ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 4, 2009

DOI: 10.1089/ars.2008.2231

Forum Review Article

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

Srikanth Pendyala, Peter V. Usatyuk, Irina A. Gorshkova, Joe G.N. Garcia, and Viswanathan Natarajan[1]

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 ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), 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

[1]Department of Medicine, The University of Chicago, Chicago, Illinois.

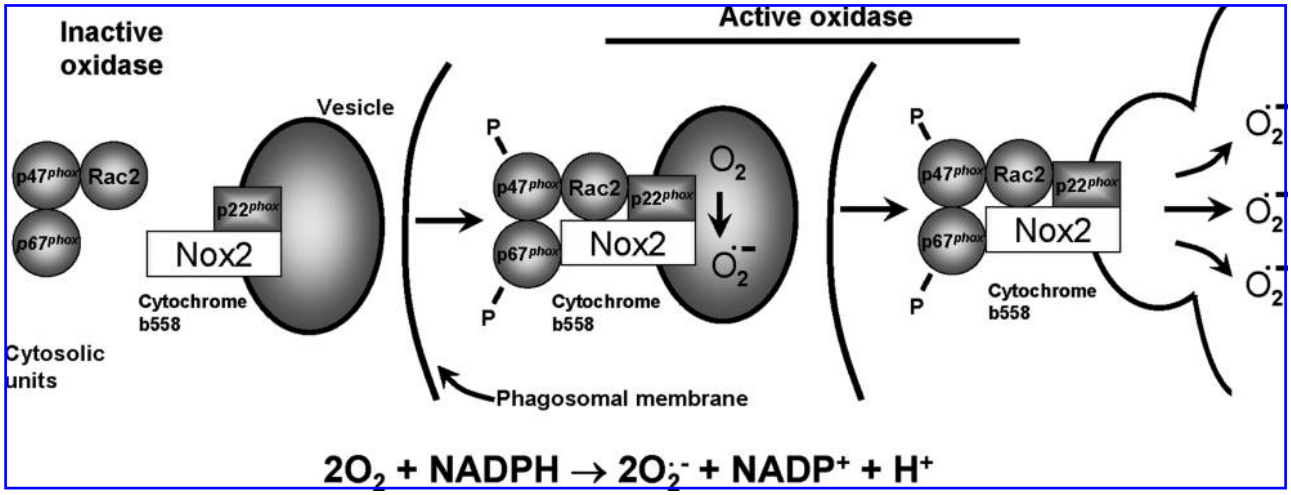

FIG. 1. Schematic diagram of assembly and activation of NADPH oxidase. Stimulus-mediated activation of phagocytic NADPH oxidase requires posttranslation modification, translocation of the cytosolic components p47^{phox}, p67^{phox}, and Rac2 to membrane-associated cytochrome *b558* components of gp91^{phox} (Nox2) and p22^{phox}. This facilitates one-electron reduction of molecular O_2 to $O_2^{\cdot -}$ by using NADPH 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-κB 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, $O_2^{\cdot -}$ 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 O_2. In mammalian cells, in addition to mitochondrial electron transport, the other potential 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 $O_2^{\cdot -}$, instrumental in the killing of invading pathogens, solely by the NADPH oxidase (30, 83, 195). Deficiency of $O_2^{\cdot -}$ 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 $O_2^{\cdot -}$ 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 H_2O_2 (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

Nox/Duox isoforms	*Physiological function*	*Tissue/cell expression*	*Subcellular localization*	*References*
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 ~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 nonmitochondrial 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 PGF2α 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 $O_2^{\cdot-}$/ROS Production in Vascular/Lung Cells and Tissues under Normal and Pathologic Conditions

Nox1 generates $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$/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 $O_2^{\cdot-}$/ROS formation in vascular SMCs and ECs (61). In human pulmonary artery smooth muscle cells (PASMCs), human 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 H_2O_2, oleoylacetyglycerol, and free arachidonic acid (AA) stimulated ROS production that was attenuated by the nonspecific inhibitor, DPI, and antioxidants through pathways involving phospholipase A_2 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 $O_2^{\cdot-}$/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 O_2 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-1α (136); thereby raising the

potential link between hypoxia, HIF-1α, 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-2α transcriptional activity in von Hippel–Lindau renal carcinoma (132), suggesting a potential role for HIF-1α/HIF-2α 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 proinflammatory 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-κB 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 $O_2^{\cdot -}$ 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 $O_2^{\cdot -}$/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 H_2O_2-induced activation of Nox4 in mesangial cells or cardiac fibroblasts (40), respectively, was dependent on stimulation of PLA_2, release of AA, and increased ROS production. The peroxide, oleoylacetylglycerol, and arachidonic acid–mediated ROS generation in cardiac fibroblasts was insensitive to inhibitors of PKC (40); however, PKCδ 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 nonphagocytic 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 $O_2^{\cdot-}$ 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 A_2, 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 A_2, phospholipase C, and phospholipase D (62, 137, 146, 149, 167). Activation of PLA_2 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 PLA_2/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 PIP_2-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 PIP_2-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 transphosphatidylation 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 A_1 or phospholipase A_2 (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 PKCζ (127), which can phosphorylate either $p47^{phox}$ or $p67^{phox}$ or $p22^{phox}$. Also, PA can regulate PIP_2 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)P_2 to form PI3P and PI(3,4,5)P_3, respectively, which may then serve as signaling molecules (71, 214). Pharmacologic agents LY294002 and wortmannin, well-known inhibitors of PI3 kinase, attenuated IgG- and FcγR-mediated phagocytosis in activated PMNs, thus biochemically linking P13 kinase activation to phagocytosis (223). Inhibition of class IA PI3K prevented TNF-α–induced $O_2^{\cdot-}$ production in neutrophils, which was confirmed with PI3K$\gamma^{-/-}$ mice (72). Further, in human lung ECs, TNF-α–induced activation of PI3Kγ was upstream to PKCζ-dependent NADPH oxidase assembly and stimulation of $O_2^{\cdot-}$ (69). The role of PI(3,4,5)P_3, 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 PI3Kγ may elicit secondary priming and activation of PI3Kδ-dependent PI(3,4,5)P_3 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 $O_2^{\cdot-}$ production was mediated by PI3K regulation of $p47^{phox}$ phosphorylation *via* diacylglycerol-dependent PKCδ 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 cosedimentation 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 sphingosine-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 $ROS/O_2^{\cdot -}$ 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 phallacidin 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

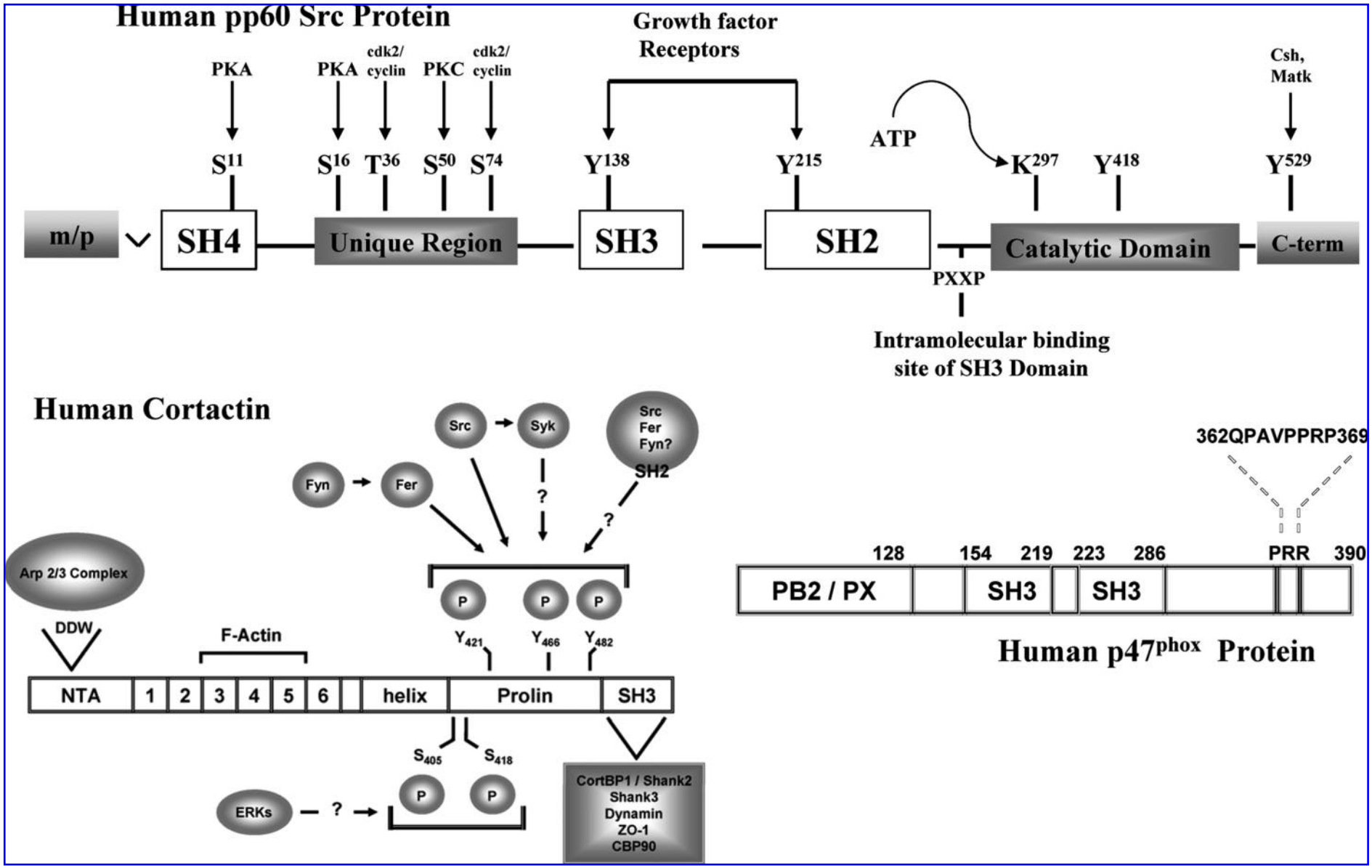

FIG. 2. Schematic diagram depicting domains in Src, cortactin, and p47^{phox} proteins involved in assembly of NADPH oxidase components. Potential serine/threonine and tyrosine phosphorylation sites in Src and cortactin and individual SH2, SH3, proline-rich PRR, and PX domains are indicated in Src, cortactin, and p47^{phox}. Mutations at Y421, Y466, and Y482 residues affect assembly of p47^{phox} and formation of ROS/$O_2^{\cdot -}$ 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 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 Noxa1 ($p51^{phox}$) 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).

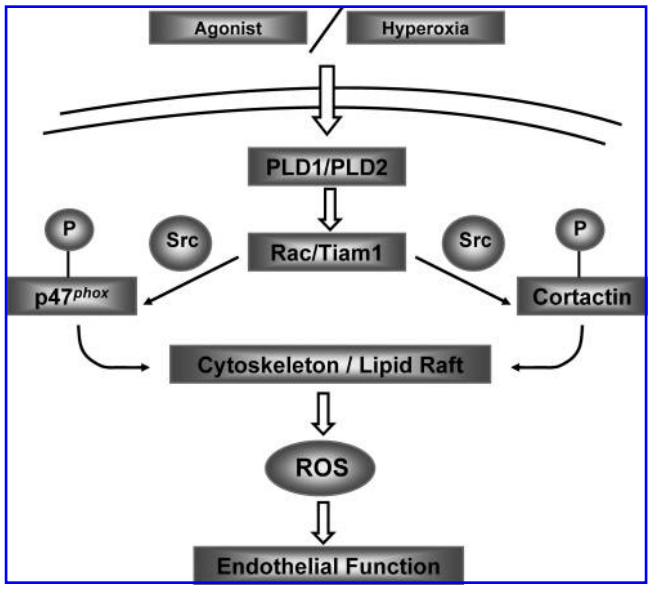

FIG. 3. Role of Rac1 in regulating NADPH oxidase activation, ROS generation, and endothelial barrier function. Stimulation of vascular cells with an agonist such as angiotensin II or exposure to hyperoxia leads to the activation of PLD1/PLD2, and generation of phosphatidic acid (PA). PA regulates Rac1/Tiam1 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 calponin 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–, $F_c\gamma$-, 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

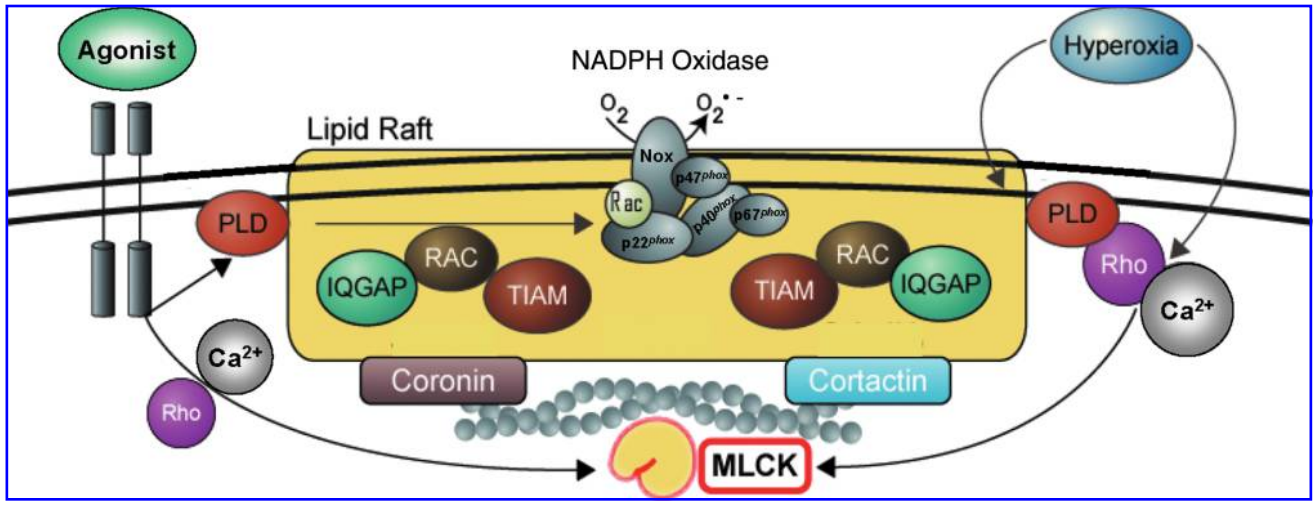

FIG. 4. Role of lipid rafts in the assembly and activation of NADPH oxidase. Lipid rafts are cholesterol- and sphingolipid-rich microdomains that serve as platforms for the assembly and compartmentalization of signaling proteins. Stimulation of vascular cells with an agonist or exposure of cells to hyperoxia 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 nonphagocytic 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 oxidase, diamide, or 5,5′ dithiobis(2-nitrobenzoic acid) or *p*-chloromercuryphenylsulfonic 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 IκB 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 evidence 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 in 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

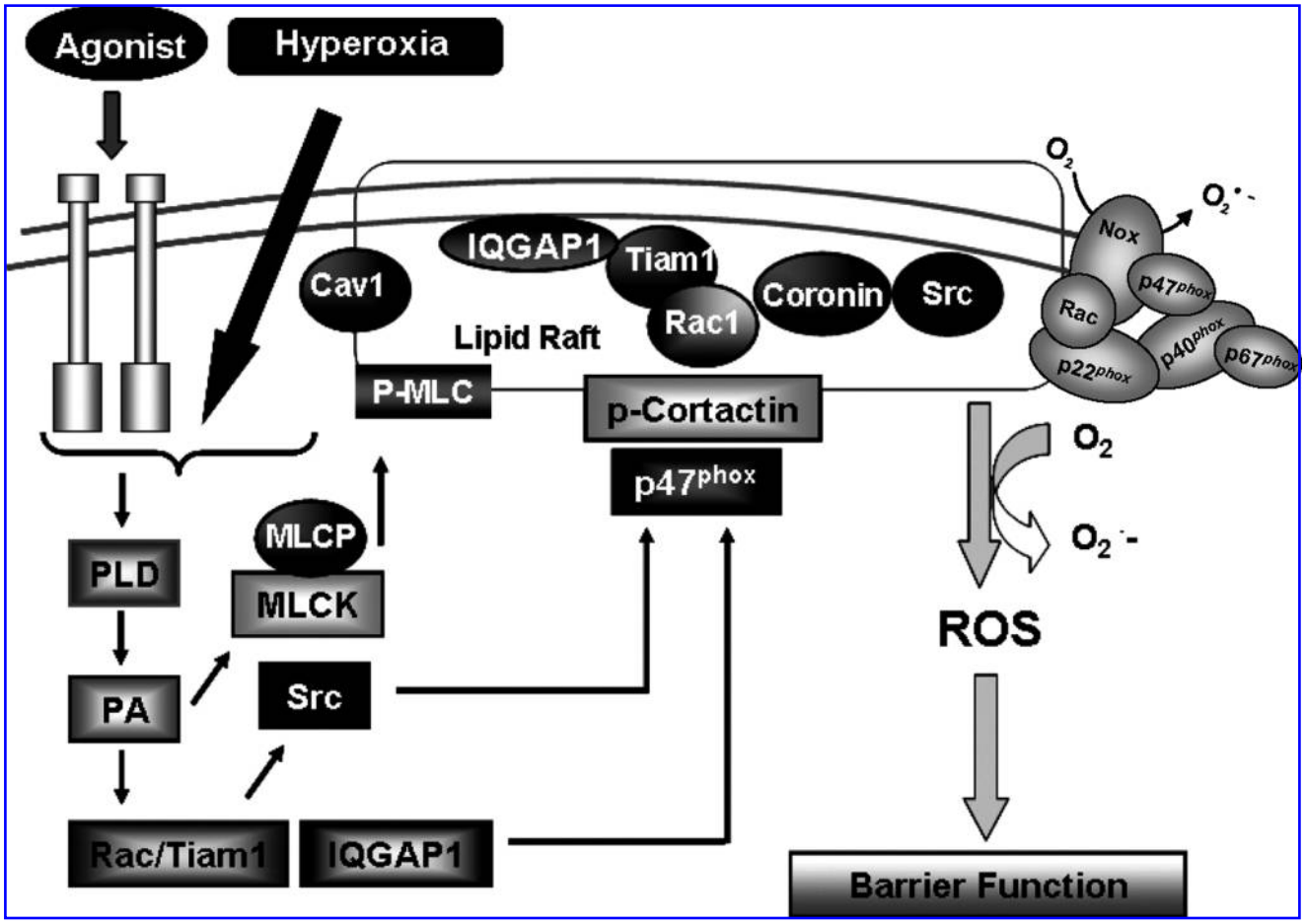

FIG. 5. Regulation of assembly and activation of NADPH oxidase by PLD, Rac1/Tiam1/IQGAP1, and cytoskeletal proteins in vascular cells. Stimulation of vascular cells with an agonist or exposure of cells to hyperoxia results in generation of phosphatidic acid (PA) through PLD signal transduction. Intracellularly generated PA functions like a second messenger and regulates Rac1 and IQGAP1 activation, tyrosine phosphorylation of cortactin, MLC phosphorylation mediated by the long form of endothelial MLCK, and $p47^{phox}$ tyrosine phosphorylation. Agonist- or hyperoxia-triggered phosphorylation of cytoskeletal proteins and NADPH oxidase components facilitates assembly of these scaffold proteins and NADPH oxidase in lipid rafts for ROS generation. Coronin, another actin-binding protein, counteracts the role of cortactin, by negatively regulating NADPH oxidase activation in response to stimuli.

activation. Additionally, membrane lipid rafts are involved in recruiting NADPH oxidase subcomponents and cytoskeletal proteins forming redox signaling platforms in regulating ROS production and endothelial function/dysfunction (Fig. 5). In contrast to excess ROS being toxic, low levels of ROS/$O_2^{\cdot-}$ generated in the vasculature, under basal and stimulated conditions, function as signaling molecules mediating cellular responses, such as growth, apoptosis, and migration. ROS generated in the vasculature are short lived and easily diffusible; thus, specific subcellular compartmentation of ROS is critical to targeted signal transduction of oxidant-sensitive proteins, which regulate different cellular responses. The recent identification of several homologues of Nox proteins, Nox1 to 5, in vascular cells, and their ability to generate ROS has further increased the complexity of localizing Nox-derived ROS and cellular functions. Furthermore, nuclear localization of Nox4 in certain types of endothelial cells raises a question on the role of nuclear ROS in regulating nuclear signals and redox-dependent gene expression. Future studies on temporal and spatial localization of Nox 1 to 5, $p47^{phox}$/$p67^{phox}$ and Rac in ROS generation, and assembly of the oxidase components with cytoskeletal proteins in lipid rafts will provide new insights into our understanding of the role of NADPH oxidase and ROS in the pathophysiology of lung diseases such as COPD, ARDS, and pulmonary hypertension. A combination of genetically engineered murine models of Nox 1 to 5 proteins with knockdown of targeted Nox proteins with siRNA/shRNA in cell-culture systems will likely provide molecular mechanisms regulating the assembly and activation of this multiprotein complex involved in various cellular responses.

Acknowledgments

We thank Dr. Michael Burns for his contributions to certain aspects of studies related to the cytoskeletal regulation by Coronin. The technical assistance of Ms. Donghong He is gratefully acknowledged. This work was supported by National Institutes of Health grants RO1 HL 08533 to V.N., and PO1 HL58064 to V.N. and J.G.N.

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-κB, nuclear factor κB; 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; $O_2^{\cdot -}$, superoxide; $OH^{\cdot}$, hydroxyl radical; $OONO^-$, peroxynitrite; 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.

References

1. Abo A, Webb MR, Grogan A, and Segal AW. Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem J* 298: 585–591, 1994.
2. Adams V, Linke A, Krankel N, Erbs S, Gielen S, Mobius-Winkler S, Gummert JF, Mohr FW, Schuler G, and Hambrecht R. Impact of regular physical activity on the NAD(P)H oxidase and angiotensin receptor system in patients with coronary artery disease. *Circulation* 111: 555–562, 2005.
3. Adelstein RS. Regulation of contractile proteins by phosphorylation. *J Clin Invest* 72: 1863–1866, 1983.
4. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, and Iida M. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation* 109: 227–233, 2004.
5. Agwu DE, McPhail LC, Sozzani S, Bass DA, and McCall CE. Phosphatidic acid as a second messenger in human polymorphonuclear leukocytes: effects on activation of NADPH oxidase. *J Clin Invest* 88: 531–539, 1991.
6. Allen LA, DeLeo FR, Gallois A, Toyoshima S, Suzuki K, and Nauseef WM. Transient association of the nicotinamide adenine dinucleotide phosphate oxidase subunits p47phox and p67phox with phagosomes in neutrophils from patients with X-linked chronic granulomatous disease. *Blood* 93: 3521–3530, 1999.
7. Al-Mehdi AB, Zhao G, Dodia C, Tozawa K, Costa K, Muzykantov V, Ross C, Blecha F, Dinauer M, and Fisher AB. Endothelial NADPH oxidase as the source of oxidants in lungs exposed to ischemia or high K+. *Circ Res* 83: 730–737, 1998.
8. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, and Kaibuchi K. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* 271: 20246–20249, 1996.
9. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, and Brandes RP. Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J Biol Chem* 279: 45935–45941, 2004.
10. Babior BM. NADPH oxidase: an update. *Blood* 93: 1464–1476, 1999.
11. Babior BM, Lambeth JD, and Nauseef W. The neutrophil NADPH oxidase. *Arch Biochem Biophys* 397: 342–344, 2002.
12. Banfi B, Molnar G, Maturana A, Steger K, Hegedus B, Demaurex N, and Krause KH. A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem* 276: 37594–37601, 2001.
13. Bayraktutan U, Blayney L, and Shah AM. Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arterioscler Thromb Vasc Biol* 20: 1903–1911, 2000.
14. Bedard K and Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87: 245–313, 2007.
15. Bengis-Garber C and Gruener N. Involvement of protein kinase C and of protein phosphatases 1 and/or 2A in p47 phox phosphorylation in formylmet-Leu-Phe stimulated neutrophils: studies with selective inhibitors RO 31-8220 and calyculin A. *Cell Signal* 7: 721–732, 1995.
16. Bengtsson T, Dahlgren C, Stendahl O, and Andersson T. Actin assembly and regulation of neutrophil function: effects of cytochalasin B and tetracaine on chemotactic peptide-induced O_2- production and degranulation. *J Leukoc Biol* 49: 236–244, 1991.
17. Bengtsson T, Orselius K, and Wettero J. Role of the actin cytoskeleton during respiratory burst in chemoattractant-stimulated neutrophils. *Cell Biol Int* 30: 154–163, 2006.
18. Billah MM and Anthes JC. The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem J* 269: 281–291, 1990.
19. Billah MM, Lapetina EG, and Cuatrecasas P. Phospholipase A2 activity specific for phosphatidic acid: a possible mechanism for the production of arachidonic acid in platelets. *J Biol Chem* 256: 5399–5403, 1981.
20. Birukov KG, Birukova AA, Dudek SM, Verin AD, Crow MT, Zhan X, DePaola N, and Garcia JG. Shear stress-mediated cytoskeletal remodeling and cortactin translocation in pulmonary endothelial cells. *Am J Respir Cell Mol Biol* 26: 453–464, 2002.
21. Blanchetot C and Boonstra J. The ROS-NOX connection in cancer and angiogenesis. *Crit Rev Eukaryot Gene Expr* 18: 35–45, 2008.
22. Bokoch GM and Knaus UG. NADPH oxidases: not just for leukocytes anymore! *Trends Biochem Sci* 28: 502–508, 2003.
23. Bokoch GM and Zhao T. Regulation of the phagocyte NADPH oxidase by Rac GTPase. *Antioxid Redox Signal* 8: 1533–1548, 2006.

24. Brar SS, Kennedy TP, Sturrock AB, Huecksteadt TP, Quinn MT, Whorton AR, and Hoidal JR. An NAD(P)H oxidase regulates growth and transcription in melanoma cells. *Am J Physiol Cell Physiol* 282: C1212–C1224, 2002.
25. Brown MD and Sacks DB. IQGAP1 in cellular signaling: bridging the GAP. *Trends Cell Biol* 16: 242–249, 2006.
26. Burns MPS, Gorshkova, IA, Bear JE, Usatyuk PV, and Natarajan V. *Coronin 1B negatively regulates hyperoxia-induced reactive oxygen species generation in human lung endothelial cells.* San Francisco: American Thoracic Society, p. A530.
27. Byrne JA, Grieve DJ, Bendall JK, Li JM, Gove C, Lambeth JD, Cave AC, and Shah AM. Contrasting roles of NADPH oxidase isoforms in pressure-overload versus angiotensin II-induced cardiac hypertrophy. *Circ Res* 93: 802–805, 2003.
28. Cai H, Griendling KK, and Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci* 24: 471–478, 2003.
29. Cai L, Holoweckyj N, Schaller MD, and Bear JE. Phosphorylation of coronin 1B by protein kinase C regulates interaction with Arp2/3 and cell motility. *J Biol Chem* 280: 31913–31923, 2005.
30. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, and Shah AM. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 8: 691–728, 2006.
31. Chabot F, Mitchell JA, Gutteridge JM, and Evans TW. Reactive oxygen species in acute lung injury. *Eur Respir J* 11: 745–757, 1998.
32. Chen J, He R, Minshall RD, Dinauer MC, and Ye RD. Characterization of a mutation in the Phox homology domain of the NADPH oxidase component p40phox identifies a mechanism for negative regulation of superoxide production. *J Biol Chem* 282: 30273–30284, 2007.
33. Chen K, Kirber MT, Xiao H, Yang Y, and Keaney JF Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* 181: 1129–1139, 2008.
34. Chen Q, Powell DW, Rane MJ, Singh S, Butt W, Klein JB, and McLeish KR. Akt phosphorylates p47phox and mediates respiratory burst activity in human neutrophils. *J Immunol* 170: 5302–5308, 2003.
35. Chen Z, Keaney JF Jr, Schulz E, Levison B, Shan L, Sakuma M, Zhang X, Shi C, Hazen SL, and Simon DI. Decreased neointimal formation in Nox2-deficient mice reveals a direct role for NADPH oxidase in the response to arterial injury. *Proc Natl Acad Sci U S A* 101: 13014–13019, 2004.
36. Cheng JJ, Chao YJ, Wung BS, and Wang DL. Cyclic strain-induced plasminogen activator inhibitor-1 (PAI-1) release from endothelial cells involves reactive oxygen species. *Biochem Biophys Res Commun* 225: 100–105, 1996.
37. Chose O, Sansilvestri-Morel P, Badier-Commander C, Bernhardt F, Fabiani JN, Rupin A, and Verbeuren TJ. Distinct role of nox1, nox2, and p47phox in unstimulated versus angiotensin II-induced NADPH oxidase activity in human venous smooth muscle cells. *J Cardiovasc Pharmacol* 51: 131–139, 2008.
38. Chowdhury AK, Watkins T, Parinandi NL, Saatian B, Kleinberg ME, Usatyuk PV, and Natarajan V. Src-mediated tyrosine phosphorylation of p47phox in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells. *J Biol Chem* 280: 20700–20711, 2005.
39. Clark RA. Activation of the neutrophil respiratory burst oxidase. *J Infect Dis* 179(suppl 2): S309–S317, 1999.
40. Colston JT, de la Rosa SD, Strader JR, Anderson MA, and Freeman GL. H_2O_2 activates Nox4 through PLA_2-dependent arachidonic acid production in adult cardiac fibroblasts. *FEBS Lett* 579: 2533–2540, 2005.
41. Contos JJ, Ishii I, and Chun J. Lysophosphatidic acid receptors. *Mol Pharmacol* 58: 1188–1196, 2000.
42. Cummings R, Parinandi N, Wang L, Usatyuk P, and Natarajan V. Phospholipase D/phosphatidic acid signal transduction: role and physiological significance in lung. *Mol Cell Biochem* 234–235: 99–109, 2002.
43. Dammanahalli KJ and Sun Z. Endothelins and NADPH oxidases in the cardiovascular system. *Clin Exp Pharmacol Physiol* 35: 2–6, 2008.
44. Dang PM, Cross AR, Quinn MT, and Babior BM. Assembly of the neutrophil respiratory burst oxidase: a direct interaction between p67phox and cytochrome b558 II. *Proc Natl Acad Sci U S A* 99: 4262–4265, 2002.
45. D'Angio CT and Maniscalco WM. The role of vascular growth factors in hyperoxia-induced injury to the developing lung. *Front Biosci* 7: d1609–d1623, 2002.
46. Datla SR, Peshavariya H, Dusting GJ, Mahadev K, Goldstein BJ, and Jiang F. Important role of Nox4 type NADPH oxidase in angiogenic responses in human microvascular endothelial cells *in vitro*. *Arterioscler Thromb Vasc Biol* 27: 2319–2324, 2007.
47. De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE, and Miot F. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J Biol Chem* 275: 23227–23233, 2000.
48. de Hostos EL. The coronin family of actin-associated proteins. *Trends Cell Biol* 9: 345–350, 1999.
49. Delles C, Miller WH, and Dominiczak AF. Targeting reactive oxygen species in hypertension. *Antioxid Redox Signal* 10: 1061–1077, 2008.
50. Dhaunsi GS, Paintlia MK, Kaur J, and Turner RB. NADPH oxidase in human lung fibroblasts. *J Biomed Sci* 11: 617–622, 2004.
51. Divecha N, Roefs M, Halstead JR, D'Andrea S, Fernandez-Borga M, Oomen L, Saqib KM, Wakelam MJ, and D'Santos C. Interaction of the type Ialpha PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4, 5-bisphosphate in the regulation of PLD2 activity. *EMBO J* 19: 5440–5449, 2000.
52. Djordjevic T, BelAiba RS, Bonello S, Pfeilschifter J, Hess J, and Gorlach A. Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells. *Arterioscler Thromb Vasc Biol* 25: 519–525, 2005.
53. Donkor J, Sariahmetoglu M, Dewald J, Brindley DN, and Reue K. Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *J Biol Chem* 282: 3450–3457, 2007.
54. Dor Y, Porat R, and Keshet E. Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. *Am J Physiol Cell Physiol* 280: C1367–1374, 2001.
55. Dudek SM, Birukov KG, Zhan X, and Garcia JG. Novel interaction of cortactin with endothelial cell myosin light chain kinase. *Biochem Biophys Res Commun* 298: 511–519, 2002.
56. Dudek SM and Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 91: 1487–1500, 2001.
57. Dudek SM, Jacobson JR, Chiang ET, Birukov KG, Wang P, Zhan X, and Garcia JG. Pulmonary endothelial cell barrier enhancement by sphingosine 1-phosphate: roles for cor-

tactin and myosin light chain kinase. *J Biol Chem* 279: 24692–24700, 2004.
58. El Benna J, Faust RP, Johnson JL, and Babior BM. Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping: phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase. *J Biol Chem* 271: 6374–6378, 1996.
59. El Jamali A, Valente AJ, Lechleiter JD, Gamez MJ, Pearson DW, Nauseef WM, and Clark RA. Novel redox-dependent regulation of NOX5 by the tyrosine kinase c-Abl. *Free Radic Biol Med* 44: 868–881, 2008.
60. Ella KM, Meier KE, Kumar A, Zhang Y, and Meier GP. Utilization of alcohols by plant and mammalian phospholipase D. *Biochem Mol Biol Int* 41: 715–724, 1997.
61. Ellmark SH, Dusting GJ, Fui MN, Guzzo-Pernell N, and Drummond GR. The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc Res* 65: 495–504, 2005.
62. English D, Brindley DN, Spiegel S, and Garcia JG. Lipid mediators of angiogenesis and the signalling pathways they initiate. *Biochim Biophys Acta* 1582: 228–239, 2002.
63. Fan CY, Katsuyama M, and Yabe-Nishimura C. PKCdelta mediates up-regulation of NOX1, a catalytic subunit of NADPH oxidase, via transactivation of the EGF receptor: possible involvement of PKCdelta in vascular hypertrophy. *Biochem J* 390: 761–767, 2005.
64. Fan J, Frey RS, Rahman A, and Malik AB. Role of neutrophil NADPH oxidase in the mechanism of tumor necrosis factor-alpha -induced NF-kappa B activation and intercellular adhesion molecule-1 expression in endothelial cells. *J Biol Chem* 277: 3404–3411, 2002.
65. Farquhar MJ, Powner DJ, Levine BA, Wright MH, Ladds G, and Hodgkin MN. Interaction of PLD1b with actin in antigen-stimulated mast cells. *Cell Signal* 19: 349–358, 2007.
66. Fisher AB, Al-Mehdi AB, Wei Z, Song C, and Manevich Y. Lung ischemia: endothelial cell signaling by reactive oxygen species: a progress report. *Adv Exp Med Biol* 510: 343–347, 2003.
67. Fontayne A, Dang PM, Gougerot-Pocidalo MA, and El-Benna J. Phosphorylation of p47phox sites by PKC alpha, beta II, delta, and zeta: effect on binding to p22phox and on NADPH oxidase activation. *Biochemistry* 41: 7743–7750, 2002.
68. Fresquet F, Pourageaud F, Leblais V, Brandes RP, Savineau JP, Marthan R, and Muller B. Role of reactive oxygen species and gp91phox in endothelial dysfunction of pulmonary arteries induced by chronic hypoxia. *Br J Pharmacol* 148: 714–723, 2006.
69. Frey RS, Gao X, Javaid K, Siddiqui SS, Rahman A, and Malik AB. Phosphatidylinositol 3-kinase gamma signaling through protein kinase Czeta induces NADPH oxidase-mediated oxidant generation and NF-kappaB activation in endothelial cells. *J Biol Chem* 281: 16128–16138, 2006.
70. Frey RS, Rahman A, Kefer JC, Minshall RD, and Malik AB. PKCzeta regulates TNF-alpha-induced activation of NADPH oxidase in endothelial cells. *Circ Res* 90: 1012–1019, 2002.
71. Fry MJ. Structure, regulation and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* 1226: 237–268, 1994.
72. Gao XP, Zhu X, Fu J, Liu Q, Frey RS, and Malik AB. Blockade of class IA phosphoinositide 3-kinase in neutrophils prevents NADPH oxidase activation- and adhesion-dependent inflammation. *J Biol Chem* 282: 6116–6125, 2007.
73. Garcia JG, Lazar V, Gilbert-McClain LI, Gallagher PJ, and Verin AD. Myosin light chain kinase in endothelium: molecular cloning and regulation. *Am J Respir Cell Mol Biol* 16: 489–494, 1997.
74. Garcia JG, Schaphorst KL, Verin AD, Vepa S, Patterson CE, and Natarajan V. Diperoxovanadate alters endothelial cell focal contacts and barrier function: role of tyrosine phosphorylation. *J Appl Physiol* 89: 2333–2343, 2000.
75. Gatfield J, Albrecht I, Zanolari B, Steinmetz MO, and Pieters J. Association of the leukocyte plasma membrane with the actin cytoskeleton through coiled coil-mediated trimeric coronin 1 molecules. *Mol Biol Cell* 16: 2786–2798, 2005.
76. Geiszt M, Kapus A, and Ligeti E. Chronic granulomatous disease: more than the lack of superoxide? *J Leukoc Biol* 69: 191–196, 2001.
77. Geiszt M, Kopp JB, Varnai P, and Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* 97: 8010– 8014, 2000.
78. Geiszt M and Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem* 279: 51715–51718, 2004.
79. Ginnan R, Guikema BJ, Halligan KE, Singer HA, and Jourd'heuil D. Regulation of smooth muscle by inducible nitric oxide synthase and NADPH oxidase in vascular proliferative diseases. *Free Radic Biol Med* 44: 1232–1245, 2008.
80. Goldstein BJ, Mahadev K, and Wu X. Redox paradox: insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signaling targets. *Diabetes* 54: 311–321, 2005.
81. Gorzalczany Y, Sigal N, Itan M, Lotan O, and Pick E. Targeting of Rac1 to the phagocyte membrane is sufficient for the induction of NADPH oxidase assembly. *J Biol Chem* 275: 40073–40081, 2000.
82. Griendling KK. NADPH oxidases: new regulators of old functions. *Antioxid Redox Signal* 8: 1443–1445, 2006.
83. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86: 494–501, 2000.
84. Grogan A, Reeves E, Keep N, Wientjes F, Totty NF, Burlingame AL, Hsuan JJ, and Segal AW. Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *J Cell Sci* 110: 3071–3081, 1997.
85. Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, Jasti AC, Harris CE, Lee AW, Prabhakar R, Atkinson SJ, Kwiatkowski DJ, and Williams DA. Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. *Science* 302: 445–449, 2003.
86. Gu Y, Xu YC, Wu RF, Souza RF, Nwariaku FE, and Terada LS. TNFalpha activates c-Jun amino terminal kinase through p47(phox). *Exp Cell Res* 272: 62–74, 2002.
87. Haeusler LC, Hemsath L, Fiegen D, Blumenstein L, Herbrand U, Stege P, Dvorsky R, and Ahmadian MR. Purification and biochemical properties of Rac1, 2, 3 and the splice variant Rac1b. *Methods Enzymol* 406: 1–11, 2006.
88. Hammond SM, Altshuller YM, Sung TC, Rudge SA, Rose K, Engebrecht J, Morris AJ, and Frohman MA. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J Biol Chem* 270: 29640–29643, 1995.
89. Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q, Cook S, Nozawa Y, Prestwich GD, Frohman MA, and Morris AJ. Characterization of two alternately spliced forms of phospholipase D1: activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric

GTP-binding proteins and protein kinase C-alpha. *J Biol Chem* 272: 3860–3868, 1997.
90. Hanna IR, Hilenski LL, Dikalova A, Taniyama Y, Dikalov S, Lyle A, Quinn MT, Lassegue B, and Griendling KK. Functional association of nox1 with p22phox in vascular smooth muscle cells. *Free Radic Biol Med* 37: 1542–1549, 2004.
91. Haurani MJ, Cifuentes ME, Shepard AD, and Pagano PJ. Nox4 oxidase overexpression specifically decreases endogenous Nox4 mRNA and inhibits angiotensin II-induced adventitial myofibroblast migration. *Hypertension* 52: 143–149, 2008.
92. Heller M. Phospholipase D. *Adv Lipid Res* 16: 267–326, 1978.
93. Henderson LM and Chappel JB. NADPH oxidase of neutrophils. *Biochim Biophys Acta* 1273: 87–107, 1996.
94. Heumuller S, Wind S, Barbosa-Sicard E, Schmidt HH, Busse R, Schroder K, and Brandes RP. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. *Hypertension* 51: 211–217, 2008.
95. Heyworth PG, Bohl BP, Bokoch GM, and Curnutte JT. Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox: evidence for its interaction with flavocytochrome b558. *J Biol Chem* 269: 30749–30752, 1994.
96. Heyworth PG, Erickson RW, Ding J, Curnutte JT, and Badwey JA. Naphthalenesulphonamides block neutrophil superoxide production by intact cells and in a cell-free system: is myosin light chain kinase responsible for these effects? *Biochem J* 311: 81–87, 1995.
97. Hoidal JR, Brar SS, Sturrock AB, Sanders KA, Dinger B, Fidone S, and Kennedy TP. The role of endogenous NADPH oxidases in airway and pulmonary vascular smooth muscle function. *Antioxid Redox Signal* 5: 751–758, 2003.
98. Hoidal JR, Xu P, Hueckstead T, Sanders KA, Pfeffer K, and Sturrock AB. Lung injury and oxidoreductases. *Environ Health Perspect* 106(suppl 5): 1235–1239, 1998.
99. Hopf HW, Gibson JJ, Angeles AP, Constant JS, Feng JJ, Rollins MD, Zamirul Hussain M, and Hunt TK. Hyperoxia and angiogenesis. *Wound Repair Regen* 13: 558–564, 2005.
100. Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* 98: 453–462, 2006.
101. Hornstein I, Alcover A, and Katzav S. Vav proteins, masters of the world of cytoskeleton organization. *Cell Signal* 16: 1–11, 2004.
102. Hoyal CR, Gutierrez A, Young BM, Catz SD, Lin JH, Tsichlis PN, and Babior BM. Modulation of p47PHOX activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase. *Proc Natl Acad Sci U S A* 100: 5130–5135, 2003.
103. Hu CD, Kariya K, Okada T, Qi X, Song C, and Kataoka T. Effect of phosphorylation on activities of Rap1A to interact with Raf-1 and to suppress Ras-dependent Raf-1 activation. *J Biol Chem* 274: 48–51, 1999.
104. Huang C, Ni Y, Wang T, Gao Y, Haudenschild CC, and Zhan X. Down-regulation of the filamentous actin cross-linking activity of cortactin by Src-mediated tyrosine phosphorylation. *J Biol Chem* 272: 13911–13915, 1997.
105. Ikeda S, Yamaoka-Tojo M, Hilenski L, Patrushev NA, Anwar GM, Quinn MT, and Ushio-Fukai M. IQGAP1 regulates reactive oxygen species-dependent endothelial cell migration through interacting with Nox2. *Arterioscler Thromb Vasc Biol* 25: 2295–2300, 2005.
106. Inanami O, Johnson JL, McAdara JK, Benna JE, Faust LR, Newburger PE, and Babior BM. Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of p47PHOX on serine 303 or 304. *J Biol Chem* 273: 9539–9543, 1998.
107. Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CX, and Laurindo FR. Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells. *J Biol Chem* 280: 40813–40819, 2005.
108. Janiszewski M, Pedro MA, Scheffer RC, van Asseldonk JH, Souza LC, da Luz PL, Augusto O, and Laurindo FR. Inhibition of vascular NADH/NADPH oxidase activity by thiol reagents: lack of correlation with cellular glutathione redox status. *Free Radic Biol Med* 29: 889–899, 2000.
109. Jiang Y, Dai A, Li Q, and Hu R. Hypoxia induces transforming growth factor-beta1 gene expression in the pulmonary artery of rats via hypoxia-inducible factor-1alpha. *Acta Biochim Biophys Sin (Shanghai)* 39: 73–80, 2007.
110. Jin S, Zhang Y, Yi F, and Li PL. Critical role of lipid raft redox signaling platforms in endostatin-induced coronary endothelial dysfunction. *Arterioscler Thromb Vasc Biol* 28: 485–490, 2008.
111. Johar S, Cave AC, Narayanapanicker A, Grieve DJ, and Shah AM. Aldosterone mediates angiotensin II-induced interstitial cardiac fibrosis via a Nox2-containing NADPH oxidase. *FASEB J* 20: 1546–1548, 2006.
112. Jones SA, O'Donnell VB, Wood JD, Broughton JP, Hughes EJ, and Jones OT. Expression of phagocyte NADPH oxidase components in human endothelial cells. *Am J Physiol* 271: H1626–H1634, 1996.
113. Kawahara T, Quinn MT, and Lambeth JD. Molecular evolution of the reactive oxygen-generating NADPH oxidase (Nox/Duox) family of enzymes. *BMC Evol Biol* 7: 109, 2007.
114. Kawahara T, Ritsick D, Cheng G, and Lambeth JD. Point mutations in the proline-rich region of p22phox are dominant inhibitors of Nox1- and Nox2-dependent reactive oxygen generation. *J Biol Chem* 280: 31859–31869, 2005.
115. Kirkham P and Rahman I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacol Ther* 111: 476–494, 2006.
116. Krause KH. Tissue distribution and putative physiological function of NOX family NADPH oxidases. *Jpn J Infect Dis* 57: S28–S29, 2004.
117. Lambeth JD, Kawahara T, and Diebold B. Regulation of Nox and Duox enzymatic activity and expression. *Free Radic Biol Med* 43: 319–331, 2007.
118. Lang ML, Chen YW, Shen L, Gao H, Lang GA, Wade TK, and Wade WF. IgA Fc receptor (FcalphaR) cross-linking recruits tyrosine kinases, phosphoinositide kinases and serine/threonine kinases to glycolipid rafts. *Biochem J* 364: 517–525, 2002.
119. Laplante MA, Wu R, Moreau P, and de Champlain J. Endothelin mediates superoxide production in angiotensin II-induced hypertension in rats. *Free Radic Biol Med* 38: 589–596, 2005.
120. Laurindo FR, Fernandes DC, Amanso AM, Lopes LR, and Santos CX. Novel role of protein disulfide isomerase in the regulation of NADPH oxidase activity: pathophysiological implications in vascular diseases. *Antioxid Redox Signal* 10: 1101–1113, 2008.
121. Lazar V and Garcia JG. A single human myosin light chain kinase gene (MLCK; MYLK). *Genomics* 57: 256–267, 1999.
122. Lee YM, Kim BJ, Chun YS, So I, Choi H, Kim MS, and Park JW. NOX4 as an oxygen sensor to regulate TASK-1 activity. *Cell Signal* 18: 499–507, 2006.

123. Li Q, Zhang Y, Marden JJ, Banfi B, and Engelhardt JF. Endosomal NADPH oxidase regulates c-Src activation following hypoxia/reoxygenation injury. *Biochem J* 411: 531–541, 2008.
124. Li S, Tabar SS, Malec V, Eul BG, Klepetko W, Weissmann N, Grimminger F, Seeger W, Rose F, and Hanze J. NOX4 regulates ROS levels under normoxic and hypoxic conditions, triggers proliferation, and inhibits apoptosis in pulmonary artery adventitial fibroblasts. *Antioxid Redox Signal* 10: 1687–1698, 2008.
125. Li WG, Miller FJ Jr., Zhang HJ, Spitz DR, Oberley LW, and Weintraub NL. H(2)O(2)-induced O(2) production by a non-phagocytic NAD(P)H oxidase causes oxidant injury. *J Biol Chem* 276: 29251–29256, 2001.
126. Li Y, Liu J, and Zhan X. Tyrosine phosphorylation of cortactin is required for H2O2-mediated injury of human endothelial cells. *J Biol Chem* 275: 37187–37193, 2000.
127. Limatola C, Schaap D, Moolenaar WH, and van Blitterswijk WJ. Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. *Biochem J* 304: 1001–1008, 1994.
128. Liu JQ, Zelko IN, Erbynn EM, Sham JS, and Folz RJ. Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox). *Am J Physiol Lung Cell Mol Physiol* 290: L2–L10, 2006.
129. Lum H and Roebuck KA. Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol* 280: C719–C741, 2001.
130. Mahadev K, Motoshima H, Wu X, Ruddy JM, Arnold RS, Cheng G, Lambeth JD, and Goldstein BJ. The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H2O2 and plays an integral role in insulin signal transduction. *Mol Cell Biol* 24: 1844–1854, 2004.
131. Manoury B, Nenan S, Leclerc O, Guenon I, Boichot E, Planquois JM, Bertrand CP, and Lagente V. The absence of reactive oxygen species production protects mice against bleomycin-induced pulmonary fibrosis. *Respir Res* 6: 11, 2005.
132. Maranchie JK and Zhan Y. Nox4 is critical for hypoxia-inducible factor 2-alpha transcriptional activity in von Hippel-Lindau-deficient renal cell carcinoma. *Cancer Res* 65: 9190–9193, 2005.
133. Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC, and Knaus UG. Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal* 18: 69–82, 2006.
134. Matthay MA, Bhattacharya S, Gaver D, Ware LB, Lim LH, Syrkina O, Eyal F, and Hubmayr R. Ventilator-induced lung injury: *in vivo* and *in vitro* mechanisms. *Am J Physiol Lung Cell Mol Physiol* 283: L678–L682, 2002.
135. May RC and Machesky LM. Phagocytosis and the actin cytoskeleton. *J Cell Sci* 114: 1061–1077, 2001.
136. McMahon S, Grondin F, McDonald PP, Richard DE, and Dubois CM. Hypoxia-enhanced expression of the proprotein convertase furin is mediated by hypoxia-inducible factor-1: impact on the bioactivation of proproteins. *J Biol Chem* 280: 6561–6569, 2005.
137. McPhail LC, Qualliotine-Mann D, Agwu DE, and McCall CE. Phospholipases and activation of the NADPH oxidase. *Eur J Haematol* 51: 294–300, 1993.
138. McPhail LC, Waite KA, Regier DS, Nixon JB, Qualliotine-Mann D, Zhang WX, Wallin R, and Sergeant S. A novel protein kinase target for the lipid second messenger phosphatidic acid. *Biochim Biophys Acta* 1439: 277–290, 1999.
139. Medhora M, Chen Y, Gruenloh S, Harland D, Bodiga S, Zielonka J, Gebremedhin D, Gao Y, Falck JR, Anjaiah S, and Jacobs ER. 20-HETE increases superoxide production and activates NAPDH oxidase in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 294: L902–L911, 2008.
140. Mehta D and Malik AB. Signaling mechanisms regulating endothelial permeability. *Physiol Rev* 86: 279–367, 2006.
141. Meyer JW, Holland JA, Ziegler LM, Chang MM, Beebe G, and Schmitt ME. Identification of a functional leukocyte-type NADPH oxidase in human endothelial cells: a potential atherogenic source of reactive oxygen species. *Endothelium* 7: 11–22, 1999.
142. Milovanova T, Chatterjee S, Manevich Y, Kotelnikova I, Debolt K, Madesh M, Moore JS, and Fisher AB. Lung endothelial cell proliferation with decreased shear stress is mediated by reactive oxygen species. *Am J Physiol Cell Physiol* 290: C66–C76, 2006.
143. Mittal M, Roth M, Konig P, Hofmann S, Dony E, Goyal P, Selbitz AC, Schermuly RT, Ghofrani HA, Kwapiszewska G, Kummer W, Klepetko W, Hoda MA, Fink L, Hanze J, Seeger W, Grimminger F, Schmidt HH, and Weissmann N. Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature. *Circ Res* 101: 258–267, 2007.
144. Morris AJ, Frohman MA, and Engebrecht J. Measurement of phospholipase D activity. *Anal Biochem* 252: 1–9, 1997.
145. Nagata M. Inflammatory cells and oxygen radicals. *Curr Drug Targets Inflamm Allergy* 4: 503–504, 2005.
146. Natarajan V, Taher MM, Roehm B, Parinandi NL, Schmid HH, Kiss Z, and Garcia JG. Activation of endothelial cell phospholipase D by hydrogen peroxide and fatty acid hydroperoxide. *J Biol Chem* 268: 930–937, 1993.
147. Nauseef WM. Biological roles for the NOX family NADPH oxidases. *J Biol Chem* 283: 16961–16965, 2008.
148. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* 308: 693–698, 1984.
149. Nixon JB and McPhail LC. Protein kinase C (PKC) isoforms translocate to Triton-insoluble fractions in stimulated human neutrophils: correlation of conventional PKC with activation of NADPH oxidase. *J Immunol* 163: 4574–4582, 1999.
150. Nozik-Grayck E and Stenmark KR. Role of reactive oxygen species in chronic hypoxia-induced pulmonary hypertension and vascular remodeling. *Adv Exp Med Biol* 618: 101–112, 2007.
151. O'Luanaigh N, Pardo R, Fensome A, Allen-Baume V, Jones D, Holt MR, and Cockcroft S. Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. *Mol Biol Cell* 13: 3730–3746, 2002.
152. Palicz A, Foubert TR, Jesaitis AJ, Marodi L, and McPhail LC. Phosphatidic acid and diacylglycerol directly activate NADPH oxidase by interacting with enzyme components. *J Biol Chem* 276: 3090–3097, 2001.
153. Papaiahgari S, Kleeberger SR, Cho HY, Kalvakolanu DV, and Reddy SP. NADPH oxidase and ERK signaling regulates hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells. *J Biol Chem* 279: 42302–42312, 2004.
154. Paravicini TM, Chrissobolis S, Drummond GR, and Sobey CG. Increased NADPH-oxidase activity and Nox4 expression during chronic hypertension is associated with enhanced

cerebral vasodilatation to NADPH *in vivo*. *Stroke* 35: 584–589, 2004.
155. Paravicini TM and Touyz RM. NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 31(suppl 2): S170–S180, 2008.
156. Parinandi NL, Kleinberg MA, Usatyuk PV, Cummings RJ, Pennathur A, Cardounel AJ, Zweier JL, Garcia JG, and Natarajan V. Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 284: L26–L38, 2003.
157. Park HS, Chun JN, Jung HY, Choi C, and Bae YS. Role of NADPH oxidase 4 in lipopolysaccharide-induced proinflammatory responses by human aortic endothelial cells. *Cardiovasc Res* 72: 447–455, 2006.
158. Pedruzzi E, Guichard C, Ollivier V, Driss F, Fay M, Prunet C, Marie JC, Pouzet C, Samadi M, Elbim C, O'Dowd Y, Bens M, Vandewalle A, Gougerot-Pocidalo MA, Lizard G, and Ogier-Denis E. NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells. *Mol Cell Biol* 24: 10703–10717, 2004.
159. Pendyala S, Gorshkova I, Usatyuk PV, Kalari SK, Burns M, He D, and Thannickal VJ, Natarajan V. *Role of Nox4 in endothelial cell signaling, ROS generation and motility*. Toronto, Canada: American Thoracic Society, 2008, p. A847.
160. Pendyala S, Gorshkova I, Usatyuk PV, Kalari SK, Burns M, He D, Thannickal VJ, Natarajan V. Role of nox4 in endothelial cell signaling, ROS generation and motility. *Proceedings of American Thoracic Society.* Toronto Canada: American Thoracic Society, 2008, p. A847.
161. Pendyala S, Natarajan V, Gorshkova IA, Usatyuk P, He D, Pennathur A, Lambeth JD, and Thannickal VJ. Role of nox4 and nox2 in hyperoxia-induced reactive oxygen species generation and migration of human lung endothelial cells. A*ntioxid Redox Signal* 2008 (in press).
162. Petry A, Djordjevic T, Weitnauer M, Kietzmann T, Hess J, and Gorlach A. NOX2 and NOX4 mediate proliferative response in endothelial cells. *Antioxid Redox Signal* 8: 1473–1484, 2006.
163. Price MO, Atkinson SJ, Knaus UG, and Dinauer MC. Rac activation induces NADPH oxidase activity in transgenic COSphox cells, and the level of superoxide production is exchange factor-dependent. *J Biol Chem* 277: 19220–19228, 2002.
164. Rahman I. Redox signaling in the lungs. *Antioxid Redox Signal* 7: 1–5, 2005.
165. Rahman I, Biswas SK, Jimenez LA, Torres M, and Forman HJ. Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxid Redox Signal* 7: 42–59, 2005.
166. Ray R and Shah AM. NADPH oxidase and endothelial cell function. *Clin Sci (Lond)* 109: 217–226, 2005.
167. Regier DS, Waite KA, Wallin R, and McPhail LC. A phosphatidic acid-activated protein kinase and conventional protein kinase C isoforms phosphorylate p22(phox), an NADPH oxidase component. *J Biol Chem* 274: 36601–36608, 1999.
168. Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, and Choi AM. Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* 9: 49–89, 2007.
169. Schroder K, Helmcke I, Palfi K, Krause KH, Busse R, and Brandes RP. Nox1 mediates basic fibroblast growth factor-induced migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 27: 1736–1743, 2007.
170. Segal AW. How neutrophils kill microbes. *Annu Rev Immunol* 23: 197–223, 2005.
171. Sergeant S, Waite KA, Heravi J, and McPhail LC. Phosphatidic acid regulates tyrosine phosphorylating activity in human neutrophils: enhancement of Fgr activity. *J Biol Chem* 276: 4737–4746, 2001.
172. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, and Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 91: 406–413, 2002.
173. Shao D, Segal AW, and Dekker LV. Lipid rafts determine efficiency of NADPH oxidase activation in neutrophils. *FEBS Lett* 550: 101–106, 2003.
174. Shatwell KP and Segal AW. NADPH oxidase. *Int J Biochem Cell Biol* 28: 1191–1195, 1996.
175. Siflinger-Birnboim A and Johnson A. Protein kinase C modulates pulmonary endothelial permeability: a paradigm for acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 284: L435–L451, 2003.
176. Sohn HY, Keller M, Gloe T, Morawietz H, Rueckschloss U, and Pohl U. The small G-protein Rac mediates depolarization-induced superoxide formation in human endothelial cells. *J Biol Chem* 275: 18745–18750, 2000.
177. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, and Griendling KK. Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 105: 1429–1435, 2002.
178. Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, Karwande SV, Stringham JC, Bull DA, Gleich M, Kennedy TP, and Hoidal JR. Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 290: L661–L673, 2006.
179. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, and Lambeth JD. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401: 79–82, 1999.
180. Sumimoto H, Miyano K, and Takeya R. Molecular composition and regulation of the Nox family NAD(P)H oxidases. *Biochem Biophys Res Commun* 338: 677–686, 2005.
181. Suzuki YJ and Ford GD. Redox regulation of signal transduction in cardiac and smooth muscle. *J Mol Cell Cardiol* 31: 345–353, 1999.
182. Szocs K, Lassegue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, and Griendling KK. Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arterioscler Thromb Vasc Biol* 22: 21–27, 2002.
183. Tamura M, Kai T, Tsunawaki S, Lambeth JD, and Kameda K. Direct interaction of actin with p47(phox) of neutrophil NADPH oxidase. *Biochem Biophys Res Commun* 276: 1186–1190, 2000.
184. Tao F, Gonzalez-Flecha B, and Kobzik L. Reactive oxygen species in pulmonary inflammation by ambient particulates. *Free Radic Biol Med* 35: 327–340, 2003.
185. Thannickal VJ and Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 279: L1005–L1028, 2000.
186. Touyz RM. Apocynin, NADPH oxidase, and vascular cells: a complex matter. *Hypertension* 51: 172–174, 2008.
187. Touyz RM, Yao G, Quinn MT, Pagano PJ, and Schiffrin EL. p47phox associates with the cytoskeleton through cortactin

in human vascular smooth muscle cells: role in NAD(P)H oxidase regulation by angiotensin II. *Arterioscler Thromb Vasc Biol* 25: 512–518, 2005.
188. Touyz RM, Yao G, and Schiffrin EL. c-Src induces phosphorylation and translocation of p47phox: role in superoxide generation by angiotensin II in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 23: 981–987, 2003.
189. Uruno T, Liu J, Zhang P, Fan Y, Egile C, Li R, Mueller SC, and Zhan X. Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat Cell Biol* 3: 259–266, 2001.
190. Usatyuk PV, Fomin VP, Shi S, Garcia JG, Schaphorst K, and Natarajan V. Role of Ca2+ in diperoxovanadate-induced cytoskeletal remodeling and endothelial cell barrier function. *Am J Physiol Lung Cell Mol Physiol* 285: L1006–L1017, 2003.
191. Usatyuk PV, Gorshkova I, Pendyala S, Kalari SK, Kamp SM, Dudek SM, Garcia JGN and Natarajan V. *Role of phospholipase D in hyperoxia-mediated myosin light chain kinase activation, reactive oxygen species formation and cytoskeleton organization.* Washington, DC: FASEB, 2007, p. A818.
192. Usatyuk PV, Kalari S, Pendyala S, He D, Gorshkova IA, Camp SM, Moitra J, Dudek SM, Garcia JGN and Natarajan V. *Interactions between myosin light chain kinase, p47phox and cortactin regulate hyperoxia-induced NADPH oxidase activation and reactive oxygen species generation in human lung endothelial cells.* Toronto, Canada: American Thoracic Society, 2008, p. A926.
193. Usatyuk PV, Romer LH, He D, Parinandi NL, Kleinberg ME, Zhan S, Jacobson JR, Dudek SM, Pendyala S, Garcia JG, and Natarajan V. Regulation of hyperoxia-induced NADPH oxidase activation in human lung endothelial cells by the actin cytoskeleton and cortactin. *J Biol Chem* 282: 23284–23295, 2007.
194. Usatyuk PV, Vepa S, Watkins T, He D, Parinandi NL, and Natarajan V. Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. *Antioxid Redox Signal* 5: 723–730, 2003.
195. Ushio-Fukai M. Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc Res* 71: 226–235, 2006.
196. Ushio-Fukai M and Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. *Mol Cell Biochem* 264: 85–97, 2004.
197. Ushio-Fukai M, Tang Y, Fukai T, Dikalov SI, Ma Y, Fujimoto M, Quinn MT, Pagano PJ, Johnson C, and Alexander RW. Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. *Circ Res* 91: 1160–1167, 2002.
198. Vallet P, Charnay Y, Steger K, Ogier-Denis E, Kovari E, Herrmann F, Michel JP, and Szanto I. Neuronal expression of the NADPH oxidase NOX4, and its regulation in mouse experimental brain ischemia. *Neuroscience* 132: 233–238, 2005.
199. Van Buul JD, Fernandez-Borja M, Anthony EC, and Hordijk PL. Expression and localization of NOX2 and NOX4 in primary human endothelial cells. *Antioxid Redox Signal* 7: 308–317, 2005.
200. van der Vliet A. NADPH oxidases in lung biology and pathology: host defense enzymes, and more. *Free Radic Biol Med* 44: 938–955, 2008.
201. Vaquero EC, Edderkaoui M, Pandol SJ, Gukovsky I, and Gukovskaya AS. Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. *J Biol Chem* 279: 34643–34654, 2004.
202. Verin AD, Lazar V, Torry RJ, Labarrere CA, Patterson CE, and Garcia JG. Expression of a novel high molecular-weight myosin light chain kinase in endothelium. *Am J Respir Cell Mol Biol* 19: 758–766, 1998.
203. Verin AD, Patterson CE, Day MA, and Garcia JG. Regulation of endothelial cell gap formation and barrier function by myosin-associated phosphatase activities. *Am J Physiol* 269: L99–L108, 1995.
204. Waite KA, Wallin R, Qualliotine-Mann D, and McPhail LC. Phosphatidic acid-mediated phosphorylation of the NADPH oxidase component p47-phox: evidence that phosphatidic acid may activate a novel protein kinase. *J Biol Chem* 272: 15569–15578, 1997.
205. Wang CL, Kang J, and Li ZH. [Increased expression of NADPH oxidase p47-PHOX and p67-PHOX factor in idiopathic pulmonary fibrosis]. *Zhonghua Jie He He Hu Xi Za Zhi* 30: 265–268, 2007.
206. Webb BA, Zhou S, Eves R, Shen L, Jia L, and Mak AS. Phosphorylation of cortactin by p21-activated kinase. *Arch Biochem Biophys* 456: 183–193, 2006.
207. Weed SA, Karginov AV, Schafer DA, Weaver AM, Kinley AW, Cooper JA, and Parsons JT. Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *J Cell Biol* 151: 29–40, 2000.
208. Weed SA and Parsons JT. Cortactin: coupling membrane dynamics to cortical actin assembly. *Oncogene* 20: 6418–6434, 2001.
209. Weintraub NL. Nox response to injury. *Arterioscler Thromb Vasc Biol* 22: 4–5, 2002.
210. Wendt T, Harja E, Bucciarelli L, Qu W, Lu Y, Rong LL, Jenkins DG, Stein G, Schmidt AM, and Yan SF. RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. *Atherosclerosis* 185: 70–77, 2006.
211. Wolfson M, McPhail LC, Nasrallah VN, and Snyderman R. Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: potential role in the activation of the respiratory burst enzyme. *J Immunol* 135: 2057–2062, 1985.
212. Wolin MS, Gupte SA, and Oeckler RA. Superoxide in the vascular system. *J Vasc Res* 39: 191–207, 2002.
213. Woodman RC, Ruedi JM, Jesaitis AJ, Okamura N, Quinn MT, Smith RM, Curnutte JT, and Babior BM. Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *J Clin Invest* 87: 1345–1351, 1991.
214. Wymann MP and Pirola L. Structure and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* 1436: 127–150, 1998.
215. Xu J, Liu D, Gill G, and Songyang Z. Regulation of cytokine-independent survival kinase (CISK) by the Phox homology domain and phosphoinositides. *J Cell Biol* 154: 699–705, 2001.
216. Yamamori T, Inanami O, Nagahata H, Cui Y, and Kuwabara M. Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. *FEBS Lett* 467: 253–258, 2000.
217. Yamaoka-Tojo M, Tojo T, Kim HW, Hilenski L, Patrushev NA, Zhang L, Fukai T, and Ushio-Fukai M. IQGAP1 mediates VE-cadherin-based cell-cell contacts and VEGF signaling at adherence junctions linked to angiogenesis. *Arterioscler Thromb Vasc Biol* 26: 1991–1997, 2006.

218. Yang B, Oo TN, and Rizzo V. Lipid rafts mediate H2O2 prosurvival effects in cultured endothelial cells. *FASEB J* 20: 1501–1503, 2006.
219. Yao H, Edirisinghe I, Yang SR, Rajendrasozhan S, Kode A, Caito S, Adenuga D, and Rahman I. Genetic ablation of NADPH oxidase enhances susceptibility to cigarette smoke-induced lung inflammation and emphysema in mice. *Am J Pathol* 172: 1222–1237, 2008.
220. Yao H, Yang SR, Kode A, Rajendrasozhan S, Caito S, Adenuga D, Henry R, Edirisinghe I, and Rahman I. Redox regulation of lung inflammation: role of NADPH oxidase and NF-kappaB signalling. *Biochem Soc Trans* 35: 1151–1155, 2007.
221. Zhan Y, He D, Newburger PE, and Zhou GW. p47(phox) PX domain of NADPH oxidase targets cell membrane via moesin-mediated association with the actin cytoskeleton. *J Cell Biochem* 92: 795–809, 2004.
222. Zhang AY, Yi F, Zhang G, Gulbins E, and Li PL. Lipid raft clustering and redox signaling platform formation in coronary arterial endothelial cells. *Hypertension* 47: 74–80, 2006.
223. Zhang J, Guo J, Dzhagalov I, and He YW. An essential function for the calcium-promoted Ras inactivator in Fcgamma receptor-mediated phagocytosis. *Nat Immunol* 6: 911–919, 2005.
224. Zhang Q, Chatterjee S, Wei Z, Liu WD, and Fisher AB. Rac and PI3 kinase mediate endothelial cell-reactive oxygen species generation during normoxic lung ischemia. *Antioxid Redox Signal* 10: 679–689, 2008.
225. Zhang Q, Matsuzaki I, Chatterjee S, and Fisher AB. Activation of endothelial NADPH oxidase during normoxic lung ischemia is KATP channel dependent. *Am J Physiol Lung Cell Mol Physiol* 289: L954–L961, 2005.
226. Zuo L, Ushio-Fukai M, Ikeda S, Hilenski L, Patrushev N, and Alexander RW. Caveolin-1 is essential for activation of Rac1 and NAD(P)H oxidase after angiotensin II type 1 receptor stimulation in vascular smooth muscle cells: role in redox signaling and vascular hypertrophy. *Arterioscler Thromb Vasc Biol* 25: 1824–1830, 2005.

Address reprint requests to:
Viswanathan Natarajan, Ph.D.
Department of Medicine
Section of Pulmonary and Critical Care Medicine
The University of Chicago
CIS Building, Suite W408B
929 East 57th Street
Chicago, IL-60637

E-mail: vnataraj@medicine.bsd.uchicago.edu

Date of first submission to ARS Central, ; accepted.

This article has been cited by:

1. Maria C. Litterio, Grayson Jaggers, Gulcin Sagdicoglu Celep, Ana M. Adamo, Maria A. Costa, Patricia I. Oteiza, Cesar G. Fraga, Monica Galleano. 2012. Blood pressure-lowering effect of dietary (-)-epicatechin administration in L-NAME-treated rats is associated with restored nitric oxide levels. *Free Radical Biology and Medicine* . [CrossRef]
2. Ping Song, Ming-Hui Zou. 2012. Regulation of NAD(P)H oxidases by AMPK in cardiovascular systems. *Free Radical Biology and Medicine* **52**:9, 1607-1619. [CrossRef]
3. G. Douglas, J. K. Bendall, M. J. Crabtree, A. L. Tatham, E. E. Carter, A. B. Hale, K. M. Channon. 2012. Endothelial-specific Nox2 overexpression increases vascular superoxide and macrophage recruitment in ApoE-/- mice. *Cardiovascular Research* . [CrossRef]
4. Jennifer Streeter, William Thiel, Kate Brieger, Francis J. Miller Jr.. 2012. Opportunity Nox: The Future of NADPH Oxidases as Therapeutic Targets in Cardiovascular Disease. *Cardiovascular Therapeutics* no-no. [CrossRef]
5. Yuxing Zhang , Yanzhi Du , Weidong Le , Kankan Wang , Nelly Kieffer , Ji Zhang . 2011. Redox Control of the Survival of Healthy and Diseased Cells. *Antioxidants & Redox Signaling* **15**:11, 2867-2908. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
6. X. Fu, J. Chen, R. Gallagher, Y. Zheng, D. W. Chung, J. A. Lopez. 2011. Shear stress-induced unfolding of von Willebrand factor accelerates oxidation of key methionine residues in the A1A2A3 region. *Blood* . [CrossRef]
7. Phillip Factor. 2011. Is it the inflammation or the free radicals that cause ischemia/reperfusion lung injury?. *Translational Research* **158**:1, 14-16. [CrossRef]
8. Shan Zhang, Beidong Chen, Wei Wu, Li Bao, Ruomei Qi. 2011. Ginkgolide B Reduces Inflammatory Protein Expression in Oxidized Low-density Lipoprotein-stimulated Human Vascular Endothelial Cells. *Journal of Cardiovascular Pharmacology* **57**:6, 721-727. [CrossRef]
9. Aron B. Fisher. 2011. Oxidant Stress in Pulmonary Endothelia. *Annual Review of Physiology* **74**:1, 110301101907077. [CrossRef]
10. Hong Li, Qiang Liu, Ningfu Wang, Jian Xu. 2011. Correlation of Different NADPH Oxidase Homologues with Late Endothelial Progenitor Cell Senescence Induced by Angiotensin II: Effect of Telmisartan. *Internal Medicine* **50**:16, 1631-1642. [CrossRef]
11. Srikanth Pendyala, Viswanathan Natarajan. 2010. Redox regulation of Nox proteins#. *Respiratory Physiology & Neurobiology* **174**:3, 265-271. [CrossRef]
12. Shahab Shahnazari, Wei-Lien Yen, Cheryl L. Birmingham, Jessica Shiu, Anton Namolovan, Yiyu T. Zheng, Keiko Nakayama, Daniel J. Klionsky, John H. Brumell. 2010. A Diacylglycerol-Dependent Signaling Pathway Contributes to Regulation of Antibacterial Autophagy. *Cell Host & Microbe* **8**:2, 137-146. [CrossRef]