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Forum Review

NADPH Oxidase-Dependent Signaling in Endothelial Cells: Role in Physiology and Pathophysiology

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Abstract

Reactive oxygen species (ROS) including superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are produced endogenously in response to cytokines, growth factors; G-protein coupled receptors, and shear stress in endothelial cells (ECs). ROS function as signaling molecules to mediate various biological responses such as gene expression, cell proliferation, migration, angiogenesis, apoptosis, and senescence in ECs. Signal transduction activated by ROS, "oxidant signaling," has received intense investigation. Excess amount of ROS contribute to various pathophysiologies, including endothelial dysfunction, atherosclerosis, hypertension, diabetes, and acute respiratory distress syndrome (ARDS). The major source of ROS in EC is a NADPH oxidase. The prototype phagaocytic NADPH oxidase is composed of membrane-bound gp91phox and p22hox, as well as cytosolic subunits such as $p47^{phox}$, $p67^{phox}$ and small GTPase Rac. In ECs, in addition to all the components of phagocytic NADPH oxidases, homologues of $gp91^{phox}$ (Nox2) including Nox1, Nox4, and Nox5 are expressed. The aim of this review is to provide an overview of the emerging area of ROS derived from NADPH oxidase and oxidant signaling in ECs linked to physiological and pathophysiological functions. Understanding these mechanisms may provide insight into the NADPH oxidase and oxidant signaling components as potential therapeutic targets. *Antioxid. Redox Signal*. 11, 791–810.

Introduction

THE ENDOTHELIUM IS THE THIN LAYER OF CELLS that line the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells (ECs) line the entire circulatory system, from the heart to the smallest capillary. These cells reduce friction of the flow of blood, allowing the fluid to be pumped further. Normal functions of endothelial cells include mediation of coagulation, platelet adhesion, immune function, control of volume and electrolyte content of the intravascular and extravascular spaces. Endothelial dysfunction, characterized by impaired endothelium-dependent vasodilatation, is implicated in various pathophysiologies, including atherosclerosis, hypertension, and diabetes mellitus. A more specific alteration in endothelial function is the change in endothelial phenotype characterized by the expression of cell-surface adhesion molecules and other proteins involved in cell–cell adhesions and endothelial permeability. Endothelial activation is important in the context of the angiogenesis inflammatory response as well as hypertension, atherosclerosis, ischemia/reperfusion, sepsis, and acute lung injury (ARDS) (136). Thus, understanding the mechanisms of endothelial activation and the development of endothelial dysfunction is critically important.

ECs produce reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$) and H_2O_2 similar to other types of nonphagocytic cells. Although excess amounts of ROS contribute to EC death and apoptosis, ROS function at physiological concentrations and act as signaling molecules to mediate various biological responses. ROS are generated from a number of sources, including the mitochondrial electron transport system, xanthine oxidase, cytochrome p450, NADPH oxidase, uncoupled NO synthase (NOS), and myeloperoxidase. NADPH oxidase appears to be a major source of ROS produced by ECs. Molecular O2 is converted to $O_2^{\cdot -}$by NADPH oxidase, and $O_2^{\cdot -}$can be converted to H_2O_2 by superoxide dismutase (SOD), or to highly reactive $OH^{\cdot -}$ by the Fenton

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or Haber–Weiss reactions (73), or to peroxinitrite (OONO-) by reacting with nitric oxidase (NO) (Fig. 1). This enzyme system is activated by numerous stimuli including growth factors, G-protein coupled receptor agonists, cytokines, shear stress, and ischemia/reperfuson. ROS derived from NADPH oxidase are involved in endothelial dysfunction and permeability, inflammation, vascular remodeling, cell growth and migration, apoptosis and senescence, which contribute to hypertension, atherosclerosis, diabetes, and acute lung injury/sepsis (Fig 1). In addition, disease may cause increased ROS production (Fig 1). ROS produced by NADPH oxidase activate diverse redox signaling pathways by activation of kinases and/or oxidative inactivation of protein phosphatases, resulting in increasing tyrosine and serine/threonine phosphorylation signaling events (Fig 3). Oxidant signaling activates redox-sensitive transcription factors which are involved in regulating redox-sensitive gene expression, leading to various physiological and pathophysiological responses. This review describes what is known about NADPH oxidase and antioxidant enzymes, activation mechanism of NADPH oxidase, oxidant signaling events activated by these enzymes, and biological and pathophysiological functions with focusing on ECs.

ROS Generating Systems and Antioxidant Enzymes in ECs

NADPH oxidase in endothelial cells

The major source of ROS in ECs is the NADPH oxidase system (10). However, there are several other enzymatic sources of ROS in mammalian cells, depending on the tissue and environmental context that include the mitochondrial electron transport chain, xanthine oxidase, cytochrome p450, and dysfunctional or uncoupled eNOS (136). There may be complex interactions among different sources of ROS and feedback and feedforward regulation of ROS accumulation (136). NADPH oxidase is activated in ECs by growth factors, cytokines, shear stress, hypoxia, and G-protein coupled agonists (86). In mammalian neutrophils, NADPH oxidase consists of the membrane-bound cytochrome b558 comprising the catalytic subunit $gp91^{phox}$ (Nox2) and regulatory subunit $p22^{phox}$, as well as cytosolic subunits, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, and the GTPase, Rac (50). The neutrophil NADPH oxidase releases large amounts of $O_2^{\cdot -}$ in bursts, whereas the nonphagocytic NADPH oxidase(s) continuously produce low levels of $O_2^{\cdot -}$ intracellularly in basal state, yet it can be further stimulated acutely by various agonists and growth factors. In nonphagocytic cells, several human homologs of $gp91^{phox}$ (also termed as Nox2) have been identified including Nox1, Nox3, Nox4, Nox5, and the dual oxidases (Duox1 and Duox2) (130). In ECs Nox1, Nox2, Nox4, and Nox5 are mainly expressed (16, 114) and Nox family members share the common binding sites for FAD, heme, and NADPH, and six transmembrane domains. Nox2 is the critical component of endothelial NADPH oxidase (Fig 2). The regulation of Nox1 activity is appears to require $p22^{phox}$, as does Nox2 (5, 219), and NoxO1 (Nox organizer 1) and NoxA1 (Nox activator 1)-respective homologs of $p47^{phox}$ and $p67^{phox}$ (219). Similar to $p47^{phox}$, NoxO1 contains an N-terminal phox homology (PX) domain that binds phosphoinositides and an SH3 domain in the central portion of the protein. To our knowledge, expression of NoxO1 and NoxA1 in ECs has not been demonstrated, and thus is the subject of future investigation.

Antioxidant Enzymes in Endothelial Cells

Superoxide dismutases (SOD)

Intracellular ROS levels are regulated by the balance between ROS generating enzymes and antioxidant enzymes that include superoxide dismutases (SOD), catalase, glutathione peroxidase (GPx), heme oxygenases, and thioredoxin system. In mammals, three isoforms of superoxide dis-

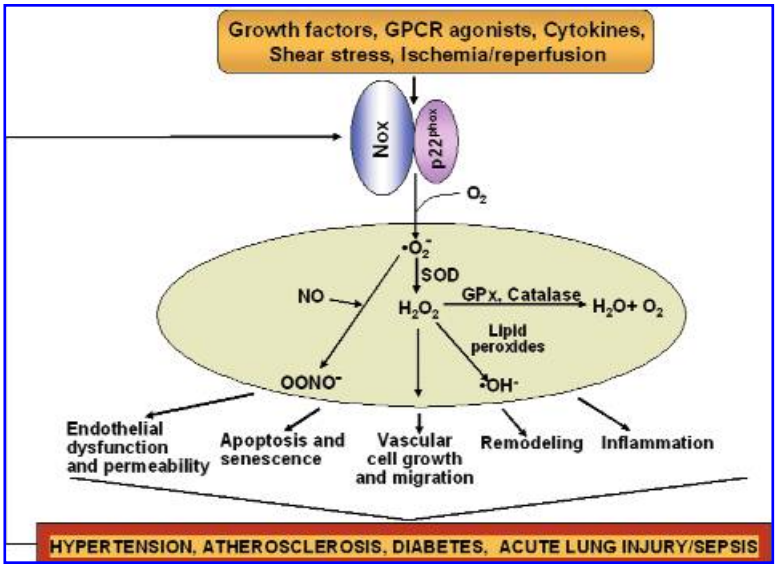

FIG. 1. Role OF NADPH Oxidase in endothelial cells. Growth factors, GPCR agonists, cytokines, shear stress, and ischemia/reperfusion activate NADPH oxidase. Oxygen can be converted to superoxide by NADPH oxidase which subsequently leads to the conversion to $OH\cdot -$ radical by lipid peroxides, to H_2O_2 via superoxide dismutase (SOD), and to H_2O and O_2 by glutathione peroxidase (GPx) and catalase. $O_2\cdot -$ and NO generated from e-NOS can combine to generate ONOO− promoting NOS uncoupling and further O2·− production. The generation of oxidants leads to endothelial dysfunction and permeability, apoptosis and senescence, vascular cell growth and migration, remodeling, and inflammation. The underlying disease progression increases susceptibility to hypertension, atherosclerosis, diabetes, and acute lung injury/sepsis. Feedback mechanisms may also exist in which an existing disease leads to an increase in ROS production. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

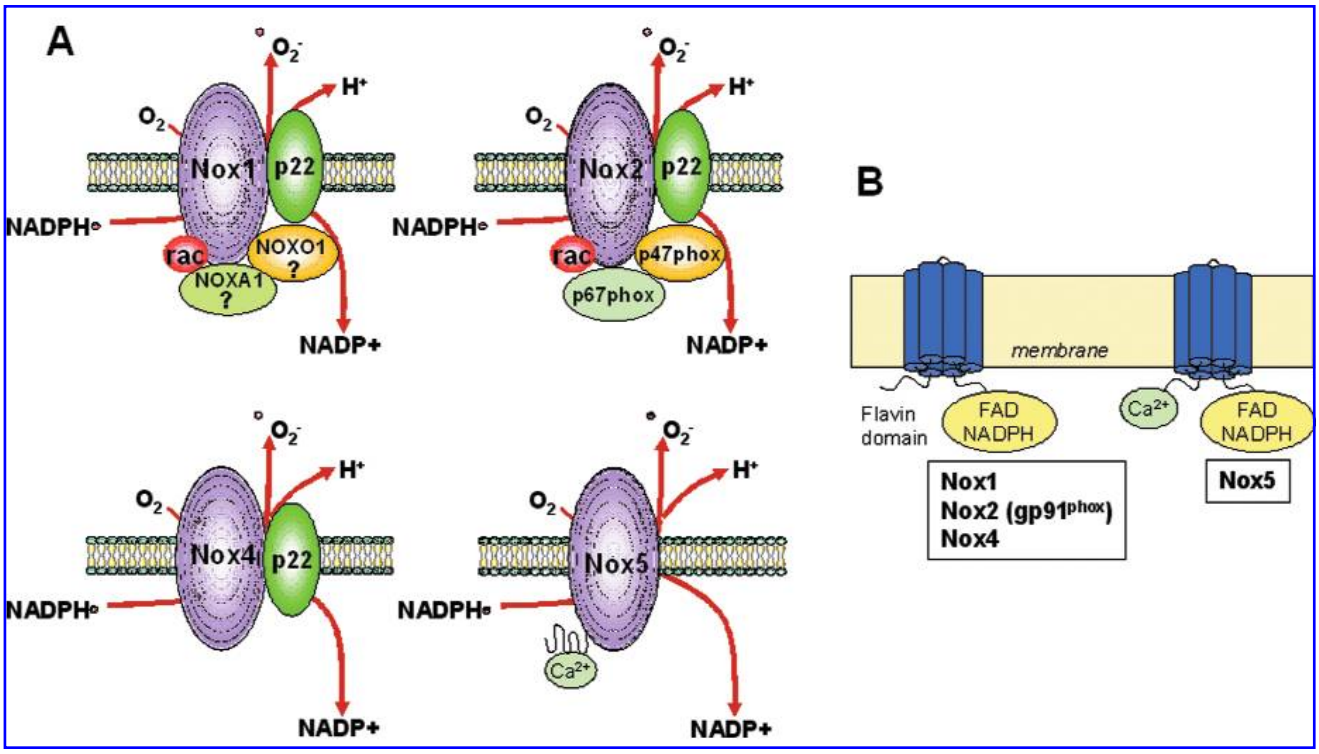

FIG. 2. Schematic diagram of the structure of endothelial NADPH oxidase. (**A**) Nox1 binds adapter subunits, NoxO1 and NoxA1, in place of the initially characterized gp91^{phox} (Nox2) adapter proteins p47^{phox} and p67^{phox}, respectively, as well as Rac and p22^{phox}. Nox4 activation does not involve p47^{phox}, p67^{phox}, or Rac, while Nox5 has EF hands that bind Ca^{+2}. ?: It is unclear if these proteins are expressed in ECs. **Transmembrane topology of Nox enzymes.** (**B**) The predicted transmembrane α-helices contain conserved histidine residues which comprise binding sites for hemes. The carboxyl-terminal domain folds within the cytoplasm and binds to flavin adenine dinucleotide (FAD) and NADPH. The enzymes catalyze the transfer of electrons from NADPH to molecular oxygen, to form O2·− across the membrane. The amino terminal calcium-binding domain of Nox5 enzyme is on the cytosolic side of the membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

mutase exist: cytoplasmic CuZnSOD (SOD1), mitochondrial MnSOD (SOD2), and extracellular Cu/ZnSOD (SOD3, ecSOD) (119). The main function of SODs is to convert $O_2^{\cdot -}$ to H_2O_2 which is then converted to water by catalase or glutathione peroxidase. EcSOD is the major SOD in the vascular EC extracellular space and is also produced by ECs, as well as by vascular smooth muscle cells and fibroblasts. It is secreted and anchored to the extracellular matrix and endothelial cell surface through binding to the heparin sulfate, collagen, and fibulin-5. Transfection of cells with ecSOD reduces $O_2^{\cdot -}$ and functions to restore the impairment of endothelium-dependent relaxation, resulting in decreased arterial pressure in a genetic model of hypertension (39).

Glutathione peroxidase (GPx)

GPx is a ubiquitously expressed selenium-dependent antioxidant enzyme present in the cytosol and mitochondria. To date, five isoenzymes are present with GPx-1 (a cytosolic form) being the most abundant and ubiquitously expressed isoform (126). It regulates the levels of hydrogen and lipid peroxides. In the absence of GPx, ROS levels increase tissue damage and may cause atherogenesis (134). Reduced levels of GPx lead to increased LDL oxidation and increased intima-media thickness that is linked to four single nucleotide polymorphisms in the GPx gene (94). Mice deleted of GPx-1 rarely have any significant phenotype (103), whereas double knockout of GPx-1 and GPx-2 resulted in inflammatory bowel disease and increased intestinal cancer incidence (38).

Heme oxygenase (HO)-1

Erythrocytes contain heme in a concentration of 20 m*M* and can release heme and iron into the vasculature that can easily enter ECs and mediate ROS-induced EC injury. HOs catalyze the conversion of heme into carbon monoxide, Fe^{2+}, bilirubin, and biliverdin. There are three human isozymes of HO, including HO-1 (inducible), HO-2 (constitutive), and HO-3 (constitutive). Cardiac-specific expression of HO-1 protects against inflammation and oxidative damage in hearts subjected to ischemia and reperfusion injury *in vivo* (248). ECs isolated from HO-$1^{-/-}$ mice showed an oxidative stress phenotype *in vitro* (173). Increased HO-1 activity leads to an increase in ecSOD expression (124) and HO-2 regulates ecSOD protein expression (225). Injection of HO-2 siRNA into rats increased apoptosis signal-regulating kinase (ASK-1) protein expression while reducing Akt phosphorylation (225). Thus, HO-2 can participate in heme homeostasis and is involved in oxidant signaling.

Thioredoxin (TRX) reductase

TRX in combination with TRX reductase and NADPH form redox-sensitive machinery that functions in an indirect signaling fashion by controlling the levels of oxidized cysteine

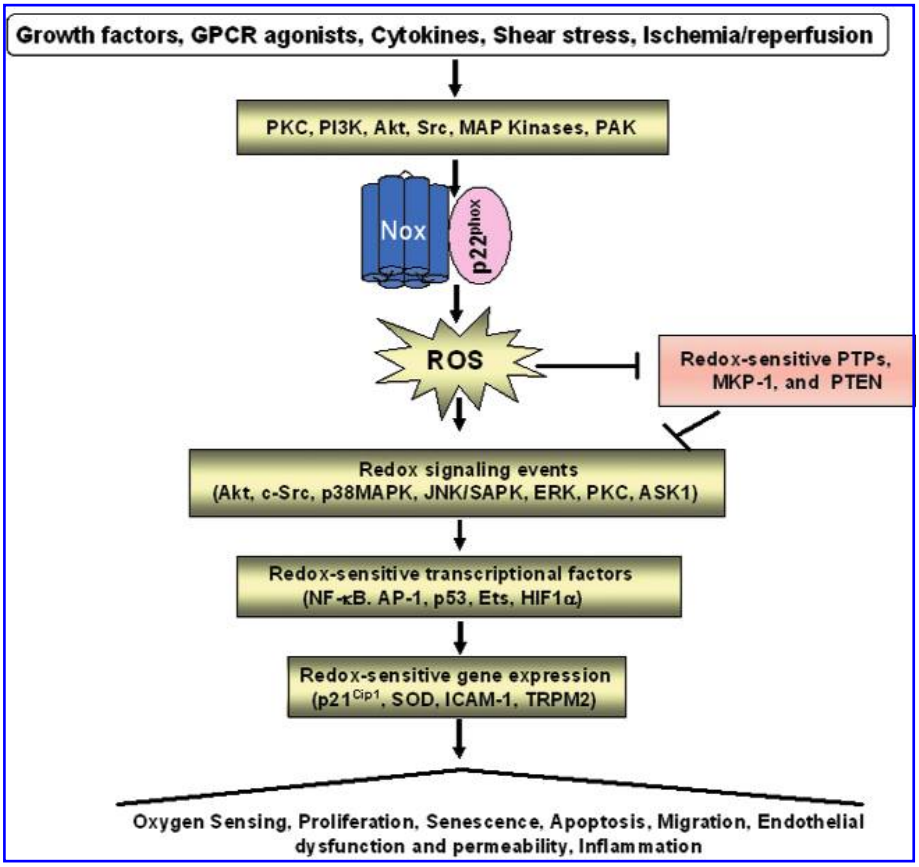

FIG. 3. Role of ROS derived from NADPH oxidase in REDOX-sensitive signaling pathways. Growth factors, GPCR agonists, cytokines, shear stress, and ischemia/reperfusion can activate PKC, PI3K, Akt, Src, MAP kinases, and PAK which stimulate NADPH oxidase to produce ROS. NADPH oxidase-induced ROS can induce oxidative inactivation of protein tyrosine phosphatases, MKP-1 and PTEN to promote downstream redox signaling events. These events are converged and integrated to induce various redox-sensitive transcriptional factors and gene expression, which are involved in EC oxygen sensing, proliferation, senescence, apoptosis, endothelial dysfunction, and permeability and inflammation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

on proteins. The more direct antioxidant properties of TRX are due to TRX peroxidase in which TRX reduces the oxidized form of TRX peroxidase, and the reduced peroxidase scavenges H_2O_2 (116). TRX exists in two isoforms, TRX–I and TRX–II. Thioredoxin contains a conserved −Cys–Gly–Pro–Cys active site which is essential for the redox regulatory function of TRX. Thioredoxin-I functions as a redox-sensitive binding protein that controls the activity of NF-κB through reducing the Cys62 on the p50 of NF-κB (148). Nuclear TRX increases transcription factor binding to antioxidant response elements; TRX increases Fos and Jun DNA binding activity via binding to a nuclear redox protein, redox factor 1 (102), that in turn reduces the conserved cysteines in Fos and Jun (241). The anti-apoptotic effects of H_2O_2 depend on nuclear TRX in ECs (197). Thioredoxin also binds to the MAPKK kinase ASK-1. ASK1 is activated by stress- and cytokine-related stimuli and activates JNK and p38 MAPK. TRX can also regulate vitamin D3-up-regulated protein (VDUP1) which function as an endogenous inhibitor of TRX and subsequently results in a negative feedback loop whereby VUDP1 expression is reduced by H_2O_2 (34). TRX serves to protect ECs from oxidant-induced apoptosis. Haendeler *et al*. showed that. H_2O_2 also resulted in TRX degradation, thereby decreasing reactive oxygen radical scavenging ability and increasing apoptosis (92).

Mechanisms of NADPH Oxidase Activation in Endothelial Cells

NADPH oxidase is activated by diverse stimuli including G-protein-coupled receptor agonists (angiotensin II and thrombin); cytokines (tumor necrosis factor α and transforming growth factor β); growth factors [VEGF (vascular endothelial growth factor), angiopoietin-1, PDGF, EGF, fibroblast growth factor and insulin]; hypoxia-reoxygenation or ischemia-reperfusion; and mechanical stimuli (oscillatory shear) (136). The molecular mechanism of NADPH oxidase activation in ECs is best characterized for the Nox2-based oxidase and Nox1. In general, Nox2 oxidase activation of ECs involves a translocation of cytosolic oxidase components ($p47^{phox}$, $p67^{phox}$, and Rac1) to the plasma membrane and association with cytochrome b_{558}, which initiates the electron transfer process. The key post-translational modifications involved in oxidase activation are the phosphorylation of $p47^{phox}$ and Rac activation (136, 228). PKC (protein kinase C) isoforms are believed to be the major kinases responsible for $p47^{phox}$ phosphorylation, although other kinases such as Src kinases, PI3 kinase (PI3K), Akt, mitogen-activated protein kinases (MAP kinases) including p38 MAPK, JNK/SAPK, and ERK, and PAK (p21-activated kinase) may also play a role depending on the stimulus (71, 136) (Fig. 3). These signaling mechanisms in NADPH oxidase activation are reviewed in detail below.

Protein kinase C (PKC)

PKC represents a family of 12 members to date that perform a variety of functions. PKC isoforms are classified into three groups based on their structure and activation mechanisms: phosphatidylserine -, diacylglycerol (DAG)-, and Ca^{2+}-dependent conventional PKC (cPKC; α, βI, βII,and γ),

Ca^{2+}-independent novel PKC (nPKC; δ, ε, μ, θ, and η) isoforms, and DAG-, and Ca^{2+}-independent atypical PKC (aPKC; ζ, and λ/ι) isoforms. Tissue distribution of PKC,-α, -δ, and -ζ is widespread, whereas the others are localized in a tissue- and cell type–specific manner. In addition to PKC-α, -δ, and -ζ, ECs also express the PKC-β, -ε, -η, and -θ isoforms (177). TNF-α–induced oxidant generation via NADPH oxidase requires the activation of PKCζ (177) the atypical PKC isoform abundantly expressed in ECs. The mechanism of activation involves PKCζ-induced phosphorylation of $p47^{phox}$ and its targeting to the membrane where it associates with Nox2 to generate the active NADPH oxidase complex. The PKC isoforms β, δ, and ζ are suggested to be the major kinases responsible for $p47^{phox}$ phosphorylation. In addition, $p67^{phox}$ and p22phox are also phosphorylated during NADPH oxidase activation, although the relevance of this remains unclear.

Src

c-Src is an important upstream kinase that regulates NADPH oxidase-induced ROS production (201). Exposure of cultured ECs to LDL stimulated ROS formation, which was completely inhibited by Src kinase inhibitor PP1 (161). Src mediates phosphorylation of $p47^{phox}$ and its translocation to the membrane in hyperoxia-induced activation of NADPH oxidase in lung ECs (37). Src is constitutively associated with $p47^{phox}$ and $p67^{phox}$, and hyperoxia increased the association of Src with $p47^{phox}$ (37, 165, 166). Cortactin is a substrate of c-Src, and transfection of HPAECs with myristoylated cortactin Src homology domain 3 blocking peptide attenuated hyperoxia-induced translocation of $p47^{phox}$ to the cell periphery and ROS production (226). In neutrophils, other Src-related tyrosine kinase family members, Hck and Lyn, are involved in the activation of the NADPH oxidase (24) and formation of PtdIns (3,4,5)P_3 via stimulation of phosphatidylinositol 3-kinase (PI3K) (175).

PI3K

Four mammalian PI3K type 1 isoforms, p110α, p110β, p110γ, and p110δ, have been identified (232), and of these, p110γ has distinct properties. Type 1A PI3Ks, p110α, p110β, and p110δ, associate with one of the five regulatory subunits: p50α, p55α, and p85α (products of alternative splicing of a single gene) and p55γ and p85β. In contrast, type 1B PI3K (or PI3Kγ), the catalytic subunit p110γ binds to the p101 adaptor molecule or the G$\beta\gamma$-activated regulatory subunit p84 (214). Type 1A PI3Ks are activated by interactions with tyrosine-phosphorylated molecules, whereas p110γ is activated by heterotrimeric G proteins Gα and G$\beta\gamma$ that bind to the pleckstrin homology domain found in the NH_2-terminal region of PI3Kγ (209). p110 is also activated by pro-inflammatory cytokines such as TNFα (26). Expression of PI3Kγ is largely confined to leukocytes, and there is a growing appreciation of its important role in immunity and host defense (96, 138). Studies also demonstrated the presence of the PI3Kγ isoform in EC (84, 176). PI3Ks catalyze the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is involved in the recruitment and activation of a variety of regulatory proteins via interactions with their pleckstrin homology and *phox* homology domains (64). Phox domains, present in two subunits of the NADPH oxidase complex, $p47^{phox}$ and $p40^{phox}$, bind to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol trisphosphate (both breakdown products of PIP_3) (64, 172). Degradation of PIP3 occurs by either PTEN (3′-phosphatase and tensin homolog deleted on chromosome 10) or SH2-containing phosphatidyl inositol phosphatases (SHIP-1 and SHIP-2) (125). PI3K and Rac are involved in the activation of endothelial cell NADPH oxidase that is associated with the acute loss of shear stress (250). TNFα induces PIP_3 production through PI3Kγ activation of PKCζ and that PI3Kγ plays a crucial role in activation of NADPH oxidase required for NF-κB activation and ICAM-1 expression in ECs (71). How TNFα receptor signals to increase PI3Kγ, NF-κB, and ICAM-1 expression remains unknown.

Akt (Protein kinase B):

There are currently three known isoforms Akt1, Akt2, and Akt3 (17). Akt1 is the predominant isoform expressed in ECs from lung and the aorta (31). Akt1 was shown to phosphorylate Ser1177 and activate eNOS in ECs (57), and to increase NO synthesis and cGMP elevation during platelet activation and thus promoting platelet secretion and aggregation (211). In phagocytic cells, Akt phosphorylates p47phox, and thereby increases ROS production (36). However, fMLF-induced $p47^{phox}$ phosphorylation in PMNs was not blocked by inhibitors of Akt (245). Similarly, angiopoietin-1-induced rise in H_2O_2 was not affected by the expression of an inactive Akt phosphorylation mutant in HUVECs (98). Akt phosphorylates $p47^{phox}$ on S304 and S328, suggesting its role in NADPH oxidase activity; however, phosphorylation of S359 or S370 on $p47^{phox}$ is not required for oxidase activation by Akt (106). $Akt1^{-/-}$ EC monolayers were 50% more permeable compared to WT EC monolayers (31), suggesting that Akt increases endothelial permeability through activating NADPH oxidase.

Mitogen-activated protein kinases (MAPKs)

The sustained superoxide production and increased NADPH oxidase activity by angiotensin II (Ang II) can be blocked by PD98059, an inhibitor of the p42/44 MAPK pathway (242). In addition, Ang II infusion led to endothelial NADPH oxidase activation and ROS production which were blocked by SB239063, a p38MAPK inhibitor (12). Ang II-induced hypertension was also significantly attenuated in MAPKAP kinase-2 knockout mice (12). These results suggest that Ang II-induced hypertension, organ damage, and ROS production are mediated by p38 MAPK. Chronic inhibition of p38 MAP kinase reduced MAPKAPK-2 phosphorylation, preserved acetylcholine-induced relaxation, and reduced vascular superoxide formation (236). Exposure of HPAECs to hyperoxia activated p38 MAPK and ERK, and inhibition of p38 MAPK and MEK1/2 attenuated the hyperoxia-induced ROS generation (165). Thus, inhibition of MAPK family members may offer a therapeutic approach for cardiovascular disease (12). In contrast, transducing HUVECs with retroviruses expressing a dominant negative JNK-1 had no effect on angiopoietin-1-induced H_2O_2 production (98). Of note, expression of the NADPH oxidase subunit $p22^{phox}$ is also regulated by ROS through p38 MAPK and PI3K/Akt (58), creating a positive feedback loop that accentuates NADPH oxidase activation.

PAK (p21-activated kinase)

PAK is a serine/threonine kinase involved in cytoskeletal dynamics, cell migration, neurogenesis, angiogenesis, mitosis, apoptosis, and transformation (113). To date, six different PAK isoforms have been discovered (112). Activation of PAK1 is initiated by the high-affinity binding of the small Rho GTPases, Rac1-3, or Cdc42, to the p21-binding domain (PBD/CRIB) (123). $p47^{phox}$ can be phosphorylated by PAK at several sites (122). Using a combination of 2-D mapping followed by HPLC and mutational analysis to identify the phosphoserines in $p47^{phox}$, Knaus and colleagues showed that serines 303, 304, 320, and 328 were targets of PAK-induced phosphorylation with the highest incorporation of ^{32}P at serine 328 on $p47^{phox}$ (142). Furthermore, active PAK can directly associate with Nox2 (142) but does not directly phosphorylate $p67^{phox}$ or $p22^{phox}$ (142).

Oxidant Signaling in ECs

ROS can act as a signaling molecule for activation of diverse signaling pathways by oxidation of reactive cysteine on the specific target molecules including kinases, phosphatases, and redox sensitive transcription factors. NADPH oxidase-derived ROS activate various redox-sensitive kinases such as Akt, Src, and MAPKs, as well as transcription factors, including NF-κB, AP-1 p53, Ets and HIF-1, thereby increasing redox-sensitive gene expression. Thus, these oxidant signaling events may contribute to various physiological and pathophysiological responses (Fig. 3). Studies using $gp91^{phox}$ (Nox2) knock-out mice have shown that the Nox2-derived ROS stimulate oxidant signaling (3, 72), and thus are involved in NF-κB activation and expression of adhesion molecules such as ICAM-1. Studies have also suggested an important role for NADPH oxidase in promoting the migration of leukocytes across the vascular endothelial barrier (143). These findings support the concept that oxidants promote transendothelial PMN migration and sequestration in tissue. Thus, NADPH oxidase-derived ROS play a critical role in signaling linked to NF-κB activation and ICAM-1 expression, thereby promoting inflammatory responses.

The best established direct molecular targets of ROS are protein tyrosine phosphatases (PTPs). Protein tyrosine phosphorylation is a major mechanism for post-translational modification of proteins that plays a critical role in regulating cell functions. The level of tyrosine phosphorylation is controlled by the balance between protein tyrosine kinases (PTK) and PTP activity. The reversible oxidative inhibition of PTPs by ROS is an important mechanism through which ROS increase tyrosine phosphorylation-dependent signaling events. The catalytic region of PTPs includes cysteines (192) that are extremely susceptible to oxidative inactivation (53). Thus, ROS decrease phosphatase activity that enhances protein tyrosine phosphorylation and thereby influences signal transduction (132). Myristoylated TRAF4 and $p47^{phox}$ target to nascent focal complex–like structures, which induces local oxidative inactivation of PTP-proline (P), glutamic acid (E), serine (S), and threonine (T) rich sequence (PEST) (240). Inhibition of PTP-PEST in turn activates Rac1 and its effector kinase PAK1, thereby promoting $p47^{phox}$ phosphorylation and creating a positive feedback loop that facilitates NADPH oxidase activation, local ROS production, and Rac1 activation (240). H_2O_2 also activate Akt through oxidative inhibition of PTEN (133) that acts as a tumor suppressor and a negative regulator of PI3K signaling. PTEN is constitutively active, unlike the 5′ lipid phosphatase Src-homology domain 2 inositol phosphate phosphatase-2 (SHIP2) which is activated in response to growth factors and cytokines (60). SHIP2 controls $PI(3,4,5)P_3$ levels and PKB/Akt activity in response to H_2O_2 (249).

PKC is also a target for redox modification by oxidants and antioxidants. Oxidants selectively oxidize N-terminal regulatory domains that contain zinc-binding, cysteine-rich motifs, and thus stimulate PKC activity. In ECs, H_2O_2 increases PKC activity and diacylglycerol formation (216), thereby promoting endothelial permeability (206). VCAM-1 activates endothelial cell NADPH oxidase to generate ROS, resulting in oxidative activation of PKCα and then activate protein tyrosine phosphatase 1B (PTP1B) (48). ROS can also inactivate MAPK phosphatase-1 (MKP-1). SAPK/JNK itself is regulated by an inhibitory interaction with a member of the glutathione S transferase family, GSTpi (2). H_2O_2 stimulates oligomerization of GSTpi, releasing SAPK, with an increase in activity without a change in phosphorylation of the activation loop of SAPK. (2). MEKK1 and apoptosis signaling kinase 1 (ASK1) are also regulated by oxidative stress. MEKK1 is inhibited by site-specific glutathionylation of a critical cysteine residue in the ATP binding domain (45). TRX inhibits ASK1 by physical binding (140). In response to H_2O_2 or TNF, TRX is oxidized, promoting dissociation from ASK1, and resulting in activation of the kinase (140). Treatment of cells with H_2O_2 activates p38 MAPK (58), ERK1/2, and Akt, and requires tyrosine kinase activity of insulin receptor and c-Src (151). Another study showed that atrial natriuretic peptide-induced $O_2^{\cdot -}$ activates JNK through regulating MKP-1 expression without activating PKC, ERK, or p38 MAPK (77), suggesting that ROS inactivate phosphatases, leading to increasing phosphorylation and activation of protein kinases.

ROS Regulation of Transcription Factors

In addition to PTPs, proteins with low-pK_a cysteine residues which can be oxidized by ROS, include transcription factors nuclear factor-κB (NF-κB) (196), AP-1 (162), hypoxia-inducible factor-1α (HIF-1α) (233), p53 (178), and Ets transcription factor, Ets-1 (160).

NF-κB

NF-κB is a transcription factor consisting of a group of five proteins, namely c-Rel, RelA (p65), Rel B, NF-κB1 (p50 and p105), and NF-κB2(p52). ROS serve as common intracellular messengers of NF-κB activation (196). We and others have shown that H_2O_2 is an activator of NF-κB in ECs and that overexpression of catalase blocks NF-κB activation induced by TNFα (187, 224). How H_2O_2 activates NF-κB is not fully understood (195). It was shown that H_2O_2-induced NF-κB activation occurred without degradation of IκBα (218). Syk protein-tyrosine kinase can induce tyrosine phosphorylation of IκBα, leading to NF-κB activation (218). The DNA binding activity of the p50 NF-κB subunit is inhibited by glutathionylation or sulfenic acid oxidation of a critical cysteine in the DNA binding domain (170). Furthermore, Nox2 transcription is dependent on NF-κB; two potential cis-acting elements in the murine Nox2 promoter control NF-κB-dependent regulation (7).

AP-1 (c-Jun and c-Fos)

Transcription factor activator protein-1 (AP-1) consists of c-Jun and c-Fos. At the transcriptional level, phosphorylation of c-Jun, which is mediated by JNK and p38 MAPK, is increased by agents that increase ROS (222). Oxidants can also induce the mRNA expression of c-fos, and c-jun (181, 182). Fra-2 is a member of the Fos family of genes, most of which are rapidly induced by second messengers. Although the role and biology of Fra-2 are less understood than those of its relatives, c-Fos, Fra-1, and FosB, it was shown that elevated Fra-2 is associated with cellular differentiation in response to the redox modifier homocysteine (163). In cardiac fibroblasts, hyperoxia induces Fra-2 mRNA and protein (188). MAPK phosphatase-1 (MKP-1) induction is dependent on the activation of AP-1 (77). The DNA binding domain of c-jun contains a critical cysteine residue that is glutathionylated *in vitro* in response to altered GSH/GSSG ratios, thus reducing DNA binding activity (121). This may be due to activation of signaling pathways upstream of ERK1/2 kinase or to an indirect effect secondary to inhibition of phosphatase activity by ROS (77, 222).

HIF-1

HIF-1 is composed of an inducible α-subunit (HIF-1α) and a constitutive ·-subunit (200). HIF-1α, upon hydroxylation by specific prolyl hydroxylases, binds the von Hippel Lindau protein, leading to HIF-1α ubiquitinylation and degradation by the 26S proteasome. During hypoxia, prolyl hydroxylase loses its activity, which blocks a von Hippel Lindau tumor suppressor gene that acts as the recognition component of an E3-ubiquitin ligase enzyme (150, 189), resulting in HIF-1α stabilization and binding to hypoxia-response elements in the promoter of target genes. As a consequence, HIF-α protein levels are low in the presence of oxygen and rise dramatically as oxygen levels decrease. HIF-1α is upregulated in response to thrombin, LPS, angiotensin II, or cytokines (19, 85, 91). However, ROS can also upregulate HIF-1α transcription under certain conditions by activating NF-κB, thus linking this important pathway to oxidant signaling (20).

Ets

Ets-1 functions as a critical downstream transcriptional mediator of Ang II-induced ROS generation by regulating the expression of NADPH oxidase subunits such as $p47^{phox}$ (160, 237). To evaluate the potential of inhibiting Ets-1, dominant negative Ets-1 membrane-permeable peptides were administered systemically into mice infused with Ang II. Ang II–induced ROS production and medial hypertrophy in the thoracic aorta were markedly diminished as a result of blocking Ets-1 (160) In addition, Ets-1 is transcriptionally upregulated by H_2O_2 via an antioxidant response element (237).

p53

p53, primarily considered as a tumor suppressor, is deactivated by hyperphosphorylation. p53 transactivation of the cell cycle inhibitor $p21^{Cip1/waf1}$ leads to inactivation of cyclin-dependent kinases (Cdks). Inhibition of Cdks leads to dephosphorylation and inactivation of retinoblastoma protein (Rb) which releases the transcription factor E2F from its inhibitory binding, allowing it to activate gene transcription required for DNA synthesis (223). Some cell cycle pathways, governed by the cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, are regulated by p53-dependent and p53-independent pathways. p53 has also received attention because it is induced by oxidative stress and plays an important role in cell cycle regulation. Once it is induced, p53 can also act as a transcriptional activator for $p21^{Cip1}$ (202). ROS derived from Nox2 (but not Nox4) are functionally involved in the regulation of the cell cycle inhibitors $p21^{Cip1}$ and p53 and participate in EC cell cycle regulation and apoptosis (135). MnSOD is a primary antioxidant enzyme whose transcription is regulated by Sp1, NF-κB and p53 (55). Angiotensin II activates p53 through the phosphorylation of Ser15 and Ser20, residues that are commonly phosphorylated in response to DNA damage. It is proposed that angiotensin II promotes the oxidation of DNA, which in turn activates p53, resulting in the mediation of apoptosis (87). After exposure to oxidative stress, nuclear IKKalpha regulates the transcription activity of the p53 by phosphorylation at Ser20 (244). H_2O_2 is cytotoxic at high concentrations and activates p53 independent of NO (220). Thus, the bioavailability of NO and superoxide is a crucial factor in the amount of p53 expressed and in the regulation of the EC cell cycle.

Physiological and Pathophysiological Roles of ROS Signaling in ECs

Oxygen sensing

Oxygen sensing is a process required for normal functioning of cells. Impaired oxygen sensing in humans has been associated with pathologic states including cancer, hypertension, sleep apnea, heart failure, stroke, and sudden infant death syndrome (205). Oxygen-sensing mechanisms at both the organ and cell levels are interdependent, such that an increase in hypoxia stimulates cellular metabolism as well as increased ventilation through increased breathing rate. The carotid body situated in the carotid artery is the primary sensor of oxygen concentration in mammals. At the molecular level, oxygen "sensing" is poorly understood. Sensing of oxygen may involve cellular mitochondria, membranes which chelate iron and potassium channels, outward movement of ATP at the afferent nerves, generation of ROS, nitric oxide, carbon monoxide, and neurotransmitters (127). NADPH oxidase itself has also been suggested as a possible oxygen sensor (198).

EC migration

EC migration is important in inflammation, vascular injury, angiogenesis, and other vascular disorders. The initial polarization of the cell towards the direction of intended migration involves reorganization of the cytoskeleton and has been shown to require Rac1 and ROS production (155). Actin filament reorganization following exposure of EC to hypoxia-reoxygenation is also ROS dependent (44). Pretreatment of HUVEC with NAC abrogated serum-induced migration of HUVEC, indicating the importance of cellular ROS production for endothelial cell motility (230). The mechanism for this is not clear, but may involve the disruption of VE-cadherin-mediated EC adhesion. It is known that gaps in VE-cadherin are required for efficient endothelial cell migration (139). Previous studies have shown that Rac1 is im-

portant in its ability to regulate EC migration (86). VEGF is an important growth factor that stimulates NADPH oxidase and EC migration and overexpression of dominant negative N17Rac1 and antioxidants attenuate VEGF- and angiopoitetin-1-induced migration of ECs (228). The process of EC migration may also require degradation or modification of matrix proteins via matrix metalloproteinases (MMPs), a diverse family of mechanosensitive zinc-dependent proteases that degrade ECM components (*e.g.*, collagen, laminin, and fibronectin) and nonmatrix substrates (*e.g.*, growth factors and cell surface receptors) (180). Although there is little information on ROS regulation of MMPs, there may be elevated MMP levels in $Nox2^{-/-}$ mice (117). VEGF is also an important factor involved in EC migration. The current paradigm for VEGF-induced EC migration is through the VEGF receptor type-2 (VEGFR2). The VEGFR2 binds to IQGAP1, a scaffolding protein that binds to actin and β-catenin and is a critical effector of Rac1. IQGAP1 which is a critical regulator for VEGF-induced ROS production and EC migration (107), colocalized with VE-cadherin and VEGFR2. Furthermore, IQGAP1 associates with Nox2 and recruits Nox2 to the leading edge, which is required for directional migration after wound injury in ECs (107). Thus, ROS may serve as important molecules linking growth factor signaling, vascular injury, and angiogenesis at the site of injury, and thereby may contribute to EC migration.

EC proliferation, survival, and apoptosis

NADPH oxidase activity is required for EC proliferation (25), cell survival, and apoptosis. Initial evidence for ROS-induced cell proliferation came from studies with Nox1 (213). *In vitro* studies based on either antisense or siRNA suppression suggested a role of Nox4 and Nox1 in smooth muscle cell proliferation (152, 212), a role of Nox5 in proliferation of esophageal adenocarcinoma cells (75), and increases in transcriptional activity and stability of the p22phox gene in ECs (15). Knockdown of Nox2, but not Nox4, blocked HIV1-Tat-induced cytoskeletal rearrangement, whereas knockdown of Nox4, but not Nox2, blocked HIV1-Tat-dependent proliferation of ECs through MAPKs (239). Nox2 and Nox4, equally contribute to ROS generation and increased proliferation under basal conditions, indicating that a complex relationship between Nox homologues controls endothelial proliferation (169). Nox4 expression promotes proliferation and migration of ECs as well as reduced serum deprivation-induced apoptosis via the Erk pathway (47). Nox activation can also result in cell death. ROS can trigger apoptosis either indirectly through damage to DNA, lipids, and proteins or directly by ROS-mediated activation of signaling molecules. Such proapoptotic signaling by ROS may occur through activation of MAP kinases, such as SAPK/JNK, ERK1/2, and p38 (108). MAP kinase activation is known to occur in many instances through ROS-dependent inhibition of tyrosine phosphatase (115). At higher ROS concentrations, hydrogen peroxide can inhibit caspases and thereby lead to a switch from apoptosis to necrosis (95). In some instances, however, Nox-derived ROS have a prosurvival effect. Nox-derived ROS may act as antiapoptotic signals through activation of the NF-κB (54) or Akt/ASK1 pathway (153). It has also been suggested that superoxide is a natural inhibitor of Fas-mediated cell death (40). Thus, NADPH oxidase-induced ROS can increase proliferation through increased expression of Nox, which leads to enhanced ERK activation and increased cell proliferation, apoptosis through ROS activation of SAPK/p38 MAPK, and DNA damage, or inhibition of protein phosphatases, or antiapoptotic through NF-κB activation.

ROS can signal both increased proliferation and apoptosis in ECs, depending on the concentration and cell type (167). High concentrations of ROS directly cause damage of DNA, lipids, and proteins that result in apoptosis (78) In contrast, several studies have shown that ROS at low nontoxic levels can induce cell signaling events (137). For example, the highly reactive $OH^{\cdot}$-radical and peroxynitrite are more damaging to cells, whereas low concentrations of H_2O_2 may have a more subtle cell-proliferative function (136). Haendeler *et al*. showed that low doses of H_2O_2 (10 and 50 μM) induce antioxidant TRX protein and mRNA expression (93) and also inhibited apoptosis (93). The mechanism for this may be due to TRX enhancing the binding activity of NF-κB and/or AP-1, which results in activation of several downstream signaling targets (164, 235) The subcellular localization of NADPH oxidase and antioxidant enzymes in caveolae, endosomal, and nuclear components, can also have an effect on how a cell responds to ROS (227). In addition, the type of ECs from different tissues or origin may have differences in the way they proliferate and respond to ROS. Cells from small vessels have greater ROS production than cells from large vessels which may be due to their higher proliferative rate (120)

Endothelial permeability

ROS signaling in ECs has an important role in the regulation of endothelial permeability. The molecule primarily responsible for adhesion of ECs is the transmembrane homophilic adhesion molecule, vascular endothelial (VE)-cadherin (49). Homotypic formation of firm EC–EC junctions is mainly attributed to VE-cadherin binding to the cytoplasmic domain of VE-cadherin-binding to β-catenin, and α-catenin linked to the actin cytoskeleton. H_2O_2 production by $Nox/p22^{phox}$-based NADPH oxidase can increase NF-κB activity to increase ICAM-1 expression on the EC surface which allows for firm adhesion of PMN via β2-integrins. H_2O_2 can also increase the expression of TRPM2 Ca^{2+} channel expression on the EC surface allowing for more Ca^{2+} to enter ECs, increasing phosphorylation of VE-cadherin and β-catenin. The linkage between VE-cadherin-based adherens junctional complex and the actin cytoskeleton contributes to the strong adhesion. Disruption of the endothelial barrier and breakdown of EC paracellular junctions is a hallmark phenotypic observation that frequently occurs in the presence of high oxygen tension (210) and inflammation. EC grown to confluence under hypoxia (5% O_2) form a tighter monolayer than ECs grown under normoxia (21% O_2). This tighter barrier in hypoxic cells appears to be due, in part, to inhibition of RhoA activity (207). Conversely, a number of studies indicated that severe acute hypoxia disrupts the EC barrier and increases EC permeability (238) due to an increase of ROS (238). We recently reported that H_2O_2 activates a ROS-sensitive channel, transient receptor potential melastatin (TRPM)2 (100), an oxidant-activated channel belonging to the TRP family of cation channels, thereby increasing $[Ca^{2+}]i$ and endothelial permeability (100). Tyrosine phos-

phorylation of VE-cadherin/β-catenin which prevents binding of β-catenin to p120, another member of the catenin family, is required for a decrease in cell-cell adhesion and resulting inhibition of EC barrier function (174).

ICAM-1 or VCAM-1 mediated ROS generation may also regulate endothelial permeability. ICAM-1 engagement was shown to lead to activation of two tyrosine kinases, Src and proline-rich tyrosine kinase 2 (Pyk2), and induces phosphorylation of VE-cadherin on Tyr658 and Tyr731, respectively, which correspond to the p120-catenin and β-catenin binding sites, respectively (4). VCAM-1 engagement was shown to activate Rac1, resulting in the generation of ROS that are capable of activating Pyk2 (217, 231) and Src (4). Furthermore, Rac1-induced ROS production disrupted VE-cadherin-mediated cell–cell adhesion (230). Decreased VE-cadherin function also activates Rac1 and increases the production of ROS, which subsequently leads to the loss of cell–cell adhesion. This reduced cell–cell adhesion was accompanied by increased tyrosine phosphorylation of β-catenin, which depends on the activation of Pyk2 that regulates cell adhesion (129) and phosphatidylinositol 3-kinase/Akt pathways (184). Thus, a positive feed-forward mechanism exists whereby ROS promote endothelial permeability.

Senescence

The fact that ROS play a key role in the aging process is based on substantial evidence showing that cellular senescence is regulated by antioxidant enzymes (41). Several studies report NOX induction of cellular senescence and cell cycle arrest (97, 203). In NOX4 overexpressing fibroblasts, there was an acceleration of cell senescence (83). In ECs, the senescent phenotype can be induced by many factors, including telomere damage (which leads to chromosomal instability), oxidative stress (35, 168), and sustained mitogenic stimulation (replicative senescence). In either type of senescence, cells flatten and enlarge, acquiring a "fried egg" appearance. Several lines of evidence indicate that EC senescence may be relevant to vascular disease. Oxidative stress and expression of oncogenic GTPase Ras appear to activate the senescence program mainly by involving the p16–pRb pathway through the p38MAPK signaling cascade (23, 52). Senescence has also been shown to accompany stress induced by peroxnitrite (229), H_2O_2, ox-LDL, and TNFα(22). Senescent ECs have changes in the level of expression and phosphorylation of eNOS and decreased production of NO and enhanced adhesion to monocytes (104, 147). Low doses of ROS can induce senescence whereas high doses of ROS can induce apoptosis (18). Prematurely senescent ECs show an impaired arginine-eNOS-NO pathway similar to that seen in aging of the endothelium (229). Thus, a balance of ROS appears to be an important factor in generation of senescent phenotype with antioxidant enzymes generally helpful in premature aging.

Angiogenesis

ROS play a crucial role in vascular angiogenesis, the formation of new vessels from pre-existing vessels. Angiogenesis involves a combination of EC and pericyte migration, proliferation, and appropriate spatial orientation to form new blood vessels for the passage of blood. The process is relevant in the pathological settings of chronic ischemia. Tissue hypoxia is one of the more potent stimuli for angiogenesis and rapidly induces proangiogenic growth factors such as VEGF (204). NADPH oxidase is involved in angiogenesis, and H_2O_2, when directly applied to cultured EC at a low concentration, stimulates tubular morphogenesis (247). NADPH oxidase-derived ROS selectively modulate some but not all the effects of VEGF on endothelial cell phenotypes (1). We have demonstrated that VEGF-induced angiogenesis involved a Nox2-based oxidase since it was inhibited by transfection of antisense Nox2 oligonucleotides, flavin-containing oxidase inhibitor, DPI, or dominant negative Rac1 mutant (228). Furthermore, in an *in vivo* sponge implant assay, angiogenesis was significantly inhibited in $Nox^{2-/-}$ mice or WT mice treated with antioxidants (228). Moreover, neovascularization in a hindlimb ligation model was significantly impaired in $Nox2^{-/-}$ mice (221). Nox4 NADPH oxidase also has an important angiogenic responses in human microvascular ECs (47). Peroxynitrite mediates VEGF's angiogenic signal and function via a nitration-independent mechanism in ECs (63). Small concentrations of oxLDL induce capillary tube formation from endothelial cells via a lectin-like oxLDL receptor dependent redox-sensitive pathway (46). This receptor is responsible for binding and uptake of oxLDL in ECs (46). Optimal ROS concentration and p38 MAP kinase are required for coronary collateral growth (185). Low concentrations of ROS produced during ischemia/reperfusion or preconditioning of hearts serve as signaling molecules to mediate myocardial angiogenesis (149). NADPH oxidase modulates myocardial Akt, ERK1/2 activation, and angiogenesis after hypoxia-reoxygenation (33). Angiopoietin-1-induced angiogenesis is modulated by endothelial NADPH oxidase (32) and IQGAP1, downstream target of Rac1, mediates ROS-dependent VEGF signaling at adherence junctions linked to angiogenesis (107). Physiological level of ROS is necessary for angiogenesis, as ischemia can induce VEGF which stimulates new vessel formation. In contrast, antioxidant therapy can block new vessel formation, suggesting that excess amount of ROS rather inhibits neovascularization. Thus, NADPH oxidase-induced ROS at optimal levels are required for the process of new blood vessel formation.

Endothelial dysfunction and regulation of vascular tone

Increased production of ROS via activation of NADPH oxidase reduces NO bioavailability, and thus causes impaired endothelium-dependent (NO-dependent) vasorelaxation (27, 136). Impaired endothelial vascular tone (or relaxation) is due to a rapid interaction between NO and superoxide to produce peroxynitrite, decreased eNOS expression, and/or loss of eNOS substrate L-arginine or cofactors tetrahydrobiopterin (BH4) (61). Hypoxia stimulates the local production of a vasoconstrictor, vasodilator or which can change vascular smooth muscle tone. Release of endothelium-derived relaxing factor (EDRF) is suppressed by hypoxia. The decrease in oxygen inhibits EDRF activity and increased pulmonary artery tone (186). Superoxide may also exert direct effects on vascular tone following dismutation to H_2O_2 (28). H_2O_2 released from the endothelium may account for endothelium-derived hyperpolarizing factor (EDHF) vasodilator activity in murine and human mesenteric arteries and in human coronary arterioles (144, 145).

Hypertension

Oxidative stress is involved in maintaining elevated blood pressure and developing hypertension-induced organ damage (154). In whole blood and in mononuclear cells from hypertensive subjects, there was an increase in oxidative stress and a reduction in the activity of antioxidant mechanisms that appeared to be independent of the blood pressure values (183). Moreover, antihypertensive treatment was able to reduce ROS close to normal levels (191). Although the increased ROS contributes to hypertension, several studies have demonstrated that ROS in hypertension is the consequence of not only an increase in ROS production but also inadequate responses of some of the antioxidant mechanisms (30). Several mutations or polymorphisms have been found in the $p22^{phox}$ gene that may contribute to ROS-induced hypertension (156). Angiotensin-II is also involved in oxidative stress-induced hypertension as it can increase blood pressure by activating NADPH oxidase as well as expression of NADPH oxidase subunits (76, 179). In $p47^{phox}$ knockout mice, AngII-increased hypertension was blocked (131). Several studies suggest the involvement of Nox1 in angiotensin II-induced hypertension using Nox1-deficient mice (146) and transgenic mice with smooth-muscle specific over-expression of Nox1 (56).

Atherosclerosis and vascular aging

Atherosclerosis is an inflammatory disease, occurring preferentially in branched arterial regions exposed to disturbed flow conditions such as oscillatory shear stress. NADPH oxidase-induced superoxide production contributes to endothelial dysfunction and atherosclerosis (89). Expression of NADPH oxidase subunits has been associated with the severity of atherosclerosis (208). Nitric oxide is known to mediate anti-atherosclerotic effects by inhibiting endothelial adhesion molecule expression and smooth muscle cell proliferation (81, 118). Nitric oxide rapidly reacts with superoxide to form peroxynitrite (88), which may contribute to atherosclerosis (21). NADPH oxidase-derived ROS also promote macrophage-mediated oxidation of LDL (8). Oxidized LDL also activates NADPH oxidase which further promotes ROS generation (190). In addition, NADPH oxidase may contribute to smooth muscle cell proliferation within the atherosclerotic plaque (13). Data from apolipoprotein (Apo) E–deficient mice suggest that the levels of several antioxidant enzymes decline during atherosclerosis (215) which implies a link between reduced antioxidant capacity and increased lesion formation. Conversely, overexpression of the antioxidant catalase reduced the severity of lesions in ApoE-deficient mice (246). $ApoE^{-/-}$ $p47^{phox-/-}$ mice have lower levels of aortic ROS production and less atherosclerosis than $apoE^{-/-}$ mice (13). Other mouse models with altered levels of SOD2 (11) and p66Shc (158) have produced a consistent theme that increased levels of vascular ROS promote, whereas decreased levels reduce atherogenesis.

Older age is a major risk factor for the development of cardiovascular disease (128). Dilation of the endothelium becomes impaired with aging in adult humans (82) and is thought to contribute to age-associated increase in cardiovascular disease (128). Reduction in endothelial dilation are associated with oxidative stress (59). Thus, age-associated decreases in endothelial dilation are inversely related to plasma markers of oxidative stress (65) and reversed by administration of supraphysiological concentrations of vitamin C (66).

Diabetes

Hyperglycemia is a primary cause of macro- and microvascular complications in diabetes. Elevated blood glucose levels play an important role in increasing superoxide generation through PKC activation and membrane lipid peroxidation to produce (90) advanced glycation end products (AGEs). Furthermore, superoxide generation in diabetic models has been linked to genetic polymorphisms (C242T) in the $p22^{phox}$ gene (99). AGEs are products of nonenzymatic glycoxidation and oxidation of proteins (14). Formation of AGEs depends on the status of hypoglycemia and the level of ROS produced (74). AGEs act through receptors termed receptor for AGE (RAGE), a member of the IgG superfamily of cell surface proteins (159). RAGE is highly expressed in lung endothelial cells, but its role in acute lung injury has not been extensively studied (194). RAGE is also a receptor for S100/calgranulins and high-mobility group box-1 (HMGB1), or amphoterin members of the TLR family, and mediate the production of proinflammatory molecules leading to an increase in MAPK, NF-κB, and AP-1 activities which increases EC VCAM-1 expression, unchecked sustained inflammation, and endothelial permeability (29, 194). In terms of EPC physiology and the prevention of disease, HMGB1 increases adhesion and migration of EPCs in a manner dependent on integrins and RAGE and stimulates the homing of EPCs into tumor and ischemic tissues *in vivo* (29). Furthermore, impaired reparative angiogenesis impedes proper postischemic healing and wound closure in diabetic patients. This defect was attributed to the shortage of, or insensitivity to, angiogenic growth factors, including VEGF. The circulating EPCs that play a critical role in forming new vessels are also dysfunctional in hyperglycemia. We previously demonstrated that ROS derived from $gp91^{phox}$ (Nox2)-based NADPH oxidase are involved in the activation of VEGF signaling, leading to EC proliferation and migration as well as reparative neovascularization in response to hindlimb ischemia (221). In contrast, overproduction of ROS (oxidative stress) via enhanced expression of Nox2 in ECs and EPCs contributes to impairment of neovascularization in a type1 diabetes model of mice (62). It was also shown that ablation of Nox2 improved neovascular and EPC function in diabetic mice. These results suggest that overproduction of ROS impair EC and EPC function in diabetes while optimal concentration of ROS is required for their normal physiological function.

Diabetes, in the reverse situation, can lead to increase in vascular dysfunction, as the disease itself can lead to increased ROS (Fig. 1). High glucose levels lead to a dramatic increase in vascular dysfunction. They include high diacylglycerol levels, high PKC activities (109), and high ROS production by ECs (42). In aortas from streptozotocin-treated rats, Nox 2 expression was elevated nine-fold (101). Thus, ROS serve a very important role for susceptibility or progression of diabetes.

Acute lung injury (ALI) and sepsis

The generation of oxidants induced by the bacterial cell wall constituent, lipopolysaccharide (LPS), has an important

signaling function in ECs (68). Oxidants mediate stable ICAM-1 expression-dependent endothelial adhesivity, resulting in the arrest of polymorphonuclear leukocytes (PMNs) (110). The ICAM-1-dependent PMN binding to ECs and EC activation are critical in microbial killing, but they can also mediate lung injury (105) and tissue edema (105, 141), the hallmarks of acute lung injury (ALI) associated with severe sepsis . Studies have focused on the cellular responses of individual cells (*i.e.,* PMNs or ECs) and have emphasized the role of cytokines, chemokines, and oxidants in the pathogenesis of ALI. Although PMNs have been implicated to play an essential pathogenic role in ALI (9), little is known about how PMN–EC interactions, which normally serve an essential host defense function, can mediate lung vascular endothelial injury. It was shown that ROS generated by the PMN NADPH oxidase complex, upregulate the cell surface expression of the pathogen-associated molecular pattern recognition receptor, Toll-like receptor 2 (TLR2), in ECs (67). This observation raises the intriguing possibility that similar induction of other TLRs, specifically the LPS receptor TLR4, in ECs by the PMN NADPH complex, may amplify the responsiveness of ECs to LPS-induced TLR4 expression, which is increased under inflammatory conditions. The PMN NADPH oxidase has also been shown to be required for the upregulation of ICAM-1 expression in ECs via NF-κB (67). Moreover, in coronary atherosclerotic plaques, TLR4 colocalizes with the p65 subunit of NF-κB (243). PMN accumulation depends on TLR4 expression by ECs rather than PMNs since PMN binding is reduced in EC *TLR4*$^{-/-}$ mice (6). Thus, the expression of such pattern recognition receptors as TLR2 and TLR4 by ECs is regulated by ROS which is generated by PMN NADPH oxidase, and may contribute to ALI.

ARDS has been defined as a severe form of ALI, featuring pulmonary inflammation and increased capillary leakage (234). A well-described pathophysiologic model of ARDS is one form of acute lung inflammation mediated by inflammatory cells and mediators as well as oxidative stress (51). An imbalance in the oxidant–antioxidant system has been recognized as one of the first events that ultimately leads to inflammatory reactions in lungs (43). The bacterial overload seen with sepsis is mainly due to gram-negative bacteria that carry LPS. LPS can induce septic conditions as well as induce ROS production. Sepsis is a major factor contributing to acute lung injury resulting from ischemia-reperfusion and or infection. Although it is known that the acute lung injury associated with Gram-negative sepsis is dependent on PMN infiltration and activation (70, 79), the extent to which oxidant generation itself is a determinant of PMN infiltration in lung tissue is unclear. Some reports indicate that impaired $O^{\cdot -}_2$ production can promote increased leukocyte migration (157). For example, NADPH oxidase knockout mice (*p47*$^{phox-/-}$ and *gp91*$^{phox-/-}$) (171, 199) exhibited increased peritoneal leukocytosis in response to thioglycolate (111, 171). *p47*$^{phox-/-}$ and *gp91*$^{phox-/-}$ mice have been used to address the role of the PMN respiratory burst in regulating PMN sequestration in lung tissue and migration into airspaces and the contribution of oxidant generation in the mechanism of lung microvascular injury (80). DeLeo *et al*. (50) demonstrated that LPS-rendered neutrophils are more responsive to other stimuli as a result of increased translocation of Rac2, p47^{phox}, and p67^{phox} (i.e., "priming"). Sanlioglu *et al*. (193) also reported that LPS induced Rac1-dependent ROS production and TNFα secretion in macrophages. We observed that there is greater lung tissue PMN sequestration and transalveolar PMN migration in *p47*$^{phox-/-}$ and *gp91*$^{phox-/-}$ mice, compared with wild-type mice after live *Escherichia coli* challenge (80). However, the lack of PMN $O^{\cdot -}_2$ generation in these mice prevented lung microvascular injury. A possible explanation of this finding comes from the observation that PMN sequestration was prevented by challenging the mice with heat-inactivated *E. coli*; thus, it appears that the augmented response is secondary to impaired microbial killing in the null mice (80). Lung tissue PMN sequestration and transalveolar migration were associated with increased bacterial load and dependent on the generation of ELR$^+$ (glutamic acid–leucine–arginine motif-positive) CXC chemokine, macrophage-inflammatory protein (MIP)-2, the functional murine homolog of IL-8 (80). TLR4 signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors (69). TLR4 signaling also induces TLR2 expression in ECs via PMN NADPH oxidase (67). PMN infiltration in lung tissue can occur in the absence of overt lung microvascular injury. Moreover, increased bacterial load in NADPH oxidase deficiency is a critical factor in activating the release of chemokines, and thereby augmenting PMN sequestration and migration into lung tissue (80).

Abbreviations

AGEs, advanced glycation end products; ALI, acute lung injury; AP-1, activator protein-1; APO, apolipoprotein; ARDS, acute respiratory distress syndrome; ASK1, apoptosis signal-regulating kinase 1; BH4, tetrahydrobiopterin; DUOX, dual oxidases; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; ELR+, glutamic acid-leucine-arginine motif-positive; ECs, endothelial cells; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; HIF1α, hypoxia-inducible factor1α; HMGB1, high mobility group box 1; HO, heme oxygenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MIP, macrophage-inflammatory protein; MKP-1, MAPK phosphatase 1; MMPs, matrix metalloproteinases; NF-κB, nuclear factor-κ B; NO, nitric oxide; NOS, nitric oxide synthase; NoXA1, Nox activator protein 1; NoxO1, Nox organizer protein 1; PAK, p21-activated kinase; PI3K; phosphatidylinositol 3 kinase; PIP_3, phosphatidylinositol trisphosphate; PKC, protein kinase C; PMN, polymorphonuclear leukocyte; PTK, protein tyrosine kinase; PTPs, protein tyrosine phosphatases; PX, phox homology; Pyk2, proline-rich tyrosine kinase 2; RAGE, receptor for advanced glycation end products; Rb, retinoblastoma protein; SOD, superoxide dismutase; TLR2, toll-like receptor 2; TNFα, tumor necrosis factor α; TRPM2, transient receptor potential melastatin 2; TRX, thioredoxin; VE, vascular endothelial; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

Disclosure Statement

No competing financial interests exist.

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