

NOX Enzymes and Pulmonary Disease

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Abstract

The primary function of the lung is to facilitate the transfer of molecular oxygen (O_2 ; dioxygen) from the atmosphere to the systemic circulation. In addition to its essential role in aerobic metabolism, O_2 serves as the physiologic terminal acceptor of electron transfer catalyzed by the NADPH oxidase (NOX) family of oxidoreductases. The evolution of the lungs and circulatory systems in vertebrates was accompanied by increasing diversification of NOX family enzymes, suggesting adaptive roles for NOX-derived reactive oxygen species in normal physiology. However, this adaptation may paradoxically carry detrimental consequences in the setting of overwhelming/persistent environmental stressors, both infectious and noninfectious, and during the process of aging. Here, we review current understanding of NOX enzymes in normal lung physiology and their pathophysiologic roles in a number of pulmonary diseases, including lung infections, acute lung injury, pulmonary arterial hypertension, obstructive lung disorders, fibrotic lung disease, and lung cancer. *Antioxid. Redox Signal.* 11, 2505–2516.

Introduction

THE RESPIRATORY SYSTEM brings the ambient air that we breathe into close proximity with the systemic circulation. This allows the lungs to accomplish their primary function in the exchange of carbon dioxide for oxygen (O_2), essential for the maintenance of aerobic metabolism. The average adult human breathes in 9,000 to 15,000 L of air (6–10 L/min) daily. This exposes the lungs to a variety of potentially injurious environmental agents, both infectious and noninfectious. The normal host response is to eradicate putative pathogens/injurious agents and to repair the damage caused directly by the agent or by the associated immune/inflammatory response. Human pulmonary diseases result, in large part, when the host response to the attempted eradication of the offending agent is dysregulated or when the repair/regenerative responses to ensuing tissue injury are impaired. A number of host factors, including genetic/epigenetic factors and age, may influence the susceptibility to pulmonary disease and the clinical phenotype (*e.g.*, severity, progression) of the associated clinical syndrome.

NADPH oxidase (NOX) enzymes emerged during the evolutionary transition from unicellular to multicellular organisms, and the number of NOX/Dual oxidase (DUOX) family enzymes have increased to seven in mammals (NOX1 to 5 and DUOX1 to 2) (11, 51, 116). NOX enzymes catalyze the reduction of molecular oxygen (O_2) to superoxide ($O_2^{\bullet-}$), the

typical primary product of the reaction (10, 57). Depending on the microenvironment or cellular compartment in which it is produced, spontaneous or superoxide dismutase (SOD)-catalyzed reduction of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) may occur in association with the generation of other reactive oxygen species (ROS). ROS function as signaling molecules and regulators of cell function when they are generated in a compartmentalized and regulated manner (126). Here, we examine the roles of these ROS-generating enzymes in cellular physiology of the lung and in the pathogenesis of pulmonary diseases (Fig. 1).

NOX Enzymes in Pulmonary Infectious Disease

The lungs are well equipped to defend against myriad microbial pathogens that may be transmitted to the lungs from inhaled air, the systemic circulation, oropharyngeal aspiration, or contiguous spread from surrounding tissues. Upper airway defense mechanisms (*e.g.*, mucociliary clearance) work in concert with lower airway defenses (*e.g.*, alveolar macrophages) to combat microbial pathogens. The role of the phagocytic “respiratory burst oxidase,” NOX2, in innate immune responses is well established and suggests an archaic host defense mechanism that is conserved across multiple species (25, 42, 99). Evidence demonstrates that NOX2 mediates its antimicrobial effect, at least in part, by facilitating compartmentalized protease activation within phagosomes

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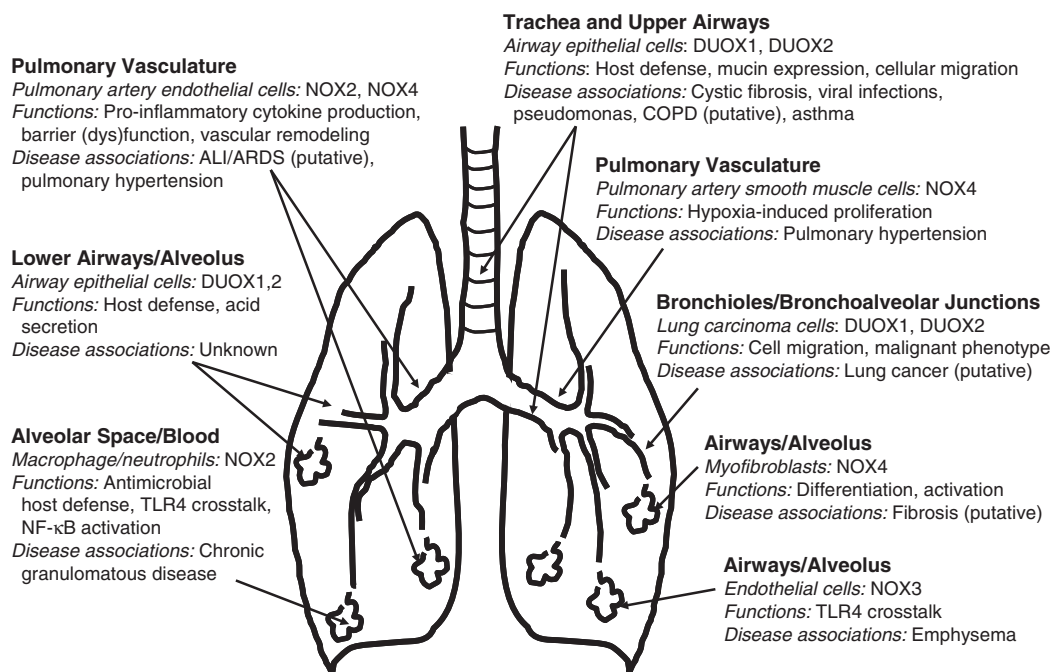


FIG. 1. NOX Enzymes in lung cellular physiology and pulmonary disease. NOX/DUOX isoforms are expressed in a number of lung cell types, including airway/alveolar epithelial, endothelial, and mesenchymal cells, extending from the proximal trachea and large airways to terminal bronchioles and alveoli. Proposed functions of NOX isoforms in various cell types and their putative roles in diverse lung diseases are indicated. Refer to text for related references and details.

through a transmembrane ion flux that is coupled to endosomal $O_2^{\bullet-}$ release (101).

Chronic granulomatous disease (CGD), characterized by susceptibility to recurrent pyogenic infections, is the prototypical example of a human disease associated with inherited loss of function of genes encoding components of the NOX2 enzymatic complex. Initially characterized as a fatal granulomatous disease of childhood, the clinical course of CGD is marked by recurrent, suppurative infections and granuloma formation (15, 106). Although CGD can be associated with a defect in any of the subunits of the multicomponent NOX2 enzyme complex, the X-linked gene mutation in the catalytic NOX2 subunit, identified and positionally cloned in 1986, represents the most common site of mutations (102). Novel mutations involving the α ($p22^{phox}$) and β ($gp91^{phox}$) transmembrane subunits of NOX2 have been reported (13, 26, 27, 63, 64, 86, 121). Pulmonary infections remain a hallmark of the disease and the leading cause of morbidity. A national CGD registry report in 2001 noted a shift in the most common infecting organisms away from staphylococci and enteric bacteria to other pathogens, with *Aspergillus pneumonia* and *Burkholderia cepacia* infections representing the leading causes of death (49). Antimicrobial prophylaxis, interferon- γ administration, and granulocyte infusions remain the current mainstay of treatment for CGD.

A murine model of X-linked CGD with targeted deletion of the NOX2 gene encoding the 91-kDa cytochrome *b* subunit has been described (99). These mice display the characteristic susceptibility to *Staphylococcus* and *Aspergillus* infections and, additionally, develop a persistent inflammatory response associated with high levels of inflammatory cytokines after challenge with sterilized *Aspergillus* hyphae (81). Studies in

mice deficient in the $p47^{phox}$ subunit indicate a role for NOX isoforms, requiring this subunit for enzyme activation in host defense against *Pseudomonas pneumonia* (103) and *M. tuberculosis pneumonia* (21); a potential role of NOX-derived ROS in suppressing neutrophilic inflammation also was suggested (21). Similar findings of a putative "antiinflammatory" role for $p47^{phox}$ /NOX2 are reported in mice challenged with intraperitoneal live *Escherichia coli* to induce sepsis (35), and in murine models of pneumococcal pneumonia (72), influenza pneumonia (109), and disseminated *Cryptococcus neoformans* infection (110).

In addition to immune defects, mice harboring mutations of $p22^{phox}$ develop vestibular dysfunction associated with otoconial malformation (85). A deficiency in neutrophil cytosolic factor-1, required for activation of NOX2, appears to protect from virus-induced acute lung injury (44). Together, these studies demonstrate the contextual role of NOX2 (and potentially other $p47^{phox}$ - and $p22^{phox}$ -requiring NOX enzymes) in modulating host inflammatory responses, in addition to its recognized role in antimicrobial killing.

Limited evidence supports the possibility that other NOX isoforms may also participate in host innate immune responses. Studies in gastric mucosal cells suggest a role for NOX1 in antimicrobial host defense (52, 122), although a similar role for NOX1 in the lung has yet to be demonstrated. More recently, the identification of DUOX1 and DUOX2 in salivary, tracheal, and bronchial epithelium has broadened the role of NOX homologues in host defense as "tissue-specific" generators of ROS (31, 36, 38, 82, 105, 108). DUOX enzymes and their roles in host defense of the upper airways are discussed elsewhere in this issue.

NOX Enzymes in Acute Lung Injury

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) represent clinical syndromes of varying severity and diverse causes that are first seen with a set of defined clinical–physiologic–radiologic criteria (141). The most common risk factor associated with ALI/ARDS is sepsis; other associated conditions include trauma, aspiration, pneumonia, acute pancreatitis, and transfusion of blood products (141). A unifying pathophysiologic feature involves disruption of the alveolar–capillary membrane, resulting in diffuse bilateral infiltrates on chest radiographs and arterial hypoxemia that is typically refractory to high concentrations of O₂ administration (141). The generation of ROS by nonenzymatic and enzymatic mechanisms, including activation of NOX enzymes, contributes to the pathobiology of ALI/ARDS (41).

NOX-dependent ROS generation by neutrophils appears to play a major role in lung injury secondary to sepsis. Lipopolysaccharide (LPS), a constituent of the outer membrane of gram-negative bacteria, primes activation of the phagocytic NOX2 enzyme. In guinea pigs, LPS-stimulated ROS generation by neutrophils and ALI were significantly reduced with apocynin, a putative inhibitor of NOX2 (136, 140). Emerging evidence suggests the presence of crosstalk between NOX enzymes and Toll-like receptors (TLRs), which cooperatively participate in the host innate immune response. High-mobility group box 1 (HMGB1), an endogenous ligand for TLR4, activates neutrophil-associated NOX, induces neutrophilic inflammation, and results in organ failure in response to hemorrhagic shock/resuscitation in mice (29). TLRs also crosstalk with nonphagocytic NOX isoforms, as demonstrated by the finding that LPS-induced ROS generation and NF- κ B activation is mediated by the interaction of TLR4 with NOX4 (93).

Endothelial barrier dysfunction in ALI/ARDS may be mediated by ROS-dependent mechanisms that involve interactions of activated neutrophils with pulmonary vascular endothelial cells (ECs) or more-direct activation of EC responses. In support of the latter concept, growing recognition indicates the expression/activation of specific NOX isoforms in vascular ECs. A role for NOX4 in LPS-induced proinflammatory responses by human aortic ECs has been reported (92); in this study, downregulation of NOX4 by transfection of NOX4 small interfering RNA (siRNA) resulted in a failure to induce ROS generation and intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-8 production in response to LPS. Pulmonary ECs have been shown to generate ROS *via* both NOX2 and NOX4 (94, 95). Circulating blood cells from septic patients generate higher levels of phorbol ester–stimulated O₂^{•−} production compared with those in control subjects, and this activation is inhibited by simvastatin, a widely used cholesterol-reducing drug (28). Further, recent data indicate that simvastatin markedly decreases LPS-induced O₂^{•−} production in human pulmonary artery ECs *via* dual inhibitory effects on RhoA and Rac1 (17). Together, these studies suggest that excessive generation of ROS by activation of phagocytic and nonphagocytic NOX enzymes may induce endothelial damage or activation with the subsequent loss of barrier function and pulmonary edema or both, key features of ALI/ARDS. Further studies are required to characterize the relative con-

tributions of different NOX isoforms in sepsis-induced ALI/ARDS and to determine mechanisms for the modulation of NOX activities by statins and other putative NOX inhibitors.

Mechanical ventilation provides life-sustaining support in critically ill patients with ALI/ARDS. However, mechanical ventilation itself may contribute to, and exacerbate, lung injury. As a result, ventilator-induced lung injury (VILI) may delay or prevent recovery from treatable clinical conditions such as sepsis. Modes of mechanical ventilation associated with greater mechanical distension/stretch enhance neutrophil infiltration and pro-inflammatory cytokine production in the lung (1, 43). Administration of N-acetylcysteine attenuates the influx of neutrophils into the alveolar space and reduces apoptosis of airway epithelial cells in rats subjected to mechanical ventilation (117). Cyclical mechanical strain of alveolar epithelial cells leads to ROS generation, *via* both mitochondrial and NOX-dependent pathways (16). Although the enzymatic source(s) have not been identified, ROS-mediated cellular damage or activation from biomechanical stress or both may augment lung injury and delay/prevent normal repair.

Patients with ALI/ARDS receiving mechanical ventilation often require high O₂ concentrations to maintain arterial oxygenation. Supraphysiologic levels of O₂ concentration (hyperoxia) and the associated generation of ROS is yet another contributor to the “biotrauma” that can worsen ALI in mechanically ventilated patients. Hyperoxia remains a particular problem in premature infants whose lungs may be ill adapted to defend against ROS (8, 144). The effect of hyperoxia on inflammation/injury has been studied in animal models and lung EC culture systems (22, 83). Exposure of mice to hyperoxia (>95% oxygen) causes lung damage characterized by inflammation, barrier dysfunction, pulmonary edema, and impaired lung function (22). The increased generation of ROS during hyperoxia may induce oxidative modifications of cellular macromolecules, including carbohydrates, nucleic acids, proteins, and lipids.

Exposure of human pulmonary artery ECs to hyperoxia (95% O₂) increases ROS production that is dependent on NOX activation and independent of the mitochondrial electron transport or xanthine/xanthine oxidase systems (91, 95). NOX4, which is expressed at relatively higher levels compared with other NOX homologues, is a major source of ROS production in vascular ECs. In cultured human pulmonary artery ECs, hyperoxia increases NOX4 mRNA and protein levels by about eight- and threefold, respectively, compared with normoxia over a 24-h period (94). Activation of lung EC-associated NOX by hyperoxia is regulated, in part, by ERK-1/2 and p38 MAPKs (91, 132). More recently, a role for Src kinase in this process was demonstrated (19); in this study, exposure of lung vascular ECs to hyperoxia stimulated tyrosine phosphorylation of p47^{phox}, which was attenuated by pharmacologic/genetic targeting of Src, suggesting Src-dependent phosphorylation of p47^{phox} in EC-associated NOX activation. In addition, evidence for *in vitro* phosphorylation of p47^{phox} by Src and interaction between Src and p47^{phox} in hyperoxia-induced O₂^{•−} generation was demonstrated (19). Interestingly, tyrosine phosphorylation of cortactin is associated with hyperoxia-induced translocation of p47^{phox} to the cell periphery and ROS generation in human lung ECs (131).

A role for NOX2 and NOX4 in hyperoxia-induced ROS generation in pulmonary vascular ECs has been demonstrated (94). Interestingly, knockdown of NOX4 or NOX2 with siRNA upregulates the mRNA and protein expression of the other homologue in human pulmonary artery ECs; a similar upregulation of NOX4 mRNA is observed in lungs of NOX2^{-/-} mice under normoxic conditions, suggesting a compensatory mechanism (94).

The role of NOX2 in hyperoxic lung injury in mice genetically deficient in NOX2 has been investigated. Exposure of wild-type mice to hyperoxia induces pulmonary edema and neutrophil influx into the alveolar space, effects that are attenuated in NOX2^{-/-} mice, thus suggesting a role for NOX2 in hyperoxia-mediated barrier dysfunction. However, the observed protection in NOX2^{-/-} is incomplete, suggesting the potential involvement of other NOX isoforms, including NOX4, in alveolocapillary barrier dysfunction (94, 95).

NOX Enzymes in Pulmonary Hypertension

Prolonged exposure to low O₂ tension induces pulmonary arterial hypertension (PAH), characterized by vascular remodeling and enhanced vasoreactivity. Accumulating evidence indicates that ROS derived from NOX isoforms, in particular NOX2 and NOX4, are involved in long-term responses of the pulmonary vasculature to hypoxia (30, 33, 66, 79). ROS generation from NOX-independent sources may also contribute to hypoxia-induced vascular dysfunction; for example, hypoxia-exposed neonatal rat pups exhibit increased serum and lung xanthine oxidase (XO) activity, increased vascular XO-derived O₂^{•-} production, and vascular nitrotyrosine formation (47). A role for NOX2 in hypoxia-induced endothelial dysfunction involving intrapulmonary arteries has been demonstrated (33). In pulmonary artery adventitial fibroblasts, hypoxia significantly upregulates NOX4 expression at the mRNA and protein levels, whereas silencing of NOX4 by siRNA reduces ROS levels and decreases cellular proliferation (65). Hypoxia-dependent development of PAH in mice has been linked to increased NOX4 expression in pulmonary artery smooth muscle cells (SMCs) (79), suggesting a key role for NOX4 in the vascular remodeling associated with hypoxia-induced PAH. Hypoxia increases the expression of TGF- β (48), production of the TGF- β -activating protein, furin (76), and NOX4 expression (114). TGF- β -induced NOX4 expression and ROS production has been implicated in proliferation of human pulmonary artery SMCs (45, 114). NOX4 has also been shown to be critical for HIF-2 α expression and transcriptional activation in renal carcinoma cells (71). Additionally, TGF- β /SMAD signaling may synergize with hypoxia/HIF-1 α (104), thereby setting up a potential feed-forward mechanism in hypoxic vascular remodeling involving HIF-1 α /HIF-2 α , transforming growth factor- β (TGF- β), and NOX4.

NOX Enzymes in Obstructive Sleep Apnea and Ischemia/Reperfusion

Obstructive sleep apnea is a clinical syndrome that is characterized by intermittent periods of hypoxemia due to partial/complete obstruction of the upper airway during sleep. Obstructive sleep apnea is also a significant cause of secondary PAH. Chronic intermittent hypoxia-induced pulmonary hypertension is associated with increased lung levels

of the NOX subunits, NOX4 and p22^{phox}, as well as activation of platelet-derived growth factor receptor- β and one of its associated downstream effectors, AKT kinase (87). In NOX2^{-/-} mice, chronic intermittent hypoxia-induced derangements, such as increased right ventricular systolic pressure, right ventricle (RV) to left ventricle + septum weight ratio, an index of RV hypertrophy, and thickness of the RV anterior wall, as measured by echocardiography, are all attenuated (87). These findings suggest that NOX2 contributes to the development of pulmonary vascular remodeling, pulmonary hypertension, and RV remodeling induced by chronic intermittent hypoxia.

ROS also play a crucial role in ischemia/reperfusion injury after lung transplantation. Normoxic lung ischemia induces EC membrane depolarization because of acute alterations in shear stress that activates EC-associated NOX activity *via* a Rac1 and phosphoinositide-3-kinase (PI3K)-dependent mechanism (150). Studies using p47^{phox} knockout mice, wild-type mice, and chimeras created by bone marrow transplantation indicate that NOX-generated ROS, specifically from bone marrow-derived cells, contribute to lung ischemia/reperfusion injury (148). Furthermore, recent studies indicate that activation of an EC-associated NOX may be the primary mechanism for ROS generation during reoxygenation after lung ischemia (5, 152). Neutrophil NOX-derived ROS also contribute to organ injury after hemorrhagic shock in mice (29). Enhanced formation of O₂^{•-} by a p47^{phox}-requiring NOX enzyme contributes to the liver injury caused by hemorrhagic shock, and inhibitors of NOX enzymes may represent a novel therapeutic approach for the treatment of hemorrhagic shock (3, 62).

NOX Enzymes in Obstructive Lung Disorders

NOX-generated ROS have long been recognized to play key roles in the pathogenesis of a number of diverse chronic lung disorders that result in obstructive physiology, in particular asthma, cystic fibrosis, and emphysema (2, 50, 53, 73, 97, 113). With the recent identification of various NOX homologues, investigators have implicated specific NOX and DUOX isoforms in the pathogenesis of obstructive lung disorders; DUOX1, DUOX2, NOX2, and NOX4, and subunits p22^{phox} and p47^{phox} have been the most frequently reported. DUOX1 has been shown to be induced by the T-helper 2 (Th2) cytokines, interleukin (IL)-4 and IL-13, key effector cytokines in asthma and allergic airways disease (38). DUOX1, in addition to host defense functions, appears to promote epithelial cell migration and maintenance of barrier function (142). NOX4 has been implicated in TGF- β -mediated proliferation and hypertrophy of human airway smooth muscle, a hallmark of airway remodeling in asthmatic patients (114). NOX subunits, p22^{phox} and p47^{phox}, were detected in airway SMCs, and NOX-dependent ROS generation mediates TNF- α -induced airway smooth muscle hyperresponsiveness, a predictor of fatal asthma (123). Interestingly, recent haplotype studies indicate that genetic variability in the gene encoding p22^{phox} (CYBA, 16q24.3) may contribute to the susceptibility to asthma (46).

Cystic fibrosis airway biopsy samples exhibit decreased DUOX2 expression, suggesting that the enhanced susceptibility to infections in cystic fibrosis may be linked to impaired DUOX-mediated host defense (146). In support of NOX-

mediated host defense in the lung, mice deficient in NOX2 have increased susceptibility to specific strains of *Burkholderia cepacia*, a pathogen commonly encountered in cystic fibrosis patients (111). *Pseudomonas aeruginosa*, another important pathogen in cystic fibrosis, appears to inhibit DUOX1-dependent antimicrobial activity *via* toxin-mediated effects on airway epithelium (100).

Smokers and those with COPD exhibit differential DUOX1 and DUOX2 depending on smoking status and type of lung epithelium sampled. For example, airway epithelium of current smokers expresses decreased DUOX1 and increased DUOX2 compared with those of never smokers, whereas former smokers (all with COPD) demonstrated down-regulation of both DUOX isoforms (84, 98). However, alveolar epithelial DUOX1 and DUOX2 were expressed at low levels and were unchanged regardless of smoking or COPD status (84). Knockout mouse models have enabled investigators to glean a functional role for NOX enzymes in obstructive lung processes. Mice deficient in $p47^{phox}$ or NOX2 exhibit increased cigarette smoke-induced lung inflammation and emphysema despite decreased ROS production compared with control mice (149). The lung responses in $p47^{phox}$ - and NOX2-null mice were associated with increased production of proinflammatory cytokines/chemokines *via* a TLR4-NF- κ B pathway, indicating that NOX2 may mediate antiinflammatory functions by restraining TLR4 activation (149). However, another group reported that $p47^{phox}$ -null mice have less inflammation, IL-6, keratinocyte-derived chemokine (KC/CXCL1), and monocyte chemoattractant protein-1 (MCP1/CCL2) in lung-lavage specimens after cigarette-smoke exposure compared with wild-type mice (56). The differences observed by these groups may be due to variability in lung-compartment sampling, cellular distributions, and chronicity of cigarette-smoke exposure. Gene-profiling studies in lung tissues from cigarette smoke-exposed mice recently revealed upregulation of NOX organizer 1 (NOXO1), which regulates NOX1 activation, indicating that other NOX isoforms may be involved in lung responses to cigarette smoke (77). Furthermore, our ability to detect specific NOX and DUOX isoforms in different lung compartments may be dependent on whether the particular isoform undergoes transcriptional or posttranscriptional regulation (88).

More recently, unexpected roles for NOX3 in the lung are being elucidated. The emergence of NOX3 in evolution corresponds with the full-time adaptation of vertebrates to the land (51), raising potential unique roles for this isoform in the adaptive physiology of this specialized "land organ." Previously, NOX3 was detected only in fetal tissues (18), and its only known physiologic role described in otolith biogenesis in the inner ear, as demonstrated by the head-tilt phenotype of mice deficient in functional NOX3 (9, 90). However, NOX3 is inducible in murine adult lung and lung endothelial cells, with the unexpected finding that NOX3 is regulated by TLR4 (151). Furthermore, NOX3 was found to be induced in aged mice with targeted deletion in TLR4 in association with lung destruction and emphysema, and these effects are reversed with chemical NOX inhibitors or NOX3 siRNA, suggesting a role for NOX3-generated ROS in age-related emphysema (151). These studies were confirmed by breeding TLR4-null with NOX3-null mice and demonstrating significant attenuation in susceptibility to emphysema (Patty Lee, unpublished data). In further support of the involvement of NOX3 in em-

physema pathogenesis, lung-targeted, inducible NOX3 transgenic mice develop emphysema in the setting of NOX3 transgene induction (Patty Lee, unpublished data). Collectively, these results reveal a previously unappreciated role for NOX3 in the pathogenesis of emphysema.

We speculate that NOX3, because of its potentially deleterious effects in the lung, requires tight suppression in adulthood (*e.g.*, by TLR4); however, aberrant or pathologic states of TLR4 deficiency may allow unrestrained NOX3 activity and ROS generation. Interestingly, recent human studies reported that aging and cigarette smoke are associated with depressed TLR4 function (69, 134), supporting the theory of a disrupted TLR-NOX3 axis in human emphysema. Additional studies in human subjects with emphysema are required to elucidate/confirm these findings further. Furthermore, the development of *in vivo*, cell-specific targeting of NOX isoforms will reveal the extent to which tissue distribution and cell specificity determine NOX-mediated responses in obstructive lung disorders.

NOX Enzymes in Pulmonary Fibrosis

Pulmonary fibrosis is a specific type of tissue-remodeling response to known (*e.g.*, environmental exposures, drugs, connective tissue diseases) or unknown (*i.e.*, idiopathic) injury that is typically recurrent or chronic in nature. Tissue-remodeling responses in fibrosis are characterized by the accumulation of activated mesenchymal cells and the deposition of extracellular matrix (ECM) (40). A subset of activated mesenchymal cells, referred to as myofibroblasts, are key effector cells in tissue remodeling and fibrotic reactions in diverse organ systems, including the lung (128). Myofibroblast differentiation is critically dependent on the action of TGF- β 1 (24, 127). In addition to the multiple fibrogenic actions, myofibroblasts generate ROS in response to TGF- β 1 (23, 125, 138). NOX4 has been identified as a source of TGF- β 1-induced ROS production in cardiac myofibroblasts and is implicated in the induction of myofibroblast differentiation (23). Although the cellular localization/compartimentalization of NOX4 has not been clarified in myofibroblasts, a unique feature of NOX4 activity is its capacity for constitutive generation of extracellular H_2O_2 (74, 107, 137). Extracellular generation of H_2O_2 by lung myofibroblasts may mediate additional fibrogenic effects in tissues by inducing epithelial cell apoptosis by a paracrine mechanism (138), or by inducing matrix-crosslinking reactions in the presence of extracellular heme peroxidases (59).

Currently limited published studies are available on *in vivo* roles for NOX4 in lung fibrosis; however, studies in kidney fibrosis (12, 89, 120, 143), vascular-remodeling/fibrosis associated with chronic hypertension (4), cardiac fibrosis (39, 112, 139), and pancreatic fibrosis (75) suggest a role for the NOX4 isoform in the fibrogenic process. Other NOX isoforms that are reported to contribute to tissue fibrosis in nonpulmonary organ systems include NOX1 (4, 75, 112, 139) and NOX2 (67, 75, 89, 112, 143). A $p47^{phox}$ -requiring NOX isoform is required for the development of fibrosis in a murine lung-injury model that is inflammation dependent, and the observed protection in $p47^{phox}/-$ mice is associated with enhanced neutrophilic inflammation and matrix metalloproteinase (MMP)-9 activity (70). Significant crosstalk occurs between the renin-angiotensin-aldosterone system and TGF- β 1 in organ fibrosis

(130, 147), and this effect is, at least in part, mediated by induction/activation of NOX1, NOX2, NOX4, or a combination of these (4, 12, 112, 120, 139, 143).

NOX Enzymes in Lung Cancer

Tumorigenesis entails a series of cellular/tissue alterations that promote cell survival and growth while usurping normal, homeostatic controls. For the malignant potential of cells to be fully realized, it has been proposed that cancer cells develop five essential capabilities: unrelenting cell proliferation, resistance to apoptosis, intrinsic growth signaling with limited response to growth-inhibitory factors, continual neovascularization (angiogenesis), and capacity for migration and tissue invasion (37). ROS have been implicated in the signaling/regulation of the malignant phenotype (34, 58), and oxidative stress-mediated epigenetic changes are increasingly recognized (32).

Early studies indicated the generation of ROS by a NOX-like flavoenzyme in several different cancer cells, although the identity of the enzymatic source(s) was not known (119). With the discovery of the different homologues of NOX/DUOX enzymes, specific isoforms have been identified in a variety of human malignancies, including colon (60, 96, 118), gastric (129), pancreatic (61, 80, 135), and prostate (14) cancers. The tumorigenic potential of NOX enzymes was first demonstrated with athymic murine models. NOX1-transfected cells produce phenotypically aggressive tumors in athymic mice (115). In a similar murine model, injection of NOX1-expressing cells resulted in rapid cell growth and tumor formation, whereas injection of cells coexpressing NOX1 and catalase failed to promote a mitogenic response or tumor formation *in vivo* (7). Recently, NOX1 was found to mediate malignant transformation of the Ras oncogene (78); in this study, RNA interference-mediated knockdown of NOX1 in K-Ras transformed cells reduced NOX1-dependent $O_2^{\bullet-}$ production, resulting in abrogation of anchorage-independent cell growth and capacity for tumor formation *in vivo* (78).

NOX1 was originally referred to as the "mitogenic oxidase" (7, 115); however, its effects on specific cell types are likely contextual and include other cellular functions. Comparing pathologic specimens of human gastric adenocarcinomas and chronic atrophic gastritis, the presence of NOX1 was demonstrated by mRNA expression and immunohistochemical staining within adenocarcinomas, whereas it was notably absent in control samples, supporting the utility of NOX1 as a marker of malignant transformation (129).

In addition to propagation of tumor cell growth, evidence supports a role for NOX1 in angiogenesis (6). Vascular endothelial growth factor (VEGF) functions as a key mediator of neovascularization within tumors. VEGF is upregulated by H_2O_2 and was found to enhance tumor cell proliferation and promote a migratory phenotype (20). Additionally, Ras-induced VEGF transcription is dependent on Sp1 phosphorylation/activation, which is mediated by a NOX1/Ras/ERK-MAPK pathway (55). Other studies support a role for the NOX2 isoform in angiogenesis (133).

Resistance to apoptosis is another hallmark of cancer cells (37). NOX4-generated ROS confer an apoptosis-resistant phenotype in pancreatic cancer cells (135). Similarly, NOX5 is

expressed in DU-145 prostate cancer cells and was found to mediate ROS production, cell proliferation, and resistance to apoptosis (14). A potential mechanism by which the NOX isoforms promote apoptosis resistance may involve ROS-mediated inactivation of protein tyrosine phosphatases (PTPs). In human pancreatic adenocarcinoma tissue samples, NOX4 colocalized with low-molecular-weight PTPs in the cytoplasm of tumor cells (61); furthermore, these investigators showed that NOX4-dependent ROS production mediates PTP inactivation and sustained phosphorylation of JAK2, a pro-survival kinase (61). In human pancreatic adenocarcinoma cells, the PI3K/AKT and apoptosis signal-regulating kinase 1 pathway has been shown to mediate NOX4-induced pro-survival signaling (80).

Recent evidence also indicates a role for epigenetic mechanisms in the regulation of NOX/DUOX enzymes in cancer cells. DUOX1 and DUOX2 and their maturation factors were found to be downregulated by promoter methylation in primary lung carcinomas (68). In this study, restoration of a functional DUOX1 altered the phenotypic profile of the lung cancer cell lines, supporting a homeostatic function for DUOX and providing the first evidence that epigenetic modification of this family of enzymes may promote malignant potential (68). An interesting aspect to the role of NOX/DUOX biology in carcinogenesis is the number and diversity of the isoforms involved in promoting the malignant phenotype, as illustrated by the finding that two different NOX enzymes function to mediate the same malignant trait—apoptosis resistance—in two different cancers (14, 135).

Conclusions

The respiratory and the cardiovascular systems co-evolved to allow transport of atmospheric O_2 to cells/tissues of internal organs in larger organisms that became dependent on aerobic metabolism, but were limited by the diffusibility of O_2 across multiple cell layers (124). The chemical properties of O_2 and ROS appear to have been exploited further in the emergence of biologic complexity; an increasing number of NOX isoforms have emerged with mammalian evolution (11, 51, 116). Gene targeting of specific NOX isoforms or related subunits in mice illustrates the complexity of these enzymatic systems *in vivo*. Although NOX enzymes serve physiologic functions in the lung, they may also contribute to disease pathogenesis. The varying roles of NOX isoforms in emphysema illustrate this pleiotropic nature. A deficiency in $p47^{phox}$ or NOX2 appears to enhance cigarette smoke-induced emphysema (149), whereas a deficiency in NOX3 is predicted to confer protection against emphysema (151). Although such observed differences may be model system-dependent or isoform specific or both, these studies raise the intriguing possibility of "antagonistic pleiotropy" of NOX genes (58). In antagonistic pleiotropy, potentially harmful genes (e.g., NOX3) are retained during evolution because they confer an early survival advantages, but their deleterious effects accrue with age and result in age-related, chronic disease (54, 58, 145). The lungs may be particularly susceptible to toxic effects of NOX activation because O_2 concentrations in lung tissues are generally higher than those in other organs systems.

Future studies on the physiological roles of specific NOX isoforms in the lung will provide important insights into their

pathologic roles in pulmonary disease and opportunities for therapeutic targeting. Because of their cell-specific and contextual effects, studies of NOX function *in vivo* will be more informative when the gene can be conditionally deleted in specific cell types (e.g., by inducible Cre recombinase under the control of a cell-specific promoter to delete a targeted/floxed gene). Animal models do not faithfully recapitulate human disease expression/phenotype because of a multitude of factors, including species-specific differences, disease heterogeneity, chronicity, and environmental influences that may result in epigenetic alterations. This highlights the importance of studying the expression and localization of NOX isoforms in lung cells/tissues derived from patients with specific lung disorders.

Acknowledgments

This work is supported in part by National Institutes of Health grants, R01 HL08533 (V.N.), P01 HL58064 (V.N.), R01 HL071595 (P.J.L.), and R01 HL67967 (V.J.T.).

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Date of first submission to ARS Central, March 21, 2009; date of acceptance, March 28, 2009.

Abbreviations Used

ALI = Acute lung injury
 ARDS = acute respiratory distress syndrome
 CGD = chronic granulomatous disease
 DUOX = dual oxidase
 EC = endothelial cell
 ECM = extracellular matrix
 HIF = hypoxia-inducible factor
 HMGB1 = high-mobility group box 1
 H₂O₂ = hydrogen peroxide
 ICAM = intercellular adhesion molecule
 IL = interleukin
 LPS = lipopolysaccharide
 MCP = monocyte chemoattractant protein
 MMP = matrix metalloproteinase
 NOX = NADPH oxidase
 O₂ = oxygen
 O₂^{•−} = superoxide anion
 PAH = pulmonary arterial hypertension
 PI3K = phosphoinositide-3-phosphate
 ROS = reactive oxygen species
 RV = right ventricle
 SMC = smooth muscle cell
 SOD = superoxide dismutase
 TGF- β = transforming growth factor- β
 Th2 = T-helper 2
 TLR = Toll-like receptor
 VEGF = vascular endothelial growth factor
 VILI = ventilator-induced lung injury
 XO = xanthine oxidase

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