3) cause oxidative damage of macromolecules, membranes, and DNA usually indirectly through the generation of more toxic (reactive) radicals such as ONOO⁻ and ·OH. Redox signaling secondary to tightly regulated ROS production by specific enzymes and the ROS-dependent inactivation of NO are fundamentally important mechanisms in the pathogenesis of several cardiovascular disorders. In this article, we review current knowledge regarding the sources of ROS generation in endothelial cells, their regulation and involvement in redox signaling, and the relevance of enhanced endothelial ROS generation and redox signaling for cardiovascular pathophysiology.

ENDOTHELIAL SOURCES OF O₂⁻.

Potential sources of endothelial O₂⁻ generation that are implicated in disease processes include mitochondria, xanthine oxidase (XO), uncoupled NO synthases, cytochrome P-450 enzymes, and NADPH oxidases. In addition, enzymes such as lipoxygenases may also generate O₂⁻ (29).

Mitochondria. The mitochondrial respiratory chain can be a major source of O₂⁻, which may then be converted to H₂O₂. During aerobic metabolism, the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP via a multicomponent NADH dehydrogenase complex (92). Molecular O₂ serves as the final electron acceptor for cytochrome (Cyt)-c oxidase (complex IV), the terminal component of the respiratory chain, and is ultimately reduced to H₂O. Up to 1–4% of O₂ may be incompletely reduced, resulting in O₂⁻ formation, mainly at complex I (NADH coenzyme Q reductase) and complex III (ubiquinol Cyt c reductase). In the presence of transition metal ions, ·OH radicals may also be formed (92).

Increased mitochondrial O₂⁻ generation in endothelial cells appears to be particularly prominent in situations of metabolic perturbation. For example, hyperglycemia induces mitochondrial O₂⁻ production, which has been shown to contribute to the activation of the hexosamine pathway and be involved in the pathogenesis of diabetic complications (31). Similarly, the adipokine leptin (which is involved in the regulation of body adiposity and weight) induces mitochondrial O₂⁻ production in cultured bovine aortic endothelial cells by increasing fatty acid oxidation via protein kinase A (171). Other settings in which mitochondrial-derived O₂⁻ radicals are increased include hypoxia-reoxygenation and ischemia-reperfusion, where the enhanced O₂⁻ is at least partially responsible for an increase in endothelial permeability (127).

XO. Xanthine oxidoreductase (XOR) is a ubiquitous metalloflavoprotein found as one of two interchangeable yet functionally distinct forms, namely xanthine dehydrogenase (XD), which is constitutively expressed in vivo, and XO, which is generated by the posttranslational modification of XD (114). Functionally, both XD and XO catalyze oxidation of hypoxanthine to xanthine and xanthine to urate (114). However, whereas XD requires NAD⁺ as an electron acceptor, XO instead requires the reduction of molecular O₂, thereby generating O₂⁻. The conversion of XD to XO occurs either through reversible thiol oxidation of sulphydryl residues on XD or via irreversible proteolytic cleavage of a segment of XD during hypoxia, ischemia, or in the presence of various proinflammatory mediators [e.g., tumor necrosis factor-α (TNF-α)] (114, 145). Of note, the former pathway provides a mechanism whereby XO activity may increase further in response to oxidative stress. Interestingly, XO may exist in a molybdenum-deficient form, in which state it is unable to use xanthine as a substrate but can nevertheless generate O₂⁻ at the expense of NADH. This state is relevant experimentally, as in this case O₂⁻ generation is not inhibited by XO inhibitors such as oxypurinol but inhibited by the flavoprotein inhibitor diphenylpyridine (DPI) (175).

XOR is expressed at high levels on the luminal surface of the endothelium of many organs including the human heart. Its expression may be transcriptionally upregulated by cytokines such as interferon-γ, although XO activity appears to be regulated mainly through the posttranslational pathways described earlier (18, 114). An area of controversy has been the apparent paradox that XO-mediated O₂⁻ production (usually assessed by inhibition by allopurinol or oxypurinol) can be documented in several pathophysiological conditions in organs where there is apparently very low or undetectable constitutive XOR activity. There are several possible explanations for this finding: 1) endothelial XO expression may be diluted and underestimated in assays of whole organs, 2) oxypurinol and allopurinol can directly scavenge ROS in some settings (i.e., their effects would not be specific for XO), and 3) it appears that XO produced in XOR-rich organs can be mobilized into the systemic circulation and then bind to endothelial cells at distant sites in a heparin-reversible manner (114).

A large body of evidence supports an important role for XO-mediated ROS generation in tissue injury during reperfusion, although there may be a relatively narrow window in which this can be therapeutically targeted (102). Cell culture studies using the ROS-generating XO system indicate that H₂O₂ may be the key promoter of tissue injury in this setting (83). Secondary to XO activation, the local accumulation and activation of neutrophils may also significantly enhance local ROS production (e.g., via neutrophil NADPH oxidase and via myeloperoxidase-mediated ROS generation). From a physiological perspective, endothelial XO-mediated ROS generation may serve as a mechanism that recruits and activates neutro-
philms as part of the microvascular inflammatory response to pathogens (114). Indeed, XO is activated by proinflammatory mediators such as TNF-α, interleukins, complement 5α, and lipopolysaccharide. Increased $O_2^\cdot$ production from XO has also been found both experimentally and clinically in several disease settings and may contribute to the genesis of vascular endothelial dysfunction as well as redox signaling, leading to altered gene/protein expression (see INVOLVEMENT OF INCREASED ENDOThelial $O_2^\cdot$ generation in cardiovascular disease).

Cytochrome P-450. Cytochrome P-450 (CYP) enzymes have traditionally been recognized as heme-containing hepatic endoplasmic reticular flavoenzymes that can oxidize, peroxidize, and/or reduce cholesterol, vitamins, steroids, and many other compounds in an oxygen- and NADPH-dependent manner (40). More recently, it has become clear that specific CYP enzymes are also expressed in extrahepatic tissues, including the cardiovascular system. CYP enzymes that metabolize arachidonic acid (namely the CYP2 and CYP4A gene families) are implicated in vascular regulation through the generation of vasodilator metabolites, vasoconstrictors, and ROS (40). For example, the compound 20-HETE generated by CYP4A in vascular smooth muscle cells is implicated in the myogenic response. Fleming and colleagues (39, 40) showed that CYP2 epoxygenases expressed on endothelial cells (notably CYP2C8/9) generate epoxygenosatrienoic acids (EETs) that account for the NO- and prostacyclin-independent endothelium-dependent hyperpolarizing factor (EDHF) activity responsible for vasodilatation in several vascular beds, including the heart and kidney (39, 40). Like all CYP enzymes, CYP2C enzymes are inhibited by NO, leading to suggestions that CYP2C-mediated vasodilator activity is only of significance in settings where NO bioavailability is reduced.

Recently, it has been appreciated that vascular CYP enzymes can also generate $O_2^\cdot$, $H_2O_2$, and $-OH$ during the CYP reaction cycle when the electrons for the reduction of the central heme iron are transferred to the activated bound $O_2$ molecule in a NADPH-dependent reaction (40). Thus the CYP2C involved in the EDHF response in porcine coronary arteries was identified as a significant source of ROS in cultured and native endothelial cells (41). Because CYP2C enzymes can generate both EDHF (a vasodilator) and ROS (which are potentially vasoconstrictor through inactivation of NO or vasodilator through conversion to $H_2O_2$), the effects of altered CYP2C activity may be quite complex. In endothelial cells, CYP2C2-derived $O_2^\cdot$-impaired NO-dependent vascular relaxation and elevated redox-sensitive nuclear factor (NF)-κB activity and VCAM-1 expression (41). Likewise, TNF-α-induced endothelial cell adhesion molecule expression was attenuated by several CYP inhibitors, e.g., ketoconazole (135). In humans with coronary artery disease, however, CYP2C9 inhibition with sulphenazole improved endothelium-dependent, NO-mediated vasodilatation, probably by reducing ROS production (37).

Endothelial CYP activity and expression are stimulated by cyclic stretch, hormonal stimuli, and HMG-CoA reductase inhibitors (statins) (39), leading to both increased ROS and increased EDHF. Pathological conditions in which CYP expression has been reported to increase include hypertension and hypercholesterolemia. In the case of statins, which also increase endothelial NO synthase (eNOS) expression, it is feasible that there is increased potential to generate $OONO\cdot$ (from reaction of NO and $O_2^\cdot$). On the other hand, oxidized LDL reduces endothelial CYP2 family expression via ROS probably generated by NADPH oxidase(s) and acting through reduced expression of the transcriptional regulator NF-1 (151).

Dysfunctional or uncoupled NOSs. eNOS is a calcium-dependent flavoenzyme that generates NO in a process that involves the oxidation of the amino acid $l$-arginine by the reduction of molecular $O_2$ (18). NOSs are complex homodimeric oxido-reductases that shuttle electrons from the reductase domain of one monomer (a CYP-like region containing the cofactors FAD, FMN, and NADPH) to the oxidase domain in the other subunit that contains the heme active site. In view of this enzymatic structure, it is not surprising that NOS can become “uncoupled,” leading to the generation of $O_2^\cdot$ rather than NO. The essential NOS cofactor tetrahydrobiopterin (BH4) appears to have a key role in regulating NOS function by “coupling” the reduction of molecular $O_2$ to $l$-arginine oxidation as well as maintaining the stability of NOS dimers (152, 158). Thus BH4 availability may be a crucial factor in the balance between NO and $O_2^\cdot$ generation by eNOS. Furthermore, BH4 itself is highly susceptible to oxidative degradation, and the initial oxidative loss of BH4 in response to increased ROS production by NADPH oxidases has been shown to amplify oxidative stress through the resulting loss of NO production and increased NOS-dependet $O_2^\cdot$ generation (86). Likewise, peroxynitrite (a product of the reaction between NO and $O_2^\cdot$) may also oxidize BH4 and represent another pathogenic cause of eNOS uncoupling (91). In addition to increased catabolism or degradation, another reason for BH4 depletion may be its reduced synthesis. Biosynthesis of BH4 occurs either via a de novo pathway in which GTP cyclohydrolase I is a rate-limiting step or via a so-called salvage pathway that uses sepiapterin as an intermediate step. The precise levels of BH4 in vivo at which eNOS becomes uncoupled and therefore supports $O_2^\cdot$ production remain unclear, but it is suggested that the ratio between reduced and oxidized BH4 metabolites may be a key regulator of ROS production by eNOS (4b, 158). The possible mechanisms involved have been reviewed elsewhere (158). A deficiency of the NOS substrate, $l$-arginine, can also result in $O_2^\cdot$ generation by the enzyme.

Elevated $O_2^\cdot$ production from uncoupled NOS has been implicated in the pathophysiology of several disorders such as atherosclerosis, diabetes, hypertension, and hypercholesterolemia (see INVOLVEMENT OF INCREASED ENDOThelial $O_2^\cdot$ generation in cardiovascular disease).

NADPH oxidases. In recent years, it has become apparent that endothelial cells and other nonphagocytic cells constitutively express an $O_2^\cdot$-generating enzyme analogous to the phagocyte NADPH oxidase of neutrophils (7, 12–14, 47, 50, 74, 88, 99). The prototypic neutrophil NADPH oxidase comprises a membrane-associated low-potential cytochrome b$_{558}$ composed of one p$_{22}^{\text{phox}}$ and one p$_{91}^{\text{phox}}$ subunit and several cytosolic regulatory subunits (p$_{47}^{\text{phox}}$, p$_{40}^{\text{phox}}$, p$_{67}^{\text{phox}}$ and the small G protein Rac1 or Rac2) that translocate to the membrane and associate with the cytochrome b$_{558}$ on neutrophil activation (8). The latter process rapidly activates the oxidase, which is normally dormant in resting neutrophils, to generate large amounts of $O_2^\cdot$ [of the order of 10 nmol·min$^{-1}·10^6$ cells$^{-1}$ (88)] in a process that requires...
NADPH as cofactor and is essential for nonspecific host defense.

All the classical neutrophil oxidase components are expressed in endothelial cells (12, 47, 74, 96, 97, 99), but the enzyme nevertheless exhibits several major differences from the neutrophil oxidase: 1) it continuously generates a low level of O$_2^-$ even in unstimulated cells, although its activity can be further increased by several agonists; 2) a substantial portion of the O$_2^-$ generated by the enzyme is produced intracellularly, whereas neutrophil oxidase O$_2^-$ generation occurs mainly in the extracellular compartment. Recently we reported that a substantial portion of NADPH oxidase subunit expression and functional activity in cultured endothelial cells is intracellular rather than plasma membrane bound. Furthermore, a significant proportion of the NADPH oxidase subunits in unstimulated cells is present as fully preassembled and more, a significant proportion of the NADPH oxidase subunits encoded for by separate genes and termed Noxs, have been identified in nonphagocytic cells (84, 88). To date, the Nox family comprises five members (Nox1–5), of which Nox2 is gp91phox or the neutrophil isoform. As mentioned above, Nox2 (gp91phox) is expressed in endothelial cells, and evidence of a functional role for this isoform in phorbol ester-induced O$_2^-$ generation and endothelium-dependent relaxation has been demonstrated in studies with gp91phox$^{-/-}$ mice (47). Nox2 is also expressed in cardiomyocytes, fibroblasts, and vascular smooth muscle of human resistance arteries (14, 126, 153). Significant levels of Nox4 mRNA (indeed, apparently greater than the expression of Nox2 mRNA) are also detectable in endothelial cells, and a recent study that used Nox4 antisense oligonucleotides suggested that an Nox4-dependent oxidase contributes functionally to basal O$_2^-$ generation and endothelium-dependent relaxation (3). Nevertheless, the relative roles of these two oxidases in endothelial cells remain to be fully elucidated. Recent studies have also reported the expression of homologues of p47$^{phox}$ and p67$^{phox}$, termed NOXO1 (Nox organizing protein 1; p47$^{nox}$) and NOXA1 (Nox activating protein 1, p51$^{nox}$) respectively, in colonic epithelium (9, 150), but their expression in endothelial cells has not been documented. The structure, function, and biological relevance of vascular NADPH oxidases and the potential roles of these homologues have been comprehensively reviewed by several authors (7, 17, 88, 99). The current article therefore focuses specifically on the endothelial oxidase, the detailed mechanisms of regulation of which are discussed in REGULATED ACTIVATION OF NADPH OXIDASE.

**Interactions among different ROS sources and ROS-dependent regulation.** Whereas numerous studies have focused on the importance of individual enzymatic sources of ROS generation, it is increasingly clear that there are in fact complex interactions among different ROS sources such that in many pathological settings multiple sources may be involved (Fig. 1). We consider several examples of such interactions.

Mitochondria, in addition to generating ROS, are themselves susceptible to oxidant damage, which can decrease respiratory enzyme activities and mitochondrial membrane potential and lead to greater ROS production (92). Indeed, the term ROS-induced ROS release was coined by Sollott and colleagues (179) to describe the phenomenon during induction of the mitochondrial permeability transition in cardiac myocytes. This phenomenon may apply not only to ROS initially produced within mitochondria but also to nonmitochondrial sources of ROS. Second, the oxidative conversion of XDH to XO (114) has already been mentioned as a mechanism for amplification of ROS production, where the initial ROS generation may derive from a separate source. Recently, this was reported to be an important mechanism contributing to endothelial cell O$_2^-$ production in response to oscillatory shear stress (113). The latter study also suggested that the level of XO was dependent on a functional NADPH oxidase. Third, the oxidative degradation of BH$_4$ (where again the initial ROS may derive from one of many sources) can serve to amplify ROS generation through NOS uncoupling. This latter phenomenon has been termed amplification via "kindling radicals" (91). Finally, it has also been suggested recently that mitochondrial ROS generation can lead to NADPH oxidase activation in endothelial cells (136).

A different type of interaction involves the ROS-dependent regulation of the activity of ROS-generating enzymes, i.e., feedback or feedforward regulation. For instance, Rac1-dependent endothelial NADPH oxidase activation and subsequent O$_2^-$ production mediates a feedback loop leading to increased proteasomal degradation of Rac1 (81). ROS-dependent down-regulation of CYP2C has already been mentioned above. On the other hand, Li et al. (101) reported a feedforward mechanism whereby exogenous exposure of smooth muscle cells or fibroblasts to H$_2$O$_2$ caused NADPH oxidase activation and endogenous O$_2^-$ generation, thereby amplifying the vascular injury process. Self-limiting feedback mechanisms may serve to restrict nonphagocytic NADPH oxidase activity to a low output state, whereas the positive feedforward mechanisms may be more important in pathological settings. These paradigms illustrate how small changes in ROS production may be amplified and/or modulated through interactions among different oxidase systems.

**TISSUE-RELATED DIFFERENCES IN ENDOTHELIAL O$_2^-$ GENERATION**

It is increasingly appreciated that there may be significant differences in the properties of endothelial cells of different origins, not only microvascular vs. macrovascular (78) but also tissue-related variations (23). However, potential tissue-related differences in endothelial O$_2^-$ generation have received very little attention. In principle, differences between tissues may be related either to primary alterations in ROS generation or may be secondary to variations in response to stimuli (e.g., cytokines) that provoke ROS production and/or differences in the production of such stimuli. An example of the first possibility is the finding that NADPH oxidase activity of cultured human microvascular endothelial cells is substantially higher than that of cultured human umbilical vein endothelial cells (HUVEC) studied under similar culture conditions (96). Greater ROS generation by microvascular vs. macrovascular endothelial cells may at least partly account for the greater proliferative capacity of microvascular cells in culture (e.g., 1, 78). As an example of altered responsiveness to ROS-generating stimuli,
It has been shown that the expression of endothelial chemokine receptors (CXCR) and of basal chemokine (e.g., CCL2, CCL5, CXCL10) secretion varies widely according to tissue (63). Similarly, the induction of adhesion molecules on endothelial cells, e.g., in diabetes, can be highly tissue specific (123). More detailed investigation of such differences among endothelia is warranted.

REGULATION OF REDOX HOMEOSTASIS IN ENDOTHELIAL CELLS

The effects of $O_2^-$ and other ROS generated within endothelial cells are critically dependent not only on the amount and sites of production but also on the processes that degrade or scavenge ROS. Several recent reviews have covered the regulation of cellular redox homeostasis (27, 29, 79, 128, 172) and we therefore provide only a relatively brief overview here.

Antioxidant systems influencing redox state comprise nonenzymatic molecules and specific antioxidant enzymes. Nonenzymatic antioxidant molecules in endothelial cells include uric acid, ascorbic acid (vitamin C), α-tocopherol (vitamin E), and glutathione (GSH) (27, 29, 79). The water-soluble vitamin C not only scavenges ROS but also protects vitamin E and GSH against oxidation in cell membranes. Vitamin E is a lipid-soluble, chain-breaking radical scavenger that is considered the most important antioxidant in cell membranes. It may also have nonantioxidant cell signaling functions, e.g., the inhibition of protein kinase C activity (29). GSH is the major low molecular weight thiol antioxidant buffer in endothelial cells (27), serving as a substrate for glutathione peroxidase to eliminate lipid hydroperoxides and $H_2O_2$, whereby it becomes converted to GSH disulfide (GSSG). Normally, GSSG is maintained at levels <1% of total GSH. The glutaredoxins have functions overlapping with those of thioredoxins (see below) and can reduce GSH mixed protein disulfides.

Important endothelial antioxidant enzymes include SODs, catalase, the thioredoxin system, glutathione peroxidase, and heme oxygenase. The SODs all efficiently convert $O_2^-$ to $H_2O_2$. The latter is then degraded to water by catalase or glutathione peroxidase. CuZnSOD is suggested to be the predominant SOD isoform in endothelium (18). Knockout of CuZnSOD in mice or inhibition of CuZnSOD results in enhanced vascular $O_2^-$ generation and profound endothelial dysfunction (28, 162). CuZnSOD expression is upregulated by shear stress and is related to cellular redox state (70). Mitochondrial MnSOD expression is also redox sensitive and can be induced by VEGF via the activation of NADPH oxidase (2). The ecSOD isoform is found primarily bound to heparan sulfate on the cell surface and may be especially relevant for regulating extracellular NO bioactivity. In mouse aorta, ecSOD expression was upregulated by angiotensin II (43), whereas on the other hand, mice lacking ecSOD developed significantly higher hypertension in response to angiotensin II infusion than wild-type controls (75).

Fig. 1. Schematic diagram showing sources of $O_2^-$ generation in endothelial cells. Potential stimuli for $O_2^-$ generation are shown at the top. Dotted lines show feedback effects of $O_2^-$ on xanthine oxidase (XO) or on endothelial nitric oxide synthase (eNOS). AGE, advanced glycation end-product; BH$_4$, tetrahydrobiopterin; ET-1, endothelin-1; GPCR, G protein-coupled receptor; GSH, glutathione; LPS, lipopolysaccharide; XDH, xanthine dehydrogenase.
Thioredoxin reductase together with thioredoxin and NADPH constitutes a ubiquitous oxidoreductase system with antioxidant and redox-sensitive regulatory roles that is abundantly expressed in endothelial cells (172). Thioredoxins efficiently reduce disulfides in proteins, peptides, and GSSG, as well as directly lowering ROS levels through their conserved -Cys-Gly-Pro-Cys- active site (172). Subsequently, the active site disulfide is itself reduced by thioredoxin reductase and NADPH. Thioredoxin has redox-sensitive signaling functions through several mechanisms: 1) selective stimulation of DNA-binding of NF-κB by reducing a specific cysteine residue in the p50 subunit (172); 2) increasing AP-1 binding activity via binding to the nuclear redox protein, redox factor 1 (Ref-1); 3) through binding to signaling molecules such as the MAPKK kinase ASK1, thereby inhibiting its activity—the oxidation of thioredoxin disrupts this binding and leads to increased ASK1 activity. Another protein that binds thioredoxin is vitamin D3-upregulated protein 1 (VDUP1), which may act as an endogenous inhibitor of thioredoxin. VDUP1 expression is reduced by biomechanical strain or H2O2, thereby increasing thioredoxin activity. Interestingly, thioredoxin expression is induced by oxidant stress (172).

Heme oxygenase has indirect antioxidant effects through the degradation of free heme (derived from many hemoproteins and which has potent pro-oxidant actions) as well as the subsequent generation of biliverdin and bilirubin, which have antioxidant properties (128). The constitutive heme oxygenase isoform, HO-2, is ubiquitously expressed in endothelial cells, whereas HO-1 is induced in response to stimuli such as heme, hypoxia, cytokines, oxidized LDL, angiotensin II, NO, peroxynitrite, and H2O2 (128). HO-1 expression in response to oxidative stress is a key manifestation of the induction of endogenous cellular antioxidant defense mechanisms.

Despite extensive experimental evidence for an important role of antioxidant systems in redox homeostasis and the finding that antioxidants such as vitamin C can acutely improve endothelial dysfunction related to oxidative stress (130), a clinically relevant beneficial effect of antioxidant supplementation remains to be demonstrated, perhaps suggesting that more specific and focused approaches may be required for therapeutic manipulation of these pathways (73).

**REDOX SIGNALING MECHANISMS**

The modulation of biological signaling pathways by ROS depends on both the upstream ligand-dependent stimulation of ROS production by different enzymatic sources and the specific interactions of ROS with individual downstream pathways. It is clear that ROS may modulate signaling pathways at multiple levels from membrane receptors and channels to various protein kinases and transcription factors in the nucleus. O2•− itself is relatively unstable in aqueous solution and is rapidly dismutated to H2O2 either spontaneously or by the action of SOD. Therefore, many O2•−-dependent signaling events are thought to be mediated through H2O2. On the other hand, NO is one of the few biomolecules that can outcompete SOD for O2•−; therefore, in settings where there are sufficiently high concentrations of NO present, O2•− reacts rapidly with NO in a near diffusion-limited fashion to form peroxynitrite (80). The latter has widely been considered to be a relatively nonspecific toxic species that can oxidize or nitrate a wide variety of biological targets. However, more recent studies indicate that, in vivo, peroxynitrite may interact directly (rather than via breakdown to -OH or -OH-like radicals) with specific biomolecules, notably thiols and metal-containing proteins, to modulate signaling events, especially in nonacidic environments (80, 160).

The precise molecules that are targeted by ROS and the exact biochemical reactions involved remain incompletely understood. A common mechanism involves redox-dependent covalent modification of specific cysteine residues on target proteins (38). In the case of tyrosine phosphatase, reversible oxidation of a cysteine residue leads to enzyme inactivation and a secondary increase in activity of tyrosine kinases (e.g., specific MAPKs). Alternatively, the reversible covalent addition of GSH to cysteine residues (or S-glutathiolation) may be involved in activating tyrosine kinases (79). In the case of peroxynitrite, in addition to oxidation of thiol and metal-containing protein centers, the tyrosine nitration of proteins could also be involved in signaling, either by negative interference with tyrosine phosphorylation of enzymes and/or by mimicking phosphorylation (80, 160). Redox signaling mechanisms that involve thioredoxin were discussed in a previous section, whereas the effects of ROS on the transcription factor HIF-1 are discussed in Oxygen sensing below. More detailed reviews of redox signaling mechanisms were published recently (29, 38, 71, 80, 82).

Although all the endothelial ROS sources discussed previously may potentially be involved in redox signaling, NADPH oxidases seem to be especially important in that they are the main source whose primary function appears to be to modulate redox signaling (15, 17, 50, 88, 99). In the next section, we therefore focus on the mechanisms of regulation of endothelial NADPH oxidase.

**REGULATED ACTIVATION OF NADPH OXIDASE**

A major attribute of nonphagocytic NADPH oxidases is that not only are they “constitutively” active but their activity is sensitively influenced by a wide variety of (patho)physiological stimuli. Endothelial NADPH oxidase activity is increased by 1) mechanical forces such as oscillatory shear stress (67); 2) hypoxia-reoxygenation (77, 136, 141), flow cessation (111), membrane depolarization (4, 140), nutrient deprivation (106), or ischemia (4, 111); 3) G protein-coupled receptor agonists such as angiotensin II (98, 107, 117, 176) or ET-1 (32); 4) phorbol esters, which activate protein kinase C (PKC) (47, 95); 5) growth factors such as VEGF (157); 6) cytokines such as TNF-α (42, 95) or IL-1 (52); 7) increased insulin (76), glucose (69), free fatty acids (34, 69), or advanced glycation end products (AGE) (164); and 8) oxidized LDL, lysophosphatidylcholine, and hypercholesterolemia (133, 144). Either a rapid posttranslational activation and/or the increased expression of oxidase subunits can be involved in the upregulation of O2•− production by NADPH oxidases. Although the altered expression of different oxidase components in response to various stimuli has been described in many studies [e.g., increased Nox2 and Nox4 mRNA expression with oscillatory shear stress but decreased expression with pulsatile flow (67)], the underlying mechanisms of transcriptional regulation remain as yet virtually unexplored. However, recent studies have begun to
shed light on the mechanisms underlying the acute activation of endothelial NADPH oxidase.

**Regulation by the p47^phox subunit.** Phosphorylation of p47^phox is known to be important for activation of the neutrophil NADPH oxidase, being required for the translocation of cytosolic subunits (p47^phox, p67^phox, p40^phox) to the membrane-located cytochrome b558 (8). Several studies using either tissues from gene-targeted mice lacking p47^phox or specific inhibitors have likewise shown a crucial role for p47^phox in endothelial NADPH oxidase activation by several agonists (angiotensin II, TNF-α, VEGF) (15, 42, 95, 98) and by chronic oscillatory shear (68). The p47^phox subunit is also required for angiotensin II-induced MAPK activation (100), TNF-α-induced JNK activation (53), and redox-mediated gene expression (15). The finding that p47^phox is essential for PMA-induced activation suggested a role for PKC (95), which was confirmed for TNF-α-induced activation of NADPH oxidase in lung vascular endothelial cells where the atypical PKC isoform, PKC-ζ, was found to phosphorylate p47^phox (42). Recently, we demonstrated that angiotensin II-stimulated endothelial NADPH oxidase activation induces the rapid serine phosphorylation of p47^phox (1–15 min), which is paralleled by increased p47^phox-p22^phox binding (i.e., increased complex formation) and increased O$_2^•^-$ generation with similar kinetics (98). These results suggest that while basal (constitutive) NADPH oxidase activity may be explained by the presence of preassembled oxidase complexes, enhancement of activity by agonists such as angiotensin II requires the formation of additional complexes. With respect to TNF-α-induced signaling, it has been reported that p47^phox associates with the TNF receptor-associated factor TRAF4, which links to downstream activation of JNK (45, 170). The binding of p47^phox to TRAF4 may therefore serve as a means to localize the ROS signal to proteins/enzymes associated with TRAF4. Whether similar protein-protein interactions involving p47^phox are used as a strategy for the spatial confinement of ROS-mediated signals generated by other agonists remains to be studied.

Studies from our group that have investigated coronary microvascular EC (CMEC) and aortae isolated from p47^phox$^{-/-}$ mice or the effects of acute depletion of p47^phox by antisense cDNA transfection suggest a more complex role for p47^phox (95, 98, 100). Surprisingly, we found that neither the chronic absence of p47^phox in knockout cells nor its acute depletion in wild-type cells resulted in a reduction in basal NADPH-dependent ROS production. On the contrary, basal ROS production was slightly but significantly higher compared with wild-type cells or aortae. Consistent with this finding, p47^phox$^{-/-}$ aortic rings had mild endothelial dysfunction and increased basal activation of ERK1/2, which were normalized by exposure to ROS scavengers (100). Nevertheless, the acute oxidative response to PMA, TNF-α, or angiotensin II was abolished in p47^phox$^{-/-}$ cells (95, 98), whereas in aortic rings angiotensin II-induced endothelial dysfunction was also abrogated (100). These results suggest that p47^phox may have a dual role in regulating endothelial NADPH oxidase activity whereby it inhibits basal, constitutive O$_2^•^-$ generation but is nevertheless essential for agonist-induced increases in ROS production. A possible explanation for these effects might be that unphosphorylated p47^phox is inhibitory when bound to the cytochrome b558 in endothelial cells, whereas phosphorylation leads to oxidative activation.

**Regulation by Rac1.** The other NADPH oxidase regulatory subunit that has been well studied in endothelial cells is the GTPase Rac1, a member of the Rho (Ras homology) family of small (20–40 kDa) GTP-binding proteins that undergo regulation through GTP binding and hydrolysis (49). Rac activity also requires a carboxy terminal geranylgeranyl moiety, which is added by posttranslational modification (isoprenylation) by geranylgeranyl transferase and is required for localization of Rac to the membrane. Geranylgeranyl groups are derived, like cholesterol, from the mevalonate pathway, and the synthesis of both these products is inhibited by HMG-CoA reductase inhibitors (statins). Thus some of the pleiotropic effects of statins may be mediated through inhibition of Rac translocation. Rac in its GTP-bound state is thought to bind to p67^phox and activate NADPH oxidase (49). Rac1 can activate NADPH oxidase in the complete absence of p47^phox in cell-free systems, but both are probably required for optimal oxidase activation (48). In endothelial cells, PMA-induced O$_2^•^-$ production [which is p47^phox dependent (95)] is reportedly inhibited by statins in a mevalonate-dependent manner (161), suggesting that while Rac1 and p47^phox are required for this response. Basal NADPH oxidase-dependent ROS production requires Rac1 as it is inhibited by a dominant negative Rac1 mutant (66). Similarly, statin withdrawal after chronic treatment in animals stimulates endothelial O$_2^•^-$ generation through Rac1-dependent activation of NADPH oxidase, suggesting that basal NADPH oxidase activity is modulated by statins through altered Rac translocation (159). NADPH oxidase-dependent ROS production stimulated by shear stress is also inhibited by dominant negative Rac1 (173), and Rac1-dependent ROS production mediates shear stress-induced endothelial cell tyrosine phosphorylation (173) and the surface expression of ICAM-1 (156). Rac1 appears important in the oxidative response to hypoxia-reoxygenation or ischemia-reperfusion. Thus Rac1 mediates oxidant production in response to hypoxia-reoxygenation in several cell types including endothelial cells (77). Likewise, in HUVEC, depolarization-induced NADPH oxidase activation (which may be relevant to ischemia) required Rac translocation (140). Rac1 is also required for oxidant-dependent expression of MCP-1 induced by nutrient deprivation (106). Another setting in which Rac1-dependent ROS production is implicated is in endothelial cell growth and survival. VEGF-induced endothelial cell migration and proliferation required Rac1-regulated O$_2^•^-$ generation (157), whereas the overexpression of a constitutively active mutant of Rac1 resulted in endothelial cell proliferation (105). In HUVEC, Rac1-dependent O$_2^•^-$ production led to protection against TNF-α-induced apoptosis (26).

**Physiological roles of endothelial O$_2^•^-$.**

Whereas O$_2^•^-$ production is implicated in many pathological processes (see later), an important question is whether ROS generation has any physiological roles. A physiological role for ROS would provide at least a teleological explanation for the widespread occurrence of ROS even in health (Fig. 2). In fact, there are several likely physiological roles for endothelial ROS, all of which involve the use of oxygen species to transmit biological information in some way.
**Oxygen sensing.** Many types of acute and chronic O₂-sensitive processes are involved in cardiorespiratory homeostasis, e.g., an acute increase in ventilation or a chronic increase in erythropoietin production in response to hypoxia (16). Likewise, O₂-sensitive alterations in endothelial function are essential for vascular homeostasis. For example, in the coronary circulation, a modest decrease in arterial Po₂ evokes a rapid increase in endothelial production of NO and vasodilator prostanooids that serve to increase blood flow and thus O₂ supply (125). Chronic hypoxia also evokes several adaptive changes in endothelial gene expression.

A substantial body of evidence implicates ROS-producing proteins in the acute sensing of changes in ambient O₂ concentration in different cell types (16). In the carotid body, an ROS-generating cytochrome similar to the cytochrome b₅₅₈ of NADPH oxidase may be involved in sensing modest hypoxia, whereas in other settings alterations in mitochondrial ROS generation are implicated. It is likely that ROS may be similarly involved in the endothelium.

Chronic changes in cellular function in many tissues, including the endothelium, involve redox-sensitive activation of the transcription factor hypoxia-inducible factor-1 (HIF-1), which may increase the expression of genes involved in angiogenesis, energy metabolism, cell proliferation, and vascular remodeling (16). The major physiological importance of these systems is attested to by the finding, for example, that gene-targeted deficiency of HIF-1 results in embryonic lethality (134). Recent studies have begun to delineate the ways in which ROS may regulate gene transcription through HIF-1 (29, 167). The HIF-1 heterodimer comprises an HIF-1α and an HIF-1β subunit. Whereas the protein level of HIF-1β is not much affected by changes in Po₂, HIF-1α undergoes rapid proteosomal degradation through its prolyl hydroxylation by specific prolyl hydroxylase enzymes. The redox regulation of HIF-1 activity appears to be mediated largely through ROS-dependent changes in HIF-1α stability as well as posttranslational regulation of HIF-1 activity. However, the precise mechanisms through which the latter regulation occurs remain unclear, with evidence for regulation through activation of the phosphatidylinositol 3-kinase/Akt pathway or through a thiol-sensitive mechanism (167).

**Regulation of vascular tone.** ROS generation may be important in the physiological regulation of vascular tone in at least two ways, first via interactions with NO and second through the direct effects of H₂O₂. It is well established that endothelium-derived NO undergoes a very rapid reaction with O₂⁻ that results in inactivation of NO (18). A fundamental aspect of the regulation of vascular tone and blood flow by NO is its rapid sensitivity to alterations in local stimuli (such as increases in shear stress) and the dependence on appropriate local vasodilator actions to achieve integrated increases and/or redistribution of blood flow among specific vascular beds. The physiological local generation of O₂⁻ is quite likely to be important in the spatial restriction of the actions of NO, together with other molecules such as hemoglobin (119). In this regard, it is of interest that increased vascular flow is a potent stimulus for the release of both O₂⁻ and NO (89). Local SOD activity (in particular edSOD) may also play an important role in regulating the NO/O₂⁻ balance.

Recent studies indicate that H₂O₂ released from the endothelium (after conversion from O₂⁻) may account for EDHF activity in murine and human mesenteric arteries and human coronary arterioles, where it is involved in flow-induced dilation (112, 115, 118). Endothelial CuZnSOD plays a pivotal role in converting O₂⁻ (generated probably mainly by NO synthase) to H₂O₂, to the extent that it was proposed to act as an EDHF synthase. These studies suggest that H₂O₂ production contributes to the physiological regulation of vascular tone in certain vascular beds.

**Other functions.** ROS can have potent effects on endothelial cell growth, migration, proliferation, and survival (see later), which have been studied mainly in pathological settings. However, it is quite conceivable that such effects may also serve important physiological functions, e.g., during development or reparative processes. Likewise, the effects of ROS on cell adhesion discussed in the next section may be physiologically relevant.
relevant in the context of the microvascular inflammatory response to pathogens.

ENDOTHELIAL CELL ACTIVATION AND INFLAMMATION

Inflammation describes the stereotyped response of vascularized tissues to injury and is a major part of innate immunity as well as being involved in the adaptive immune response. It involves the microvasculature, principally postcapillary venules, which are the main sites of vascular leak and leukocyte extravasation. The recruitment and adhesion of leukocytes to endothelial cells and their subsequent emigration from the blood across the endothelium and into the affected tissue is an early step in inflammation, which requires the regulated expression of cell-surface adhesion molecules and other proteins on one or both types of cell (i.e., cell “activation”) (46). Adhesion molecules either tether the two cells together and/or act as signals that induce changes in endothelial cell (and leukocyte) structure and function, e.g., raised intracellular Ca$^{2+}$, ROS production, cytoskeletal rearrangement. Among the adhesion molecules expressed on activated endothelial cells, ICAM-1, VCAM-1, endothelial leukocyte adhesion molecule-1 (ELAM-1 or E-selectin), and P-selectin (CD62) have been well studied. Very little ICAM-1 is normally expressed on endothelial cells but it is greatly increased by inflammatory stimuli such as lipopolysaccharide and cytokines (interleukin-1, TNF-α, interferon-γ) over a time course of hours (46). P-selectin is rapidly translocated to the cell membrane in activated endothelial cells in minutes, whereas E-selectin is newly synthesized and appears after 4–6 h of activation (46). Oscillatory shear is also a potent stimulus for the surface expression of ICAM-1, VCAM-1, and E-selectin in endothelial cells (20).

Substantial experimental data indicate that ROS are potential regulators of endothelial cell adhesion molecule expression and inflammatory microvascular dysfunction (46), for example during sepsis (129). Cytokines such as TNF-α increase VCAM-1 and chemoattractant protein-1 (MCP-1) expression through a redox-sensitive mechanism involving NF-κB, which is inhibited by antioxidants or the NADPH oxidase inhibitor apocynin (155, 165). The role of NADPH oxidase and O$_2^·$ in this process was highlighted by a study using adenosine-mediated overexpression of dominant negative Rac1 and SOD, both of which suppressed TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression, through NF-κB inhibition (22). TNF-α-induced rapid upregulation of endothelial P-selectin was also found to depend on O$_2^·$ generation from the synergistic effects of NADPH oxidase and XO (149). However, it should be noted that a significant component of the ROS released in response to TNF-α may also emaminate from neutrophil NADPH oxidase and then induce ICAM-1 expression in microvascular endothelial cells (35).

Endothelial adhesion molecule expression may also be triggered by hypercholesterolemia, ischemia-reperfusion, AGEs, and the renin-angiotensin system. Induction of VCAM-1 by AGE or angiotensin II involves NADPH oxidase and NF-κB activation (131, 164). Hypercholesterolemia-induced leukocyte-EC adhesion and leukocyte emigration also involve NADPH oxidase. Stokes et al. (144) reported that both these processes were significantly attenuated in p47$^{phox−/−}$ mice, an effect that was attributed to reduced O$_2^·$ production by both the endothelium and white cells on the basis of studies using bone marrow chimeras. Likewise, P-selectin-dependent adhesion of platelets and leukocytes in the cerebral microcirculation was blunted in hypercholesterolemic gp91$^{phox−/−}$ mice (72). In the context of microvascular dysfunction induced by ischemiareperfusion, XO-derived ROS appear to contribute to an increase in endothelial permeability (114).

ENDOTHELIAL CELL GROWTH, MIGRATION, AND APOPTOSIS

Endothelial cell proliferation, migration, and organization into tubular network structures are critical steps in angiogenesis. Endothelial cell growth and survival are dependent on several factors, including the presence of specific growth factors and cellular interactions with the extracellular matrix. In vitro, growth factor deprivation leads to apoptosis, whereas cell detachment leads to a special form of programmed cell death called anoikis.

Recent studies indicate that low-level regulated generation of ROS is necessary for the processes involved in both angiogenesis and endothelial cell survival. Thus several growth factor receptors are coupled to intracellular production of O$_2^·$ and H$_2$O$_2$ (1, 157). For example, VEGF-induced endothelial cell proliferation and migration was shown to be dependent on O$_2^·$ generation from NADPH oxidase, in that the mitogenic and chemotactic effects of VEGF were abrogated by three structurally unrelated NADPH oxidase inhibitors (1). Ushio-Fukai et al. (157) showed more specifically that VEGF-induced endothelial cell proliferation, migration, and angiogenesis were inhibited by dominant negative Rac1 or antisense gp91$^{phox}$ oligonucleotides, which reduced VEGF-induced O$_2^·$ production. Furthermore, in this study, VEGF-induced angiogenesis in an in vivo sponge implant assay was significantly attenuated in gp91$^{phox−/−}$ mice. Other stimuli that induce endothelial cell migration and/or proliferation, such as OxLDL or angiotensin II, also appear to signal these effects via NADPH oxidase-derived ROS (133). Endothelial proliferation induced by shear stress and coronary collateral vessel development are also ROS dependent (51). Hypoxia induces endothelial cell proliferation independent of paracrine effectors, and recently this was shown to be redox sensitive and to involve ROS production by both mitochondria and NADPH oxidase (136). In a perhaps more pathophysiologically relevant model, ischemia-induced neovascularization in the mouse hindlimb was found to be significantly inhibited in gp91$^{phox−/−}$ mice compared with wild type (61).

The migratory behavior of endothelial cells either after injury or during angiogenesis requires significant reorganization of the actin cytoskeleton, a process that appears to require O$_2^·$ generation. In an endothelial monolayer-wounding assay, ROS production in response to the loss of endothelial confluence was required for the actin cytoskeleton reorganization necessary for endothelial migration and regeneration (116). Likewise, Rac1 is reported to be required for shear stress-induced endothelial cell polarization, which is an important component of the migratory response (168). In the context of hypoxia-reoxygenation, it was reported that the reoxygenation of previously hypoxic endothelial cells induced a burst of O$_2^·$ that was necessary for translocation of actin filaments to the submembranous network and cytoskeletal reorganization (25).
This phenomenon was suggested to be of relevance in priming endothelial cells for angiogenesis (25).

Apoptosis (programmed cell death) may be considered as a mechanism that counterbalances the effects of cell proliferation. An increase in intracellular ROS production is often observed in apoptotic processes triggered by various stimuli. O$_2^\cdot$ generated from NADPH oxidase may play a dual role in influencing both endothelial survival and death. For example, Rac1-dependent ROS generation appears to protect endothelial cells against TNF-α-induced apoptosis (26). On the other hand, oxidized LDL or high glucose-induced NADPH oxidase activation promoted endothelial cell apoptosis (44). Likewise, anoikis resulting from detachment of endothelial cells from the extracellular matrix involved a significant rise in the intracellular ROS level (93). The mechanisms by which endothelial cells die or survive under oxidant stress remain unclear, although the downstream activation of JNK is implicated in H$_2$O$_2$ and other stress-induced apoptosis, whereas ERK activation is implicated in VEGF-induced endothelial cell survival (71). Mitochondrial-derived ROS play a central role in endothelial cell apoptosis (19, 145). The loss of cytochrome c into the cytoplasm and opening of the mitochondrial permeability transition pore are important events in the apoptotic cascade. Loss of cytochrome c leads to increased ROS generation, which may activate the mitochondrial permeability transition. Interestingly, the deficiency of mitochondrial cytochrome-c oxidase has recently also been linked causally to increased O$_2^\cdot$ generation during endothelial senescence (169).

**INVOLVEMENT OF INCREASED ENDOTHELIAL O$_2^\cdot$ GENERATION IN CARDIOVASCULAR DISEASE**

**Endothelial dysfunction.** Chronic dysfunction of the endothelium is implicated in the pathophysiology of several cardiovascular disorders including atherosclerosis, hypertension, diabetic vasculopathy, and heart failure (18, 57). Whereas endothelial dysfunction encompasses a broad range of abnormalities, a reduced bioavailability of endothelium-derived NO is the most widely studied aspect. A reduction in NO bioavailability in the vessel wall impairs endothelium-dependent vasorelaxation and reduces other beneficial effects of NO such as its inhibition of platelet and leukocyte adhesion and its anti-proliferative effects (57, 138). A decline in NO bioavailability may be caused by 1) reduced expression of eNOS; 2) deficiency of eNOS substrate (L-arginine) or cofactors (BH$_4$); and/or 3) increased inactivation of NO by O$_2^\cdot$. The latter is now recognized as a fundamentally important underlying mechanism in most settings (18, 120). It should be noted, however, that the eNOS pathway is subject to regulation by ROS at other levels too. For example, H$_2$O$_2$ increases eNOS expression through transcriptional and posttranscriptional mechanisms (30).

In many settings of oxidative stress-related endothelial dysfunction, the increased O$_2^\cdot$ generation originates not only from the endothelium but also other cell types in the vessel wall, notably vascular smooth muscle cells and adventitial fibroblasts. In the current review, we focus primarily on the role of endothelial O$_2^\cdot$ rather than these other cellular sources, which have been covered by several excellent reviews (18, 50, 57, 82, 88). The main sources of O$_2^\cdot$ that are implicated in the genesis of endothelial dysfunction are XO, NADPH oxidase, and uncoupled eNOS. An important point to be reiterated here is that in many cases multiple sources are involved, often as a consequence of ROS-dependent regulation as discussed previously. This is especially relevant in respect of O$_2^\cdot$ or peroxynitrite-induced degradation of BH$_4$, which leads to eNOS uncoupling (86, 91), as discussed in the section on Dysfunctional or uncoupled NOSs. Good in vivo evidence for a role of ROS derived from uncoupled eNOS in cardiovascular disease models has been relatively limited until recently, with studies in which exogenously administered BH$_4$ was found beneficial being limited by the fact that BH$_4$ may have direct antioxidant effects (4b). However, recent studies in which an increase in BH$_4$ levels driven by the increased gene expression of GTP cyclohydrolase I successfully reversed BH$_4$ deficiency and improved endothelial function (5, 6, 178), now provide more convincing evidence for the in vivo relevance of eNOS uncoupling.

**Atherosclerosis and coronary artery disease.** Traditional risk factors for atherosclerosis such as hypercholesterolemia and heavy smoking affect endothelial function by increasing ROS production, which decreases NO bioavailability and may convert the normal anti-inflammatory phenotype of the microcirculation to a proinflammatory “activated” phenotype. In addition, ROS production contributes to the oxidative modification of LDL, which plays a critical role in atherosclerosis. Several different ROS sources are implicated. In early atherosclerosis in heritable Watanabe hypercholesterolemic rabbits or cholesterol-fed normal rabbits, reduced NO bioavailability was attributed to increased degradation by O$_2^\cdot$ derived from endothelial NADPH oxidase activation, which was at least partly AT$_1$-receptor dependent (163). Likewise, diet-induced atherosclerosis in primates was associated with increased vascular O$_2^\cdot$ generation, which seemed to be at least partly NADPH oxidase dependent (58). In addition to endothelial dysfunction, increased NADPH oxidase-derived O$_2^\cdot$ production may also influence the development of atherosclerotic lesions. In p47 phox$^{-/-}$ mice studied on an ApoE$^{-/-}$ background, it was found that atherosclerotic lesion area was significantly reduced in the descending aorta compared with p47 phox$^{+/+}$ mice, although there was no difference in lesion area at the level of the aortic sinus (10). Consistent with these data, studies on diseased human coronary arteries have shown evidence of increased NADPH oxidase subunit expression together with increased in situ O$_2^\cdot$ generation in the plaque shoulder area (142). Guzik et al. (56) reported that vascular O$_2^\cdot$ generation in vessels from patients undergoing coronary artery bypass surgery increased as a function of the number of risk factors for coronary artery disease, with the O$_2^\cdot$ source being NADPH oxidase. On the other hand, in a recent elegant study in patients with coronary artery disease, Spiekermann et al. (143) showed that increased O$_2^\cdot$ generation leading to endothelial dysfunction derived from both XO and NADPH oxidase. XO-derived O$_2^\cdot$ generation is also implicated in endothelial dysfunction in heavy smokers (54) and in hypercholesterolemia (18, 124). NOS uncoupling, too, has been implicated in hypercholesterolemia, smoking (59), and atherosclerosis (91). In a recent study, more direct evidence for an involvement of NOS uncoupling in atherosclerosis was provided by the finding that transgenic mice with endothelium-specific overexpression of GTP cyclohydrolase had reduced aortic atherosclerosis compared with wild types when crossed with ApoE knockout mice.
(5). Finally, in patients with coronary artery disease, plasma levels of heparin-mobilizable ecSOD were found to be re-
duced, which may predispose to oxidative stress (87).

_Diabetes._ Oxidative stress has emerged as a strong patho-
genic cofactor in the development of long-term complications of type II diabetes, such as atherosclerosis, nephropathy, and retinopathy. As mentioned previously, we focus here on endo-
thelial ROS generation in diabetes. Endothelial dysfunction
attributable to increased \( \cdot O_2 \) generation is a prominent feature of diabetic vascular disease. As with hypercholesterolemia and atherosclerosis per se, multiple ROS sources are undoubtedly involved. Nevertheless, an increasing number of studies sug-
gest an important role for NADPH oxidase-derived \( \cdot O_2 \) and uncoupled NOS (reviewed in 4b; 99). In aortas from strepto-
zotocin-treated rats, the bioavailability of NO was decreased in
the face of increased eNOS expression as a result of increased
\( \cdot O_2 \) production from both uncoupled eNOS and NADPH
oxidase (64). In this study, a ninefold increase in gp91phox
expression was found, and it was suggested that this may be
driven by PKC (64). In a porcine model of streptozotocin-
induced diabetes, coronary artery oxidative stress was also
related to increased NADPH oxidase activity, although this
occurred mostly in the media and adventitia (177). In diabetic
patients undergoing coronary artery bypass surgery, it was
found that NADPH oxidase subunit expression and activity as
well as uncoupled NOS-dependent \( \cdot O_2 \) production were sig-
nificantly higher than in nondiabetics, independent of hyper-
cholesterolemia and at least partly driven by PKC (55, 56).
Recently, it has been shown that a key driver of endothelial
dysfunction may be the increased oxidation of BH4, which
results in NOS uncoupling. For example, in insulin-resistant
rats, oral administration of BH4 was able to reduce oxidative
stress and prevent endothelial dysfunction in the aorta (139).
More definitively, in streptozotocin-treated mice, endothelial
dysfunction was shown to be improved by the endothel-
ium-specific transgenic overexpression of GTP cyclohy-
drolase I (6).

Several pathogenic features of type 2 diabetes may be
involved in increasing NADPH oxidase activity, which would
then promote eNOS uncoupling. These include hyperinsulin-
emia (76), elevated blood glucose and free fatty acids (34, 69),
hypercholesterolemia (133, 144, 148), increased AGEs (164,
177), and increased activation of the renin-angiotensin sys-
tem (98, 176). Insulin resistance per se may also increase
NADPH oxidase activity, at least partly via the renin-
angiotensin system (139).

_Hypertension._ A substantial body of experimental and clin-
cal data implicates increased oxidative stress as being patho-
physiologically important in hypertension (18). In patients with
renovascular hypertension, excessive oxidative stress was
strongly suggested to contribute to impaired endothelium-
dependent vasodilatation, which improved after surgery in
conjunction with reduced indexes of oxidative stress (62). The
link between oxidative stress and hypertension is especially
robust with respect to the genesis of endothelial dysfunction,
whereas the involvement of \( \cdot O_2 \) in the development of in-
creased blood pressure itself is more contentious. It has been
reported that heparin-binding SOD reduced blood pressure in
SHR but not in normotensive rats (122) and that antioxidants
such as vitamin C and E prevented progression of hypertension
in SHR (21). Similarly, in hypertension induced by angiotensin
II infusion for up to 7 days, a significant reduction in blood
pressure was found with either infusion of liposome-encapsu-
lated SOD in rats (90) or infusion of a peptide inhibitor of
NADPH oxidase in mice (132). However, the precise mecha-
nism(s) involved in these antihypertensive effects remain to be
established and could include a direct effect on endothelial/
vascular function as well as indirect nonvascular pathways. For
example, in several studies a dissociation between altered
vascular \( \cdot O_2 \) and blood pressure has been found, e.g., in
studies in models of low renin hypertension (100b, 141b).

In the last few years, increased NADPH oxidase activity has
been reported as a major source of \( \cdot O_2 \) in the vessel wall of
experimental hypertension models, including angiotensin II-
induced hypertension, renovascular hypertension, genetic hyp-
ertension, and DOCA-salt hypertension (86, 100b, 119, 154,
174, 178). However, it should be noted that in many cases this
increased activity is found in the vascular smooth muscle and
adventitia rather than the endothelium. Studies in hypertensive
models associated with activation of the renin-angiotensin
system have convincingly shown that increased NADPH ox-
dase activity (at least in part due to increased subunit expres-
sion) contributes both to endothelial dysfunction and elevation
of blood pressure per se (21, 50, 85, 90, 100, 132). In the case
of low renin hypertension (often studied experimentally using
unilateral nephrectomy and administration of DOCA plus salt),
an increasing body of evidence implicates ET-1 driven vascu-
lar NADPH oxidase activation as being important, especially
for endothelial dysfunction (100b, 178). In rats with DOCA-
salt hypertension, Li et al. (100b) reported that an ET-1-
induced activation of arterial NADPH oxidase, which was ET\(_\alpha\)
receptor-dependent contributed to endothelial dysfunction in
carotid arteries. In this study, in vivo treatment with an ET\(_\alpha\)
antagonist reduced arterial \( \cdot O_2 \) levels and partially suppressed
systolic blood pressure. In studies of DOCA-salt hypertension
in mice, Landmesser et al. (86) reported that vascular ROS
production involved not only NADPH oxidase but also uncou-
ned NOS, with the stimulus for eNOS uncoupling suggested to
be ROS-induced oxidation of BH4 . In that study, ROS produc-
tion was inhibited by an NOS inhibitor or in eNOS \({ }^{\text{atm}}\) mice
and was also decreased by BH4 supplementation. However, in
rats, Li et al. (100b) did not find evidence for a contribution to
\( \cdot O_2 \) generation by uncoupled NOS based on lack of reduction
in ROS with NOS inhibitors. The reasons for these differences
between studies, apart from the obvious one of species, are
unclear. Interestingly, in a subsequent study from the same
group, ex vivo gene transfer of GTP cyclohydrolase I into
arterial segments was found to increase BH4 levels, increase
central NO release, and restore endothelium-dependent relaxa-
tion, implicating NOS dysfunction as an important contribu-
tory mechanism in this model (178). These latter observations
may imply that, in some settings, although a reduction in BH4
levels contributes to eNOS dysfunction this is not necessarily
associated with eNOS uncoupling and \( \cdot O_2 \) generation. There-
fore, a beneficial effect of BH4 supplementation is insufficient
on its own to prove the existence of eNOS uncoupling; better
evidence of the latter process would require more direct data
that there is ROS production from eNOS that is related to BH4
deficiency. In this regard, it is unclear how low BH4 levels
would need to drop in vivo to lead to eNOS uncoupling and
\( \cdot O_2 \) generation by the enzyme.
In addition to the studies in low renin hypertension mentioned above (141b, 178), a role for increased endothelial \( O_2^- \) generation by uncoupled eNOS has also been reported in the spontaneously hypertensive rat (24). XO-derived \( O_2^- \) has also been reported to contribute to impaired endothelium-dependent vasodilatation and increased blood pressure in the SHR (146, 122). Finally, antioxidant activities (e.g., SOD and catalase) may be reduced in the SHR.

Heart failure. Increased ROS generation and endothelial dysfunction may play important roles in the development of heart failure. Endothelial dysfunction contributes to the increased peripheral vascular resistance that is a hallmark of congestive heart failure and may be especially important in contributing to reduced exercise tolerance. Indeed, the endothelium has been suggested to be a therapeutic target in this condition (103). A reduced production of NO due to decreased expression of eNOS and a reduction in NO bioavailability due to increased \( O_2^- \) production in the endothelium have emerged as two principal mechanisms that are involved both in experimental and human heart failure (103, 120). In patients with congestive heart failure, both acute and chronic treatment with vitamin C is reported to improve systemic vascular endothelial dysfunction (33, 120). In an experimental model of heart failure induced by myocardial infarction in rats, Bausersch et al. (11) demonstrated a marked degree of aortic endothelial dysfunction despite an increased expression of eNOS, which was attributable to increased vascular \( O_2^- \) production derived from NADPH oxidase. Our own studies have shown that in experimental pressure overload cardiac hypertrophy and failure, endothelium-dependent (NO dependent) enhancement of left ventricular relaxation is impaired despite unaltered eNOS expression (110) as a consequence of enhanced \( O_2^- \) generation from NADPH oxidase (109). The increased NADPH oxidase activity was subsequently shown to be at least partly due to an increased expression of oxidase subunits (94). Furthermore, impaired endothelium-dependent cardiac function could be restored by treatment with the antioxidants vitamin C or deferoxamine (109). Stimuli that may be important in activating NADPH oxidase in cardiac hypertrophy and heart failure include angiotensin II, ET-1, cytokines, and mechanical forces (60).

A significant body of evidence also supports a role for XO-derived ROS in the systemic vascular endothelial dysfunction seen in chronic heart failure, which can be improved in patients by chronic treatment with the XO inhibitor allopurinol (36).

Ischemia-reperfusion. The increased generation of ROS during reperfusion after ischemia contributes to tissue injury, microvascular dysfunction, increased endothelial permeability, and endothelial “stunning.” Tissue injury after reperfusion may have serious consequences depending on the organ, e.g., myocardial infarction or stunning, stroke, and injury after organ transplantation or cardiac bypass surgery. The endothelial generation of \( O_2^- \) plays an important part in these processes. Increased mitochondrial \( O_2^- \) generation on reoxygenation may be involved in increasing endothelial cell permeability (108). Numerous studies support an important role for XO-mediated \( O_2^- \) generation in reperfusion injury. In addition, XO-derived \( O_2^- \) contributes to endothelial dysfunction and activation, which can persist for several days or weeks after reperfusion. Endothelial activation may serve to further enhance ROS production through the recruitment and activation of leukocytes. Increased peroxynitrite formation at reperfusion has also been implicated in cardiac reperfusion injury although the relevance of this in vivo remains to be established (104).

Several studies indicate that endothelial NADPH oxidase may contribute to ROS production during ischemia-reperfusion. Stimuli relevant to ischemia-reperfusion that activate endothelial NADPH oxidase include hypoxia-reoxygenation (77, 136, 141), 2) membrane depolarization (4, 140), 3) flow cessation (111), and 4) nutrient deprivation (106, 166). In HUVEC, increased ROS generation during reoxygenation after 8 h hypoxia was attributed to XO activation, whereas NADPH oxidase seemed to be downregulated based on studies with gp91ds-tat (141). Hoffmeyer et al. (65) investigated the effects of NADPH oxidase in ischemia-reperfusion injury using p47\(^{phox}\)−/− mice subjected to 30 min coronary occlusion and 24 h reperfusion. However, no difference was found in infarct size between wild-type and p47\(^{phox}\)−/− mice in this study (65).

Sepsis. Septic shock is characterized by severe hypotension, reduced organ perfusion, loss of vascular responsiveness (hypeoreactivity), and in many cases disseminated intravascular coagulation (DIC). An increase in oxidative stress is believed to play a major role in driving or mediating several of these abnormalities, with dysfunction of the endothelium being a major component of pathophysiology. The endothelium represents both a source and a target for ROS released in the vasculature in sepsis, although other cells in the vessel wall as well as inflammatory cells also play important roles. ROS-related endothelial dysfunction in sepsis includes the loss of physiological NO bioactivity; ROS-dependent proinflammatory events such as adhesion molecule expression, recruitment of neutrophils, and cytokine release (see ENDOTHELIAL CELL ACTIVATION AND INFLAMMATION); peroxynitrite production, which may further accentuate these proinflammatory effects and contribute to antioxidant (glutathione) depletion; and the occurrence of DIC, at least in part due to endothelial damage and apoptosis (134b). Many if not all of the main inflammatory pathways implicated in sepsis (cytokines, ET-1, platelet activating factor, etc.) can act on both phagocytes and endothelial cells (as well as other cells) to induce ROS production through NADPH oxidase activation (8, 50), whereas XO-derived ROS is also implicated (114). A major challenge for the future is to assess whether ROS-dependent pathophysiology can be specifically targeted to therapeutic benefit in septic shock.

Nitrate tolerance. Organic nitrates such as glyceryl trinitrate (GTN) are widely used for the symptomatic treatment of ischemic heart disease, where they act by causing venous and arterial dilatation and thereby reducing myocardial work and oxygen consumption. Chronic treatment with nitrates is limited by the development of nitrate tolerance and cross-tolerance to other classes of nitrovasodilators. Although the mechanisms underlying the development of nitrate tolerance are probably multifactorial, studies undertaken by Munzel and colleagues (121, 147) have clearly shown an important role for increased endothelial generation of ROS in this phenomenon. The enhanced endothelial \( O_2^- \) generation has been suggested to emanate both from NADPH oxidase (121) and from mitochondrial ROS, which may act by inhibiting the aldehyde dehydrogenase enzyme (ALDH-2), which is involved in GTN biotransformation in vivo (147).
SUMMARY

The importance of the vascular endothelium in the regulation of cardiovascular homeostasis has become increasingly evident. Endothelial cells have many different functions that are susceptible to modulation by specific local and environmental stimuli. Endothelial activation and dysfunction play a role in the pathogenesis of several cardiovascular diseases. The generation of ROS, in particular O$_2^•$, by endothelial cells is relevant to their functions both in physiological and pathophysiological settings. ROS exert their effects through several mechanisms including their interactions with NO, their modulation of redox-sensitive signaling cascades, and direct effects on cellular membranes, proteins, and DNA. Several enzymatic sources of ROS are present in endothelial cells, among which a phagocyte-type NADPH oxidase appears particularly important with respect to tightly regulated redox signaling. The effects of ROS generated within endothelial cells are dependent on the amount and site of production as well as the antioxidant balance. Understanding the mechanisms underlying the regulated generation of O$_2^•$ in endothelial cells and the downstream effects of ROS in different pathological settings may help inform therapeutic strategies to tackle endothelial activation and dysfunction in conditions such as hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure.

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REFERENCES


ENDOTHELIAL O₂⁻ PRODUCTION


Invited Review

ENDOTHELIAL O$_2^\bullet$-PRODUCTION


