Forum Review Article

Regulation of NADPH Oxidase in Vascular Endothelium: The Role of Phospholipases, Protein Kinases, and Cytoskeletal Proteins

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Abstract

The generation of reactive oxygen species (ROS) in the vasculature plays a major role in the genesis of endothelial cell (EC) activation and barrier function. Of the several potential sources of ROS in the vasculature, the endothelial NADPH oxidase family of proteins is a major contributor of ROS associated with lung inflammation, ischemia/reperfusion injury, sepsis, hyperoxia, and ventilator-associated lung injury. The NADPH oxidase in lung ECs has most of the components found in phagocytic oxidase, and recent studies show the expression of several homologues of Nox proteins in vascular cells. Activation of NADPH oxidase of nonphagocytic vascular cells is complex and involves assembly of the cytosolic (p47^{phox}, p67^{phox}, and Rac1) and membrane-associated components (Noxes and p22^{phox}). Signaling pathways leading to NADPH oxidase activation are not completely defined; however, they do appear to involve the cytoskeleton and posttranslation modification of the components regulated by protein kinases, protein phosphatases, and phospholipases. Furthermore, several key components regulating NADPH oxidase recruitment, assembly, and activation are enriched in lipid microdomains to form a functional signaling platform. Future studies on temporal and spatial localization of Nox isoforms will provide new insights into the role of NADPH oxidase-derived ROS in the pathobiology of lung diseases. *Antioxid. Redox Signal.* 11, 841–860.

Introduction

THE VASCULAR ENDOTHELIUM has been recognized as a tissue capable of producing reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (OH[•]), nitric oxide (NO), and peroxynitrite (OONO[•]) (83, 129, 140, 181, 185). ROS and RNS have emerged as important second messengers modulating signal-transduction pathways involved in endothelial cell (EC) growth, migration, and barrier function.

Recent studies suggest that low levels of ROS modulate protein phosphorylation mediated by protein kinases and phosphatases, alter intracellular calcium levels, stimulate phospholipases, and regulate transcription factors and growth factors/growth factor receptors (28, 30, 45, 54, 99). However, excessive production and accumulation of ROS/RNS are

detrimental to cells and tissues, resulting in injury and ultimately loss of viability and death through oxidative damage to cellular macromolecules. In contrast to other organs in the body, the lung exists in a high-oxygen environment and is susceptible to injury by oxidative stress. Cigarette smoking, inhalation of airborne pollutants/toxins/oxidant gases and particulate matter result in direct lung damage as well as activation of lung inflammatory responses (220). Long-term exposure of lungs to higher oxygen tension (hyperoxia), as with premature babies and critically ill patients on ventilators, causes oxidative stress and lung injury (164, 184).

Increased ROS production has been directly linked to inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and acute respiratory distress syndrome (ARDS) (31, 115). The imbalance in the ratio of oxidants produced to oxidants detoxified (*i.e.*, a change in the redox equilibrium) seems to play an important role in the

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FIG. 1. Schematic diagram of assembly and activation of NADPH oxidase. Stimulus-mediated activation of phagocytic NADPH oxidase requires posttranslational modification, translocation of the cytosolic components p47^{phox}, p67^{phox}, and Rac2 to membrane-associated cytochrome b558 components of gp91^{phox} (Nox2) and p22^{phox}. This facilitates one-electron reduction of molecular O₂ to O₂⁻ by using NADPH as an electron donor.

development of various inflammatory lung diseases (168). Increased ROS production has been directly linked to oxidation of DNA, proteins, lipids, and sugars; remodeling of the extracellular matrix; alteration of mitochondrial respiration; and apoptosis (83). Furthermore, increased levels of ROS have been implicated in initiating signaling cascades leading to activation of transcription factors (NF- κ B and AP-1), chromatin remodeling, and gene expression of proinflammatory mediators (66, 134). ROS generated by phagocytes that have been recruited to sites of inflammation and excess generation of ROS by vascular cells are a major cause of edema and lung injury (140, 185). Generation of ROS and ROS signaling in lung endothelium alters vascular permeability *in vivo* (145, 184) and in endothelial monolayers (74, 185, 190).

Mechanisms of ROS-mediated modulation of endothelial barrier function are not yet clear; however, O₂⁻ production increases endothelial barrier permeability (129, 175). Recent evidence indicates that ROS are essential for normal lung/endothelial barrier function (76), and an imbalance of the redox equilibrium seems to contribute to pulmonary edema and leakiness (164, 165). An explosion has occurred in publications and reviews related to ROS-generating NADPH oxidases in cardiovascular health and disease states (21, 43, 49, 79, 147, 150, 155, 200, 220), with a limited focus on mechanisms of regulation. In this review, we address the Nox family of NADPH oxidase as a potential source of ROS involved in lung injury and regulation of endothelial NADPH oxidase activation by protein kinases, phospholipases, and the cytoskeleton.

NADPH Oxidase as a Major Source of Vascular/Endothelial ROS

The generation of ROS by aerobic cells occurs through enzymatic and nonenzymatic reactions (185). Subcellular organelles such as mitochondria, endoplasmic reticulum, nuclear membranes, peroxisomes, plasma membranes, and the cytoplasm have enzymatic systems to transfer electrons from NADH or NADPH to molecular O₂. In mammalian cells, in addition to mitochondrial electron transport, the other po-

tential enzymatic sources of ROS include arachidonic acid metabolism by cyclooxygenase/lipoxygenase, cytochrome P450, xanthine oxidase, NADPH oxidase, NO synthase, peroxidase, and other hemoproteins (30, 83, 195). Phagocytic cells of the immune system (neutrophils, eosinophils, monocytes, and macrophages) generate O₂⁻, instrumental in the killing of invading pathogens, solely by the NADPH oxidase (30, 83, 195). Deficiency of O₂⁻ results in the genetically inherited disorder, chronic granulomatous disease, a condition in which the affected individuals are susceptible to infection (6).

Phagocytic NADPH oxidase

Professional phagocytes kill infectious pathogens with an array of antimicrobial weapons. Of particular potency in this arsenal is the local generation of ROS, which disinfect engulfed microbes within phagolysosomes. Oxygen is reduced to O₂⁻ in a reaction catalyzed by the NADPH oxidase complex by using electrons derived from cellular NADPH (170, 174). Superoxide is subsequently converted to a host of other oxidants including H₂O₂ (170, 174). Because these oxidants cannot discriminate between host and pathogen targets, NADPH oxidase activity is regulated tightly by phagocytes. NADPH oxidase activation in the absence of infection is thought to be an important contributor to inflammatory disorders and tissue injury (11). NADPH oxidase is activated when cytosolic p47^{phox}, p67^{phox}, and Rac2 translocate to the phagosomes and plasma membrane and form a complex with integral membrane cytochrome b-558, which, in turn, is a Nox2 (gp91^{phox})/p22^{phox} heterodimer (39, 93) (Fig. 1). The Nox2 subunit binds to NADPH, FAD, and two hemes and is the electron transfer engine of the oxidase. p67^{phox} greatly facilitates electron flux through the oxidase, possibly by contributing to NADPH binding to Nox2 (44). p47^{phox} is not absolutely required for phagocytic NADPH oxidase activity *in vitro*, but appears to function as a chaperone in phagocytes, facilitating translocation of p67^{phox} and proper assembly of the oxidase (81). Rac2 is a member of the Rho class of GTPases and functions to activate p67^{phox} and other subunits of the oxidase

TABLE 1. LOCALIZATION, DISTRIBUTION AND PHYSIOLOGICAL FUNCTIONS OF NOX ENZYMES

<i>Nox/Duox isoforms</i>	<i>Physiological function</i>	<i>Tissue/cell expression</i>	<i>Subcellular localization</i>	<i>References</i>
Nox1	Cell proliferation and angiogenic switch	Colon, uterus, prostate, colon carcinoma cells, and VSMCS	Punctate surface distribution along cellular margins	28, 107, 135, 153, 167
Nox2	Immune defense, oxygen sensor, regulation of blood pressure	Phagocytes, endothelial and adventitial cells	Perinuclear, cytoplasm	13, 14, 107, 135, 178
Nox3	Development of fetal kidney	Fetal kidney and cancer cell line HepG2	Plasma membrane	14, 107, 165
Nox4	Regulation of erythropoietin synthesis, oxygen sensor	Kidney, endothelial cells, heart, skeletal muscle, brain	Focal adhesions, ER, nucleus	4, 74, 107, 121, 142, 165
Nox5	Calcium dependent ROS generation	Tissue lymphocytes, testis	Endoplasmic reticulum	56, 107, 135
Duox1 and Duox2	Thyroid hormone synthesis	Thyroid, bronchi, airway epithelial cells	Plasma membrane, ER	14, 105, 107, 108

(22, 44). Assembly of phagocytic oxidase is initiated by two signals. One signal is the phosphorylation of multiple serine and some tyrosine residues in the $p47^{phox}$, which leads to unmasking of $p47^{phox}$ SH3 domains that bind to a proline-rich target in the C terminus of $p22^{phox}$ (4, 23, 176, 211). The interaction between $p47^{phox}$ and $p22^{phox}$ seems to be an essential requirement for the translocation of other cytosolic components of the oxidase. The second signal for the assembly and activation of the phagocytic oxidase is the binding of GTP to Rac2, which leads to the dissociation of Rac from Rho-GDI, binding to $p67^{phox}$, followed by translocation of $p67^{phox}$ /GTP-Rac2 to the membrane (183). Furthermore, identification of phosphoinositide binding PX domains in $p47^{phox}$ suggests the involvement of inositol phospholipids in targeting membrane translocation of NADPH oxidase components (221).

Nonphagocytic NADPH oxidase

Although the phagocytic NADPH oxidase has been well characterized, recent studies suggest that a number of tissues contain NADPH or other oxidases involved in $O_2^{\cdot-}$ /ROS generation (10, 11, 170). In contrast to micromolar to millimolar levels of oxidants produced by phagocytic cells in response to a challenge from microorganisms or cytokines, oxidant production in nonphagocytic cells is low, typically in the nanomolar to micromolar range (166). Among the nonphagocytic cells, the endothelial and smooth muscle cell (SMC) NADPH oxidase has been studied more extensively (7, 22, 28, 61, 66, 112, 141, 156, 166, 181, 187, 193, 199). Several types of ECs from bovine pulmonary artery, porcine pulmonary artery, rat coronary microvascular, human lung, and human umbilical vein have been shown to express NADPH oxidase activity (11, 82, 156). The NADPH oxidase in ECs appears to have most of the key components found in the phagocytic oxidase. In most of these studies, the presence of Nox2 (formerly known as $gp91^{phox}$) and $p22^{phox}$ was described (66, 156); however, in one report, all four components were detected both at the mRNA level and by Western blotting (141). Activation of the vascular NADPH oxidase is mediated by hormones, proinflammatory cytokines such as TNF- α , mechanical forces, ischemia/reperfusion (7, 36, 64, 141), and by hyperoxia (38, 153, 156). In most of the reported studies, the generation of $O_2^{\cdot-}$ in response to a stimulus was partly

blocked by diphenyleneiodonium (DPI) or apocynin or both; however, DPI and apocynin are not specific NADPH oxidase inhibitors but affect ROS production either by blocking flavin containing enzyme(s) or by acting as an antioxidant, respectively (94, 186). Furthermore, recent studies suggest that vascular NADPH oxidase may be responsible for excessive $O_2^{\cdot-}$ generation in atherosclerosis, ischemic lung, pulmonary hypertension, and diabetes (7, 36, 66, 80, 130, 141, 182, 198).

Nox family of NADPH oxidases

Since the identification of *mox1*, which encodes a homologue of the catalytic subunit of the superoxide-generating NADPH oxidase of phagocytes, $gp91^{phox}$ (179), and based on the current database from human genome, seven NADPH oxidase (Nox) protein family members [Nox1, Nox2 ($gp91^{phox}$), Nox3, Nox4, Nox5, Duox1 and Duox2] have been described (78, 116, 117). All the family members share a common core structure made up of six transmembrane domains containing two heme-binding regions and a long cytoplasmic C-terminus region consisting of FAD- and NADPH-binding domains. However, Nox5, Duox1, and Duox2 have an N-terminal extension, and Nox 5 consists of 4 EF-hands (78). Furthermore, consistent with the ability of the EF-hand domain to bind calcium, Nox5, Duox1, and Duox2 are activated by Ca^{2+} (12, 47). The tissue distribution and putative physiologic functions of the Nox family are summarized in Table 1.

Expression of the Nox Family of Proteins in Vascular Tissues and Cells

Current data on the expression of the Nox family of proteins in cardiovascular tissues and cells are somewhat contradictory, and the utility of the data is limited because of lack of specific antibodies, species variations, and differences in passage numbers of cultured mammalian cells. Furthermore, mRNA expression profiles of Nox isoforms do not correlate with protein expression and oxidase activity. In HPAECs and HLMVECs, Nox4 mRNA expression was $\sim 1,000$ -fold higher compared with Nox2 expression, and Western blot analyses revealed that both Nox4 and Nox2 exhibit similar levels of protein expression. Protein expression is dictated by several

factors including level of mRNA half-life, translation, and turnover rate of the protein of interest. It is evident that no direct correlation exists between mRNA expression levels and protein expression, and in many instances, an increase in mRNA expression does not necessarily translate to similar levels of protein expression. In HPAECs, hyperoxia (24 h) increased mRNA levels by about eightfold compared with normoxia; however, the protein expression was enhanced by about threefold after hyperoxia (161). For example, Nox1 mRNA is highly expressed in vascular SMCs (169) as compared with Nox4 mRNA, which is the predominant isoform in lung ECs (46). In the endothelium, the Nox4 isoform, in addition to Nox2, is emerging as a key regulator of non-mitochondrial sources of ROS production. Nox1 and Nox2 genes have almost identical numbers and lengths of exons and exhibit (~60%) sequence homology (179). In addition to its constitutive expression, Nox1 message is induced by angiotensin II, PDGF, and PGF2 α in vascular smooth muscle (14). Nox4, based on the submitted sequence to The Human Gene Bank, is a 578-amino acid protein with ~39% sequence homology to Nox2; the linear sequence of Nox4 has heme, FAD, and NADPH-binding domains (78, 116). Originally described as a renal oxidase (Renox), Nox4 is highly expressed in the kidney (77), and recent studies have shown that all of the oxidase components are present in ECs from macro- and microvascular beds (61, 97, 116, 156, 199). The EC oxidase is constitutively active at low levels under basal conditions, and the activation of the oxidase by hyperoxia (156) or TNF- α (64) or other stimuli (10, 126, 187, 188) generates moderately higher ROS; however, the oxidative burst is much smaller compared with the phagocytic enzyme (22). Although it is well established that Nox2 is expressed in ECs, including human pulmonary artery endothelial cells (HPAECs) and human lung microvascular endothelial cells (HLMVECs) (13, 156), it appears that the mRNA levels for Nox4 in some of the ECs from rat and human is much higher compared with Nox2 (4). Interestingly, in HPAECs and HLMVECs, expression of p22^{phox} under unstimulated conditions, is several-fold higher (~10- to 50-fold) compared with Nox4 expression (161). In phagocytes, the expression levels of Nox2 and p22^{phox} are much higher with relatively low expression of Nox4 (4).

Contribution of Nox1, Nox2, and Nox4 to O₂⁻/ROS Production in Vascular/Lung Cells and Tissues under Normal and Pathologic Conditions

Nox1 generates O₂⁻ at very low levels under basal and stimulated conditions and depends on cytosolic NoxO1 and Noxa1 organizing proteins for activity (113, 117). Nox2 is a highly glycosylated protein, and its activation requires interaction with other membrane (p22^{phox}) and cytosolic (p47^{phox}, p67^{phox}, p40^{phox}, and Rac1/2) components. Although Nox2 is a critical component of phagocytic NADPH oxidase-mediated O₂⁻/ROS production, the role of Nox2 in vascular NADPH oxidase activity is controversial and may depend on cell types involved within the vessel wall (116, 166, 180, 212). For example, expression of Nox2 has been reported in arteriolar SMCs but not in aortic SMCs. As ECs express higher levels of Nox4 compared with Nox2, some recent data using anti-sense oligonucleotides to Nox4 suggest that Nox2 is involved in O₂⁻/ROS formation in vascular SMCs and ECs (61). In human pulmonary artery smooth muscle cells (PASMCs), hu-

man urotensin II (hU-II) activated NADPH oxidase, which was abrogated by p22^{phox} or Nox4 anti-sense oligonucleotides (52). Furthermore, depletion of Nox4 or p22^{phox} blocked hU-II-, but not S1P-mediated cell proliferation of PASMCs, indicating an involvement of Nox4 or p22^{phox} in mitogenesis (52). In 3T3-L1 adipocytes, Nox4, but not Nox2, appeared to be a major mediator of insulin-induced ROS production that was associated with oxidative inhibition of protein tyrosine phosphatase (PTP) 1B activity (80, 130). In human aortic SMCs, 7-ketocholesterol, a component of oxidized LDL, triggers NADPH oxidase activation and overproduction of ROS via Nox4 and JNK signaling (158). In cardiac fibroblasts, exogenous H₂O₂, oleoylacylglycerol, and free arachidonic acid (AA) stimulated ROS production that was attenuated by the nonspecific inhibitor, DPI, and antioxidants through pathways involving phospholipase A₂ and Nox4 (40). However, in many of the studies concerning Nox4 and ROS production, it is unclear whether Nox4 is subjected to a posttranslational modification as a prerequisite for activation or whether it is constitutively active like eNOS. Studies with HPAECs showed that generation of O₂⁻/ROS by hyperoxia (3–24 h) does not involve mitochondrial electron transport and is dependent on NADPH oxidase activation (156). Interestingly, Nox4 siRNA did not alter the expression of Nox1 and Nox3 levels; however, expression of Nox2 mRNA was upregulated on silencing Nox4, whereas Nox4 mRNA and protein expression were enhanced after knockdown of Nox2. Similarly, siRNA-mediated knockdown of p22^{phox} increased Nox4 mRNA levels by about twofold (161). A similar compensatory mechanism between Nox4 and Nox2 after siRNA treatment was observed in human cardiac ECs; however, in primary human bronchial epithelial cells and the adenocarcinoma cell line A549, knockdown of Nox2 and Nox4 by siRNA did not upregulate the expression of Nox4 or Nox2, respectively. These data suggest for the first time the ability of lung ECs to compensate reciprocally for Nox2 or Nox4 deficiency (146).

Role of NADPH Oxidase in Lung Injury

Lung injury represents a wide spectrum of pathologic conditions, the most severe being the acute respiratory distress syndrome (ARDS). Acute lung injury is a syndrome that includes pulmonary vasoconstriction, inflammation, and increased permeability of both the alveolar capillary endothelium and epithelium, resulting in arterial hypoxemia, resistant to oxygen therapy, and the presence of diffuse infiltrates on chest radiograph (98). Various studies have implicated involvement of ROS generated by NADPH oxidase activation in the pathobiology of acute lung injury. Although Nox2 and Nox4 seem to regulate hyperoxia-induced ROS production in lung ECs (159), a role for Nox2 in ROS generation from lungs and endothelial cells derived from Nox2 gene-targeted mice in response to normoxic lung ischemia has been demonstrated (7, 142, 225). Prolonged exposure to low O₂ tension induces pulmonary hypertension (PAH), which is characterized by vascular remodeling and enhanced vasoreactivity. Accumulating evidence suggests that Nox isoforms, and in particular, Nox2 and Nox4, are involved in long-term responses of the pulmonary vasculature to hypoxia (128). Hypoxia increases the expression of TGF- β (109), production of the TGF- β -activating protein furin (136), and Nox4 expression (178). TGF- β in turn induces HIF-1 α (136); thereby raising the

potential link between hypoxia, HIF-1 α , TGF- β , and Nox4 in pulmonary arterial hypertension. Hypoxia-induced endothelial dysfunction of intrapulmonary arteries was mediated *via* Nox2/ROS pathway in Nox2^{-/-} mice (68), and TGF- β -induced Nox4 expression and ROS production was implicated in proliferation of human pulmonary artery SMCs (178). Furthermore, hypoxia-dependent development of pulmonary arterial hypertension in mice has been causally linked to increased Nox4 expression in pulmonary artery SMCs (138). These studies suggest a key role for Nox4 in vascular remodeling associated with the development of hypoxia-induced pulmonary arterial hypertension (143). Additionally, Nox4 has been shown to be critical for HIF-2 α transcriptional activity in von Hippel-Lindau renal carcinoma (132), suggesting a potential role for HIF-1 α /HIF-2 α and Nox4 in hypoxia-induced pulmonary arterial hypertension.

In addition to involvement in hyperoxia- and hypoxia-induced lung injury, a role for Nox4 in LPS-induced pro-inflammatory responses by human aortic ECs has been described. Downregulation of Nox4 by transfection of Nox4 siRNA resulted in a failure to induce ROS generation and subsequent expression of ICAM-1, MCP-1, and IL-8 secretion in response to lipopolysaccharide LPS (157). Cigarette smoke is a major risk factor for the development of COPD, and prolonged exposure to CS induces lung inflammation and injury involving enhanced recruitment of inflammatory cells in the lungs and generation of ROS *via* NADPH oxidase. Interestingly, exposure of mice with targeted genetic ablation of NADPH oxidase components (p47^{phox} and gp91^{phox}) to CS showed decreased ROS generation; however, they were more susceptible to CS-induced lung inflammation, airspace enlargement, and alveolar damage (219). This pathologic abnormality was linked to enhanced TLR4/NF- κ B signaling in response to CS in p47^{phox}- and gp91^{phox}-knockout mice. In idiopathic pulmonary fibrosis, increased expression of NADPH oxidase components, p47^{phox} and p67^{phox}, and ROS production in the development of bleomycin-induced pulmonary fibrosis have been demonstrated (131, 205). Thus, Nox2 and Nox4 seem to play a role in lung injury and pulmonary diseases associated with enhanced ROS production and inflammation.

Functional Association of Nox1, Nox2, and Nox4 with Other NADPH Oxidase Components in Vascular Cells

Nox1 uses NADPH or NADH as a substrate and requires the membrane component, p22^{phox}, for O₂⁻ production. Furthermore, involvement of Rac1 and Nox organizer proteins, Noxo1 and Noxa1, in regulating Nox1 activity has been demonstrated (14, 113). Nox2 is selective for NADPH over NADH as a substrate for electron transfer to molecular oxygen, and its activation requires translocation of the cytosolic components to the Nox2/p22^{phox} complex in the phagosome or plasma membrane (14, 113). Nox2 gene expression is inducible, and its expression is increased in response to angiotensin II, hyperoxia, arterial injury, and hypoxia (35, 37, 111, 123). Although new knowledge on Nox 4 gene expression and its role in ROS production and vascular diseases is growing, very little is known regarding potential regulation of Nox4 and its interactions with other NADPH oxidase components. It is unclear whether assembly with cytosolic components

(p47^{phox}, p67^{phox}, and Rac1) is required for Nox4-mediated ROS formation. Certainly p22^{phox} and p47^{phox} appear to be required for O₂⁻/ROS production in HPAECs (38, 156) and Nox1 plus p22^{phox} in vascular SMCs (90), whereas blocking Rac1 or p67^{phox} reduces ROS in fibroblasts (50). Recent evidence suggests that p22^{phox} directly interacts with Nox4, as a mutation of the heme-binding site in Nox4 disrupts the complex formation of Nox4 as well as Nox1 with p22^{phox} (133). However, in HEK293 and COS-phox cells, overexpression of the NADPH oxidase components or siRNA treatment reduces ROS production by Nox4 that appears to be independent of Rac1, p47^{phox}, and p67^{phox}, but dependent on p22^{phox} (133). Furthermore, overexpression of p22^{phox} stabilizes Nox4, suggesting a potential role for the interaction between these two components in ROS generation and signal transduction (9, 114). Molecular mechanisms of activation of Nox4 are still poorly defined. Angiotensin II- (91) or H₂O₂-induced activation of Nox4 in mesangial cells or cardiac fibroblasts (40), respectively, was dependent on stimulation of PLA₂, release of AA, and increased ROS production. The peroxide, oleoyl-acetyl-glycerol, and arachidonic acid-mediated ROS generation in cardiac fibroblasts was insensitive to inhibitors of PKC (40); however, PKC δ upregulated activity of Nox1 *via* transactivation of EGF-R in A7r5 rat SMCs (63).

Protein Phosphorylation and Activation of NADPH Oxidase

Activation of NADPH oxidase in phagocytic and non-phagocytic cells is complex and involves assembly of the cytosolic (p47^{phox}, p67^{phox}, and Rac) and membrane-associated components (Nox2 and p22^{phox}) (39, 93). The signaling pathways leading to the activation of phagocytic NADPH oxidase is also complex and not completely defined. However, at least two distinct pathways of activation are known: one leading to translocation of the cytosolic phox components to the membrane flavocytochrome, and the other involving the activation and translocation of Rac.

Recent studies have identified a central role for lipid-derived second messengers, activation of protein kinases, and phosphorylation of NADPH oxidase components in the activation of phagocytic/nonphagocytic NADPH oxidase (11, 22). In phagocytes as well as in vascular cells, one of the cytosolic protein components that is phosphorylated is p47^{phox}; this step is crucial for the assembly and activation of the NADPH oxidase (10, 11). A number of earlier studies in leukocytes showed that p47^{phox} is phosphorylated at several serine residues located between amino acids 303 and 379 by protein kinase C (PKC), protein kinase A (PKA), mitogen-activated protein kinases (MAPKs), and p21-activated protein kinase (PAK) (58, 67, 106). Stimulation of neutrophils with fMLP enhanced phosphorylation of p47^{phox} by PKC- α (15), and on stimulation with angiotensin II, p47^{phox} is phosphorylated at serine and tyrosine residues in vascular smooth muscle cells (188). p67^{phox} was translocated from the cytosol to membranes (32), and p40^{phox} was phosphorylated by PKC. Similarly, in activated neutrophils, both Nox2 and p22^{phox} were identified as phosphoproteins, and phosphorylation of Rap1A was dependent on PKA (103). In addition to protein kinases, phosphatidic acid (PA)-dependent phosphorylation of p47^{phox} and p22^{phox} in neutrophil NADPH oxidation has been demonstrated both *in vivo* and *in vitro* (138, 152, 167);

however, the mechanism of PA-dependent activation of NADPH oxidase remains unclear. Hyperoxia activates lung EC NADPH oxidase, which in part was mediated by ERK and p38 MAPK signaling (156, 194).

More recently, a role for Src kinase in the activation of lung EC NADPH oxidase was demonstrated (38). Exposure of HPAECs to hyperoxia stimulated tyrosine phosphorylation of p47^{phox} (38), which was attenuated by PP2, dominant-negative Src and Src siRNA, suggesting Src-dependent phosphorylation of p47^{phox}. In addition, evidence for *in vitro* phosphorylation of p47^{phox} by Src and interaction between Src and p47^{phox} in hyperoxia-induced O₂^{•-} generation was provided, confirming the *in vivo* studies (38). Thus, regulation of NADPH oxidase activation in phagocytic and nonphagocytic cells is complex and includes posttranslational modifications of p47^{phox}, p67^{phox}, Nox2, and participation of homologues of p47^{phox} and p67^{phox}, named Nox organizing (NoxO1) and activating (NoxA1) proteins (116).

The Role of Phospholipases A₂, C, and D in NADPH Oxidase Activation

In intact neutrophils, modulation of signal-transduction pathways, initiated by specific receptor–ligand interactions, activate three types of phospholipases: phospholipase A₂, phospholipase C, and phospholipase D (62, 137, 146, 149, 167). Activation of PLA₂ releases arachidonic acid (AA) from the sn-2 position of membrane phospholipids, and its subsequent conversion to oxygenated derivatives by cyclooxygenase/lipoxygenase activates NADPH oxidase activity (40). 20-Hydroxyeicosatetraenoic acid (20-HETE), generated by cytochrome P-450 ω -hydroxylation of AA, activates NADPH oxidase and increases ROS production in bovine pulmonary artery endothelial cells (139). The role of PLA₂/AA in phagocytosis is not clear; however, AA and its oxygenated metabolites such as 20-HETE activate NADPH oxidase by promoting tyrosine phosphorylation and translocation of p47^{phox} through Rac1/2 signaling mechanisms in bovine pulmonary artery endothelial cells (139) and facilitate the membrane fusion necessary for phagosome formation. Phospholipase C may also be involved in PMN NADPH oxidase activation (69, 70). Diacylglycerol (DAG), generated by PIP₂-specific PLC or PC-specific PLC can activate PKC, which may phosphorylate components of NADPH oxidase and regulate the oxidase activation (69, 70). A correlation between stimulus-induced PKC activation, its translocation to the cytoskeletal fraction, and subsequent activation of NADPH oxidase in neutrophils has been demonstrated (149). In addition to PIP₂-specific PLC, PC-specific PLC may also be activated in response to phagocytic particles in PMNs, generating additional DAG for PKC activation. Two isoforms of phospholipase D (PLD), PLD1 and PLD2, have been cloned and partly characterized in mammalian cells (88, 89). PLD catalyzes the hydrolysis of phosphatidylcholine (PC) and other membrane phospholipids to PA and releases a polar head group, such as choline or ethanolamine or serine, depending on the phospholipid substrate (18, 19, 92). PLD, in addition to exhibiting a phosphohydrolase activity, also has a phosphatidyltransferase activity that catalyzes the transfer of PA to acceptors including water and primary alcohols (60) but not secondary or tertiary alcohols (144). Such a transphosphatidyl transfer reaction to alcohols such as methanol, ethanol, or 1-butanol results in the

generation of phosphatidylmethanol, phosphatidylethanol, or phosphatidylbutanol, respectively, and can be used as an index of PLD activation in response to a stimulus in mammalian cells (42). PA generated by PLD signal transduction is a second messenger (5), which regulates several functions including vesicular trafficking, cytokine secretion, activation of transcriptional factors, NADPH oxidase assembly, ROS generation, and cell motility (65, 166). PA is metabolized to diacylglycerol (DAG) by lipid phosphate phosphatases (42) or lipins (53), and lysophosphatidic acid (LPA) by PA-specific phospholipase A₁ or phospholipase A₂ (19, 42, 148). LPA is a naturally occurring bioactive lipid that signals *via* G protein-coupled LPA receptors in ECs and other cell types (41). Thus, PA generated by the PLD pathway and subsequent metabolism of PA to DAG modulates phagocytic and nonphagocytic NADPH oxidase (42, 137, 138, 152, 167, 171); however, the molecular mechanism(s) of PA-mediated NADPH oxidase activation is(are) poorly understood. *In vitro*, PA stimulates phosphorylation of subunits of NADPH oxidase through PA-dependent kinases yet to be identified (138, 204). PA, generated in tissues and cells *via* the PLD pathway, can activate PKC ζ (127), which can phosphorylate either p47^{phox} or p67^{phox} or p22^{phox}. Also, PA can regulate PIP₂ levels in cells *via* type 1 phosphatidylinositol-4-phosphate 5 kinase (PIP5K); thus, PA can modulate interactions between NADPH oxidase subunits and actin-binding proteins, thereby altering the actin cytoskeleton (51, 65, 151).

Phosphatidylinositol-3-Kinase Signaling in NADPH Oxidase Activation

Phosphatidylinositol-3-kinases (PI3 kinases) belong to a large family of enzymes that can be classified into three classes (I, II, and III) that phosphorylate the D3 position of phosphatidylinositol (PI) and phosphatidylinositol(4,5)P₂ to form PI3P and PI(3,4,5)P₃, respectively, which may then serve as signaling molecules (71, 214). Pharmacologic agents LY294002 and wortmannin, well-known inhibitors of PI3 kinase, attenuated IgG- and Fc γ R-mediated phagocytosis in activated PMNs, thus biochemically linking PI3 kinase activation to phagocytosis (223). Inhibition of class IA PI3K prevented TNF- α -induced O₂^{•-} production in neutrophils, which was confirmed with PI3K γ ^{-/-} mice (72). Further, in human lung ECs, TNF- α -induced activation of PI3K γ was upstream to PKC ζ -dependent NADPH oxidase assembly and stimulation of O₂^{•-} (69). The role of PI(3,4,5)P₃, generated by PI3K, in the context of NADPH oxidase assembly and activation, is more complex and may involve recruitment of a variety of regulatory proteins *via* interaction with their pleckstrin homology (PH) and PX domains (215). Additionally, activation of PI3K γ may elicit secondary priming and activation of PI3K δ -dependent PI(3,4,5)P₃ required for NADPH oxidase activation (72). Furthermore, activation of PI3 kinase has been linked to PLD activation and arachidonate release in stem cells, which was inhibited by LY 294002 (51). PI3K, independent of its lipid kinase activity, phosphorylates Akt, and activation of Akt modulates p47^{phox} phosphorylation and NADPH oxidase activation *in vitro* and *in vivo* (34, 102). However, in HL-60 and RAW264 cells, fMLP-induced O₂^{•-} production was mediated by PI3K regulation of p47^{phox} phosphorylation *via* diacylglycerol-dependent PKC δ but not Akt (216). Activation of endothelial cell NADPH oxidase

during normoxic lung ischemia is linked to Rac1 and PI3K signal transduction, which is sensitive to alterations in membrane potential associated with the acute loss of shear stress (224). Thus, mechanisms of PI3K activation of NADPH oxidase assembly and activation differ, depending on the cell type and stimulus.

Regulation of NADPH Oxidase by the Actin Cytoskeleton

Actin, a major component of the cytoskeleton, is present abundantly in phagocytic and nonphagocytic cells. Accumulating evidence supports a link between the actin cytoskeleton and actin-binding proteins in the activation of phagocytic and nonphagocytic NADPH oxidase (135). Stimulation of neutrophils with phorbol-ester resulted in co-sedimentation of the oxidase activity with heavy plasma membrane fractions containing actin and fodrin (58, 213). Furthermore, stabilization of the labile oxidase by chemical cross-linking prevented its extraction by Triton X-100, suggesting that the NADPH oxidase complex is linked to the actin filaments themselves (135, 213). Stimulation of neutrophils with phorbol-ester induced translocation of $p47^{phox}$ to the cytoskeleton without altering the distribution of either $p40^{phox}$ or $p67^{phox}$ and increased the oxidase activity that co-sediments with heavy plasma membrane fractions consisting of actin and fodrin (135, 213). A similar association of $p47^{phox}$ with actin has been shown in vascular SMCs and ECs (183, 187, 193, 221). Earlier studies suggested that disruption of actin fibers at the plasma membrane and phagosomes enhanced fMLP-triggered oxidative burst in phagocytes (17). In neutrophils, cytochalasin B potentiated the fMLP-induced accumulation of diacylglycerol (DAG), which could activate NADPH oxidase *via* protein kinase C (16, 17). Pretreatment of HPAECs with cytochalasin D and latrunculin A enhanced NADPH oxidase-mediated ROS production; thus, activation of both phagocytic and nonphagocytic NADPH oxidase by actin-destabilizing agents, such as cytochalasin D, may involve multiple signaling pathways associated with actin cytoskeletal reorganization (193). In addition to actin, actin-binding proteins such as coronin and cortactin are known to interact with NADPH oxidase subcomponents and are involved in the regulation of oxidase-dependent ROS production. Interestingly, the interaction between coronin and F-actin in adherent neutrophils was markedly diminished in cells from patients lacking $p47^{phox}$ or $p67^{phox}$, suggesting malfunctioning of the cytoskeleton in different genetic forms of chronic granulomatous disease (6); however, in this study, the role of coronin in regulating phorbol-ester-dependent NADPH oxidase activity was not evaluated.

Cortactin Regulates Endothelial NADPH Oxidase Activation

Cortactin (p80/p85), is an actin-binding protein, widely expressed in adherent cells including ECs (3). Cortactin is involved in many aspects of cytoskeleton-mediated cellular functions, such as cell motility, shape change (104, 126, 189), and barrier enhancement (56, 57, 74, 190), and is targeted to sites of actin polymerization and rearrangement (104). Translocation of cortactin from the cytosol to the periphery of the cell is observed in a variety of mammalian cells in response to different stimuli (57). In HPAECs, stimulating with sphin-

gosine-1-phosphate induced translocation of cortactin to the cell periphery and membrane ruffles (57), whereas shear stress also caused translocation of cortactin to the periphery *via* Rac GTPase in ECs (20). Studies by Zhan *et al.*, (104, 126) indicated that Src-mediated tyrosine phosphorylation of cortactin modulated F-actin cross-linking and ROS-induced injury of ECs. Cortactin interacts with F-actin and a number of other cytoskeletal proteins *via* putative interaction domains such as NTA, F-actin-binding sites, praline-rich PRR, and SH3 domains. Cortactin also stimulates the actin nucleating activity of the Arp 2/3 complex (189). In addition to tyrosine phosphorylation at Y412, Y466, and Y482, cortactin can be serine/threonine phosphorylated by MAPKs; however, the role of such phosphorylation in ROS production is unclear (206–208). A role for cortactin in hyperoxia-induced translocation of $p47^{phox}$ to the cell periphery and subsequent activation of NADPH oxidase and generation of ROS/O_2^- was recently demonstrated in lung endothelium (187, 188, 193). Exposure of HPAECs to hyperoxia for 3 h induced NADPH oxidase activation, as demonstrated by enhanced superoxide production (38, 156, 193). Hyperoxia also caused a thickening of the subcortical dense peripheral F-actin band and increased the localization of cortactin in the cortical regions and lamellipodia at cell–cell borders that protruded under neighboring cells (193). Pretreatment of HPAECs with the actin-stabilizing agent phalloidin attenuated hyperoxia-induced cortical actin thickening and ROS production, whereas cytochalasin D and latrunculin A enhanced basal and hyperoxia-induced ROS formation (193). In HPAECs, a 3-h hyperoxic exposure enhanced the tyrosine phosphorylation of cortactin and the interaction between cortactin and $p47^{phox}$, a subcomponent of the EC NADPH oxidase, when compared with normoxic cells (193). Furthermore, transfection of HPAECs with cortactin siRNA or myristoylated cortactin SH3-blocking peptide attenuated ROS production and the hyperoxia-induced translocation of $p47^{phox}$ to the cell periphery. Similarly, downregulation of Src with Src siRNA attenuated the hyperoxia-mediated phosphorylation of cortactin tyrosine residues and blocked the association of cortactin with actin and $p47^{phox}$ (38, 193). In addition, the hyperoxia-induced generation of ROS was significantly lower in ECs expressing a tyrosine-deficient mutant of cortactin than in vector control or wild-type cells (193). A similar role for $p47^{phox}$ /actin interaction, through cortactin, in angiotensin II-mediated assembly, NADPH oxidase activation, and ROS generation *via* p38 MAPK signal transduction has been demonstrated in human vascular SMCs (187). These results demonstrate a potential role for SH3/praline-rich PRR domains in Src, cortactin, and $p47^{phox}$ for interaction and assembly in activation of NADPH oxidase and ROS generation in human vascular cells (Fig. 2).

Endothelial Cell MLCK, MLC Phosphorylation, and NADPH Oxidase Activation

The driving force behind actin cytoskeletal reorganization is myosin ATPase, capable of generating mechanical force by promoting translational movement across actin stress fibers (3). Among the various myosin isoforms, myosin II is the predominant nonmuscle class of myosin consisting of homodimers of heavy chains (~200 kDa) and light chains (16–20 kDa). Myosin light-chain (MLC) phosphorylation is

Human pp60 Src Protein

Human Cortactin

FIG. 2. Schematic diagram depicting domains in Src, cortactin, and p47^{phox} proteins involved in assembly of NADPH oxidase components. Potential serine/threonine and tyrosine phosphorylation sites in Src and cortactin and individual SH2, SH3, proline-rich PRR, and PX domains are indicated in Src, cortactin, and p47^{phox}. Mutations at Y421, Y466, and Y482 residues affect assembly of p47^{phox} and formation of RO5/O₂⁻ in response to hyperoxia or angiotensin II.

regulated by either myosin light-chain kinase (MLCK), a calcium/calmodulin-dependent kinase and/or by MLC phosphatase regulated by Rho/Rho kinase (8, 55, 73, 202, 203). MLC phosphorylation at serine-19 and threonine-18 initiates myosin ATPase activity and actin polymerization, an essential component of smooth muscle and non-smooth muscle tension development (8, 55, 73, 121, 202, 203). The EC MLCK has a higher molecular mass (~214 kDa) compared with the smooth muscle type, and is derived from a single gene on chromosome 3 in humans, which also encodes the smooth muscle MLCK. The EC MLCK has ~95% homology with smooth muscle MLCK but, in addition, has a unique 922 amino acid N-terminus sequence with multiple sites for protein-protein interactions as well as sites for tyrosine phosphorylation by Src. Activation of MLCK by thrombin and other agonists caused endothelial contraction and barrier dysfunction, whereas inhibition of MLCK prevented an agonist-induced increase in EC permeability (56). Tyrosine phosphorylation of MLCK seems to play a regulatory role in thrombin-induced EC barrier dysfunction by promoting the development of actomyosin contractile complex consisting of MLCK, actin, myosin, calmodulin, Src, and cortactin (56). Activation of EC MLCK also modulates ROS production, as demonstrated in neutrophils by using an inhibitor of MLCK, ML-9 (96). Studies carried out with HPAECs suggest that hyperoxia/VEGF-mediated activation of EC MLCK and MLC phosphorylation partly regulates NADPH oxidase assembly of $p47^{phox}$ with cortactin, and ROS production (192). Thus, activation of EC MLCK may provide not only a mechanism that tightly regulates cytoskeletal rearrangement and cellular contraction, but also a protein platform for ROS production.

Coronin as a Negative Regulator of NADPH Oxidase Activation and ROS Generation

Coronins are actin-associated proteins that play an important role in cytoskeletal dynamics and are characterized by their ability to bind filamentous actin and the Arp2/3 complex. Gene-disruption studies show that coronin mutants are impaired in cytokinesis, cell locomotion, and phagocytosis (48). With the exception of Coronin 7, all coronins share a structural similarity comprising an N-terminal seven-bladed β -propeller, an unstructured (flexible) linker domain, and a coiled-coil region at the C-terminus. The coiled-coil region mediates homotrimerization of coronin 1 (75) and its binding to the p35 subunit of the Arp2/3 complex. Coronin 1 inhibits Arp2/3-mediated actin nucleation (75) by apparently locking the Arp2/3 complex into an open and inactive conformation. Phosphorylation of coronin 1B at serine 2 by PKC was recently shown to regulate coronin interaction with the Arp2/3 complex (29). Coronin trimerization generates a cytoskeletal binding site comprising positively charged residues within the linker domain, whereas the β -propeller region mediates coronin binding to the plasma membrane. Thus, by virtue of these binding domains, coronin may facilitate integration of extracellular signals from membrane receptors with F-actin remodeling. Coronin also binds the cytosolic NADPH component $p40^{phox}$ (84), but the physiologic relevance of this association is currently not well understood. In HPAECs, coronin 1B is highly expressed, as evidenced by real-time RT-PCR and Western blotting (26). Exposure of HPAECs to hyperoxia (95% O_2) for 3 or 24 h had no effect on the protein

expression of coronin 1B (26). Coronin 1B was localized at the leading edge of the cell periphery and colocalized with cortactin in membrane ruffles under normoxic conditions, and exposure to hyperoxia (3 h) increased accumulation of coronin 1B and cortactin in membrane ruffles at the leading edge of the lamellipodia (26). Downregulation of coronin 1B with coronin 1B siRNA enhanced basal (~300% control) as well as hyperoxia-induced (~450% control) generation of ROS (26). These results demonstrate that coronin 1B acts as a negative modulator of hyperoxia-induced ROS production in lung ECs. The mechanism of coronin 1B-mediated modulation of hyperoxia-induced ROS generation *via* NADPH oxidase is yet to be defined.

Rac1 as a Key Regulator of Vascular NADPH Oxidase

Rac belongs to the superfamily of Ras GTPases, and four isoforms, Rac1, Rac1b, Rac2, and Rac3, have been identified (87). Rac1 is expressed in nonhematopoietic cells and is likely the activator of Nox1, Nox3, Nox4, and Nox5, whereas Rac2 is expressed mostly in hematopoietic cells and is required for the activation of Nox2 in neutrophils (100). Several studies have suggested a role for Rac1 and Rac2 in ROS production *via* Noxes in vascular cells and polymorphonuclear leukocytes (85, 172). Current studies support a complex formation between $p67^{phox}$ and activated Rac in the membrane for optimal electron transport through flavocytochrome *b-558* in $O_2^{\cdot-}$ production. Translocation of Rac-GTPase to the plasma membrane, independent of $p47^{phox}$ and $p67^{phox}$, is essential for the assembly and activation of NADPH oxidase (1, 95); however, molecular mechanisms that regulate activation of Rac GTPases in phagocytosis and ROS generation remain incompletely defined. In unstimulated and resting cells, Rac-GDP is present as a complex with Rho-GDI, a negative regulator of Rho GTPases, and this complex dissociates and forms Rac-GTP in stimulated cells (23). Activation of Rac GTPase is facilitated by GTP exchange factors (GEFs) and several GEFs, including Vav1, Vav2, Tiam1, Rex1, and β -PIX, have been implicated in Rac activation and ROS production (101). Furthermore, GEFs may regulate Rac in a complex manner with different efficiencies. In the COS-phox system, expression of Vav1, Vav2, and Tiam1 resulted in variable oxidase activation by Rac (163). In this system, whereas Tiam1 and Vav2 were most effective in exchanging Rac-GDP for Rac-GTP, Vav1 was highly efficient in inducing the oxidase activity (163). This suggests that different GEFs regulate Rac localization of activated Rac at different sites and interaction of Rac with its effectors, such as Nox1. Several Nox isoforms can be regulated through Rac and have been implicated in Rac-dependent ROS generation in vascular cells (100). However, it is unclear whether Rac1 interacts directly or requires the so-called "activators" ($p47^{phox}$) or Nox1 ($p51^{phox}$) for Rac1-dependent regulation of Nox isoforms. Furthermore, the effectiveness of Rac1-dependent regulation of Noxes may depend on the host cell type tested and the type of Rac1 mutant used. In lung microvascular ECs, hyperoxia-induced redistribution of $p47^{phox}$ to the cell periphery is dependent on Rac1 activation mediated by PLD1/PLD2 (191), and further studies are necessary to delineate the mechanism(s) of Rac1-dependent translocation of $p47^{phox}$ and signaling in the activation of endothelial NADPH oxidase (Fig. 3).

FIG. 3. Role of Rac1 in regulating NADPH oxidase activation, ROS generation, and endothelial barrier function. Stimulation of vascular cells with an agonist such as angiotensin II or exposure to hyperoxia leads to the activation of PLD1/PLD2, and generation of phosphatidic acid (PA). PA regulates Rac1/Tiam1 activation, Src-dependent phosphorylation of p47^{phox} and cortactin, and assembly of the complex in lipid microdomains for ROS generation. ROS generated by NADPH oxidase alter endothelial functions such as permeability, proliferation, and migration.

IQGAP1 in Cytoskeletal Reorganization and ROS Generation

IQGAP1 is an IQ domain containing protein, with a region containing a sequence that has homology to RasGAP (25). IQGAP1 has several interaction sites of well-established cal-

ponin homology, WW, IQ motif, GRD, and RGCT domains (25). IQGAP1 interacts with several proteins, including signaling molecules such as Cdc42, Rac1, calmodulin, β -catenin, E-cadherin, actin filaments, and microtubules. Other proteins include end-tracking proteins (CLIP170) and adenomatous polyposis coli (APC), suggesting a role in cell polarity, adhesion, and migration (25, 217). A recent study indicates that IQGAP1 may link Nox2 to actin at the cells' leading edge, thereby enhancing ROS production and contributing to cell motility in ECs (105). Further characterization of the interactions between IQGAP1 and NADPH oxidase components will enhance our understanding of the molecular mechanisms of IQGAP1 regulation of NADPH oxidase activation and ROS generation in vascular endothelium.

Lipid Rafts, NADPH Oxidase Assembly, and ROS Generation

Lipid rafts, consisting of dynamic assemblies of cholesterol/glycolipids/phospholipids/signaling proteins on the cell membrane are clustered or aggregated in response to stimuli to form a physical signaling platform. Recent studies show that lipid rafts or cholesterol-enriched detergent-resistant membrane microdomains play an important role in the fMLP-, phorbol myristate acetate-, F_{cy} -, and angiotensin II-induced activation of NADPH oxidase by triggering the recruitment of key oxidase subunits (110, 118, 173, 218, 222). Caveolin-1 (Cav1), an integral component of lipid rafts and caveolae, seems to be essential for the activation of Rac1 and NADPH oxidase after angiotensin II stimulation of vascular smooth muscle cells (226). Although formation of signaling platforms in lipid rafts has been proposed in the development of atherosclerosis, hypertension, and ischemia/reperfusion injury, very little is known about the assembly of NADPH oxidase subunits and cytoskeletal proteins in lipid rafts, the mechanisms of ROS generation and barrier function. Preliminary studies suggest enrichment of NADPH oxidase

FIG. 4. Role of lipid rafts in the assembly and activation of NADPH oxidase. Lipid rafts are cholesterol- and sphingolipid-rich microdomains that serve as platforms for the assembly and compartmentalization of signaling proteins. Stimulation of vascular cells with an agonist or exposure of cells to hyperoxia promotes recruitment of Nox isoforms, p47^{phox}, p67^{phox}, p22^{phox}, Rac1, and cytoskeletal proteins such as cortactin and MLCK into lipid rafts. Cortactin and MLCK function as scaffold proteins to target Rac1 and NADPH oxidase components to lipid rafts rich in caveolin-1, thereby stimulating ROS production and ROS-dependent signal transduction, modulating cell function. (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).

subunits, actin/actin-binding proteins, and PIP kinases in lipid rafts after hyperoxia or HGF treatment of HPAECs, and Cav1 siRNA partly blocked hyperoxia-induced ROS generation (Irina Gorshkova and V. Natarajan, unpublished data). These results suggest a role for lipid rafts in NADPH oxidase assembly, activation, ROS generation, and barrier function in the endothelium (Fig. 4).

Redox and Thiol-Dependent Regulation of Nox Isoforms of NADPH Oxidase

Current evidence suggests that the phagocytic and non-phagocytic NADPH oxidase is redox regulated. H_2O_2 induces superoxide production (125) and increases expression and activity of Nox4 (40, 133). In K562 cells expressing Nox5, H_2O_2 -dependent Nox5 activation was achieved *via* the tyrosine kinase c-Abl through a Ca^{2+} -mediated, redox signaling pathway (59). Additionally, several oxidizing or alkylating thiol reagents modulated oxidase activity. Pretreatment with *N*-acetylcysteine, phenylarsine oxide, diamide, or 5,5'-dithio-bis(2-nitrobenzoic acid) or *p*-chloromercuriphenylsulfonic acid (108) attenuated agonist- or hyperoxia-induced ROS/ $O_2^{\cdot-}$ production, indicating a role for thiol-oxidoreductase-mediated mechanisms in regulating oxidase activity. Recent studies show a novel role of protein disulfide isomerase (PDI), a thiol oxidoreductase, in the regulation of NADPH oxidase in vascular smooth muscle cells (107, 120). Although redox pathways seem to be involved in PDI-mediated modulation of vascular NADPH oxidase, mechanisms underlying this modulation of oxidase activity remain undefined. It has been postulated that PDI may be involved in a redox-mediated on/off switch of the oxidase at subcellular organelles/membranes and processing or trafficking or both of Nox components between the endoplasmic reticulum and distal secretory pathways (120).

Nox and Phox Components of NADPH Oxidase as Potential Signaling Proteins

The role of Nox1, Nox2, Nox3, and Nox4 in ROS production is well documented in vascular and other cell types (61, 116, 199), animal models of diabetes (210), brain ischemia (198), hypertension (119, 154), coronary artery disease (2, 177), and restenosis (182). Additionally, evidence exists that Nox isoforms participate as a signaling protein in several physiologic functions in the cell. Recent studies show that increased expression of Nox4 under stress may be linked to apoptosis or proliferation (27, 201, 209). Nox4, as part of growth factor-stimulated NADPH oxidase activity, protected pancreatic cancer cells and melanoma cells from apoptosis through an undefined mechanism (24). Nox4, initially identified as Renox, may have a role in oxygen sensing in the kidney cortex (24, 77, 78) and as an oxygen sensor to regulate the two-pore weakly inward-rectifying K channel (TWIK)-related acid-sensitive K channel (TASK-1) (122). In HPAECs, TNF- α - or hyperoxia-mediated cell migration and capillary tube formation were partially prevented by Nox4 siRNA and *N*-acetylcysteine, and Nox4 siRNA attenuated TNF- α -dependent phosphorylation of ERK and I κ B in HPAECs, indicating the potential participation of Nox4 in signal transduction (160). Interestingly, in HUVECs, TNF- α -mediated activation of JNK was partly dependent on the expression of p47^{phox} protein expression, as disruption of the first SH3 binding site on p47^{phox} interfered with the activation of JNK (86). Recent studies provide evi-

dence for the role of Nox2 and Nox4 in EC proliferation. In HUVECs, the VEGF-dependent ROS generation and proliferation were attenuated by Nox2 anti-sense oligonucleotides (196). A novel role of gp91(phox)-containing NAD(P)H oxidase has been implicated in vascular endothelial growth factor-induced signaling and angiogenesis (196, 197). Similarly, silencing of Nox2 with siRNA inhibited the EC proliferative response (162). Nox2 and Nox4 mediate proliferative responses in endothelial cells (162). These studies provide evidence for Nox2 activity in EC proliferation. In contrast to Nox2, a role for Nox4 in EC proliferation is unclear. Nox4 seems to be critically involved in proliferation and inhibition of apoptosis of pulmonary artery adventitial fibroblasts *via* ROS production under normoxic and hypoxic conditions (124). Furthermore, Nox4 is localized to the endoplasmic reticulum in human aortic ECs and appears to be involved in the regulation of PTP1B and also an endoplasmic resident protein through redox-mediated signaling (33). Interestingly, Nox4-mediated oxidation and inactivation of PTP1B in the endoplasmic reticulum regulated EGF signaling and EGF-R trafficking in COS-7 cells (33). Thus, the specificity of ROS-mediated signal transduction by Nox isoforms may be regulated in a spatially dependent manner by differential localization within specific subcellular organelles and the nucleus. For example, localization of Nox2 in the plasma membrane and other organelles such as the endosomes might regulate cytoskeleton and cytoskeleton-dependent motility, and antiapoptotic signaling, whereas Nox4 localization in perinuclear and within the nucleus of the EC might regulate cell proliferation and migration. Furthermore, specific interactions of the SH3 and PX domains of NADPH oxidase subcomponents with the cytoskeletal proteins may confer signaling specificity or enhance signaling efficiency through ROS-dependent and -independent mechanisms in vascular cells and tissues.

Conclusions

Endothelial activation and barrier dysfunction occur in association with lung inflammation, ischemia/reperfusion injuries, sepsis, hyperoxia, and ventilator-associated lung injury. Recent studies demonstrated that the generation of ROS by vascular endothelial cells (ECs) plays a major role in the genesis of endothelial activation and barrier dysfunction. Despite several potential sources of ROS (mitochondrial electron-transport chain, cytochrome P-450 enzymes, xanthine oxidase, nitric oxide synthases, and the myeloperoxidase system), accumulating evidence suggests that Nox enzymes are also important sources of ROS in vascular tissues and cells, and enhanced production has been linked to the pathophysiology of several vascular/pulmonary disorders. Furthermore, an increasing number of studies point to Nox-derived ROS in regulating apoptosis, stimulating branching morphogenesis in sinusoidal endothelial cells, and cell motility. Activation of vascular NADPH oxidase and ROS/ $O_2^{\cdot-}$ production is complex and is regulated by phosphorylation and assembly of oxidase subcomponents using cytoskeleton/cytoskeletal proteins as protein platforms. Specific cytoskeletal proteins such as the cortical actin-binding proteins and contractile effectors, cortactin and myosin light-chain kinase (MLCK), are essential participants in the assembly and activation of p47^{phox} involved in ROS generation and EC

Abbreviations

AP-1, activator protein 1; Cav, caveolin; DAG, diacylglycerol; Duox, dual oxidase; ECs, endothelial cells; EF-hand, named arbitrarily after the E and F regions (helix-loop-helix Ca^{2+} -binding motif) of parvalbumin; ERK, extracellular-signal-regulated kinase; GEF, guanine nucleotide exchange factor; GRD, Ras GTPase-activating protein related domain; H_2O_2 , hydrogen peroxide; HGF, hepatocyte growth factor; HLMVECs, human lung microvascular endothelial cells; HPAECs, human pulmonary artery endothelial cells; IQ-GAP1, IQ motif containing GTPase activating protein 1, JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MLCK, myosin light-chain kinase; NF- κ B, nuclear factor κ B; NO, nitric oxide; NOS, nitric oxide synthase; Noxes, NADPH oxidases; PA, phosphatidic acid; PAK, p21-activated protein kinase; PC, phosphatidylcholine; PDI, protein disulfide isomerase; phox, phagocytic oxidase; PI, phosphatidylinositol; PIP5K, phosphatidylinositol-4-phosphate 5 kinase; PI3K, phosphatidylinositol-3-kinase; PIP_2 , phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLA_2 , phospholipase A_2 ; PLC, phospholipase C, PLD, phospholipase D; PMN, polymorphonuclear leukocytes; PTP1B, protein tyrosine phosphatase 1B; $\text{O}_2^{\cdot-}$, superoxide; OH $\cdot$, hydroxyl radical; $\text{OONO}^{\cdot-}$, peroxyxynitrite; PRR, proline rich region; PTP, phosphotyrosine phosphatase; PX, phox homology; RGCT, RasGAP-C terminus; RNS, reactive nitrogen species; ROS, reactive oxygen species; SMC, smooth muscle cell; TNF- α , tumor necrosis factor alpha; VSMCs, vascular smooth muscle cells; WW domain, a protein module that binds proline-rich or proline-containing ligands.

Disclosure Statement

No competing financial interests exist.

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