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Therapeutics

FORUM REVIEW ARTICLE

NOX Modifiers—Just a Step Away from Application in the Therapy of Airway Inflammation?

Joanna Wieczfinska,[1] Milena Sokolowska,[2] and Rafal Pawliczak[1]

Abstract

Significance: NADPH oxidase (NOX) enzymes, which are widely expressed in different airway cell types, not only contribute to the maintenance of physiological processes in the airways but also participate in the pathogenesis of many acute and chronic diseases. Therefore, the understanding of NOX isoform regulation, expression, and the manner of their potent inhibition might lead to effective therapeutic approaches. ***Recent Advances:*** The study of the role of NADPH oxidases family in airway physiology and pathophysiology should be considered as a work in progress. While key questions still remain unresolved, there is significant progress in terms of our understanding of NOX importance in airway diseases as well as a more efficient way of using NOX modifiers in human settings. ***Critical Issues:*** Agents that modify the activity of NADPH enzyme components would be considered useful tools in the treatment of various airway diseases. Nevertheless, profound knowledge of airway pathology, as well as the mechanisms of NOX regulation is needed to develop potent but safe NOX modifiers. ***Future Directions:*** Many compounds seem to be promising candidates for development into useful therapeutic agents, but their clinical potential is yet to be demonstrated. Further analysis of basic mechanisms in human settings, high-throughput compound scanning, clinical trials with new and existing molecules, and the development of new drug delivery approaches are the main directions of future studies on NOX modifiers. In this article, we discuss the current knowledge with regard to NOX isoform expression and regulation in airway inflammatory diseases as well as the aptitudes and therapeutic potential of NOX modifiers. *Antioxid. Redox Signal.* 23, 428–445.

The Presence and Activation of the NADPH Oxidase in the Airway

NADPH-oxidase (EC 1.6.3.1) is the major source of nonmitochondrial cellular reactive oxygen species (ROS) and a highly regulated dynamic complex containing both membrane and cytosolic proteins. This enzyme was first discovered in phagocytes, as an essential defense mechanism against different pathogens. Currently, it is known that nonphagocytic cells also express various isoforms of NADPH oxidases and are able to produce ROS. ROS, derived from NADPH oxidases, are one of the most important mechanisms of host defense and innate immunity. However, during prolonged, unresolved inflammation, tissue injury, or dysregulated balance between ROS-generating mechanisms and antioxidants, they might lead to detrimental consequences and various diseases. Therefore, understanding mechanisms leading to the NADPH oxidase activation, expression, and regulation of their various isoforms and potential ways of effective blockade may lead to the development of more effective therapies in airway diseases.

Seven NADPH oxidase homologues form the NOX family (97): NOX1 to NOX5, dual oxidase 1 (DUOX1), and DUOX2. Their expression profile varies in various cell types in the airways (Fig. 1) and is different in the steady state or during inflammation. Depending on the NOX isoforms, the enzymatic activity of NADPH oxidases is modulated, *inter alia*, by regulatory subunits or calcium binding (126). The structure and function of NADPH oxidases have been extensively reviewed elsewhere (26, 77). The mechanisms

[1]Department of Immunopathology, Faculty of Biomedical Sciences and Postgraduate Training, Medical University of Lodz, Lodz, Poland.
[2]Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland.

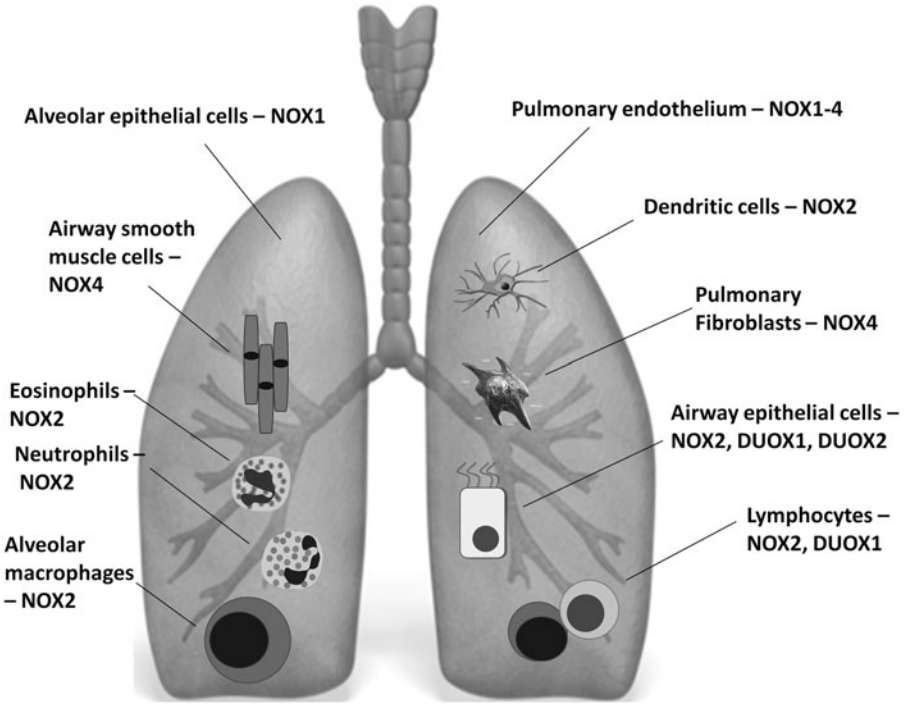

FIG. 1. NOX isoforms expression in the lung in the steady state and on pathologic conditions. Phygocytic and nonphagocytic cells of the airway express various isoforms of NADPH oxidases and are able to produce reactive oxygen species (ROS). ROS, derived from NADPH oxidases, are one of the most important mechanisms of host defense and innate immunity. However, during prolonged, unresolved inflammation, tissue injury or dysregulated balance between ROS-generating mechanisms and antioxidants, they might lead to detrimental consequences and various diseases. Refer to text for related details.

through which the activation of NADPH oxidase is regulated still remain unclear.

NOX2 (initially designated as gp91phox)—the phagocytic, first described and the most abundant isoform of NADPH oxidase, requires the assembly of several components for its activation (Fig. 2). They comprise the membrane-bound p22phox, which stabilizes the NOX protein and docks cytosolic factors and the cytosolic proteins: p47phox, p67phox, Rac, and modulatory p40phox. p47phox is regarded as an organizer protein. Cell stimulation leads to the phosphorylation and translocation of p47phox to the cell membrane. The translocation of p67phox, which is an activator subunit, requires the presence of p47phox, which has been confirmed in p47phox-deficient neutrophils (41). At the cell membrane, p67phox directly interacts with the NOX2 activating it (148). The next component Rac protein is not an NOX subunit in the strict sense, because it regulates other cellular functions; nevertheless, it is required for NADPH oxidase activation. The GTP-bound Rac protein is recruited to the membrane on cell stimulation independently of p47phox or p67phox, but at the membrane, it interacts directly with p67phox *via* the N-terminal domain (75). Finally,

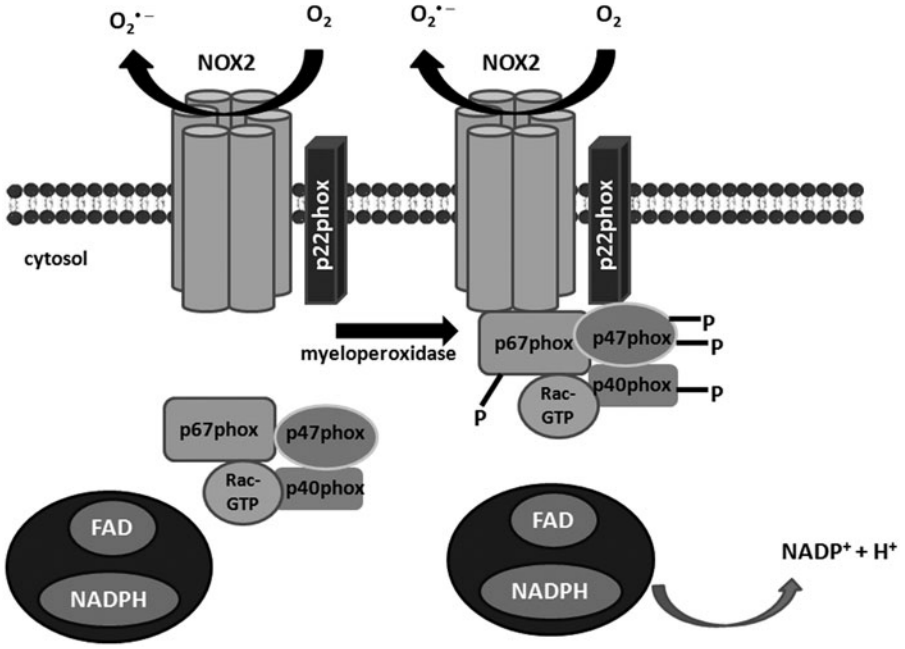

FIG. 2. Schematic illustration of NADPH oxidase NOX2 isoform association with cytosolic regulatory subunits for their activation. The oxidase is a multisubunit protein, activated by translocation of the cytosolic subunits—p47phox, p67phox, and Rac to the NOX/p22phox membrane complex. Cytosolic components phosphorylation results in the movement of the proteins and interaction with p22phox. On activation, there is an exchange of GDP for GTP on Rac, leading to its activation. FAD, flavin adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

the most recently discovered subunit p40phox appears to be modulatory, rather than obligatory, though it constantly associates with p67phox and interacts with p47phox, at least in the resting state (8, 29, 149). Together, the assembly of this complex forms the functional NOX2 enzyme. NOX2 isoforms are ubiquitously expressed, and they are present in the lungs endogenously in the steady state and are overabundant during inflammatory conditions (16, 30, 60, 156). The main NOX2-expressing cell types within the respiratory tract include alveolar macrophages, dendritic cells, and/or other resident or infiltrated inflammatory-immune cells (*e.g.,* neutrophils, eosinophils, and lymphocytes) (Fig. 1) (156). Airway epithelium and pulmonary endothelial cells also express NOX2 (157).

Pulmonary endothelial cells express NOX 1–4, while NOX4 was demonstrated in the airway smooth muscle cells (SMCs), pulmonary epithelial cells, and pulmonary fibroblasts as well (15, 65, 113, 145). NOX5 expression has been shown in immortalized human microvascular endothelial cells-1 (HMEC-1) (9) and lung cancer cells (5, 95). Its expression, though, was not confirmed in primary human artery endothelial cells or human lung microvascular endothelial cells (113), making NOX5 localization in the lungs uncertain. NOX1, similar to NOX2, depends on Rac activity (79). An exception is NOX4, which seems to require only p22phox to be active, and its activity has been described to be determined only by its mRNA/protein levels (100, 126).

The dual oxidases DUOX1 and DUOX2 are the isoforms of NADPH oxidase generating mainly H_2O_2 (122), found in the airway epithelial cells. DUOX comprises an NOX2 homology domain typical for all members of the NOX family, intracellular EF hand-type Ca^{2+}-binding pockets, and an extracellular domain that is not found in other NOXs in humans (47). In the airways, a major function of DUOX is to support lactoperoxidase (LPO) to generate $OSCN^-$ (bactericidal hypothiocyanite) (47). DUOX1 and DUOX2 do not require p22phox for their activity and are activated by agonist-induced increases in intracellular calcium and various phosphorylations (2).

Under specific inflammatory/pathologic conditions, in different parts of the airways, each member of the NOX family might be present, though the expression level might differ significantly (Fig. 1).

Recently, many reviews considering NADPH oxidase inhibitors have emerged (26, 45, 46, 82), most of which appear to be clinically irrelevant; thus in this review, we focus on the significance of specific NOX isoforms in various airway diseases, mechanisms regulating their expression and activation in the airways, and the inhibitory potential of existing molecules.

The Role of NADPH Oxidase and Subunits in Airway Diseases

There is now increasing evidence that ROS are generated by immune and structural cells of the airways and lungs, but above all—inflammatory cells. Overabundant ROS production, known as oxidative stress, is associated with acute and chronic respiratory diseases of profound inflammatory components; for example, asthma, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), pulmonary fibrosis, cystic fibrosis (CF), pulmonary arterial hypertension (PAH), pulmonary complications in sickle cell disease (SCD), and others (58, 120). ROS might initiate inflammatory responses in the airways and lungs *via* the activation of redox-sensitive transcription factors (such as activator protein 1 [AP-1], hypoxia-inducible factor 1-alpha [HIF-1α] or nuclear factor kappa-light-chain-enhancer of actived B cells [NF-κB]) and furthermore—expression of pro-inflammatory gene expression (*e.g.,* vascular cell adhesion molecule-1 [*VCAM-1*], cytosolic phospholipase A_2 [*cPLA*$_2$], intercellular adhesion molecule-1 [*ICAM-1*], matrix metalloproteinase-9 (*MMP-9*), cyclooxygenase-2 [*COX-2*], and others) (12, 13, 90, 96). In addition, prolonged and uncontrolled inflammation itself leads to aggravation of oxidative stress in the airways and lungs, and for that reason, ROS and its sources appear to be crucial factors in the control of airway inflammatory diseases, and offer potential targets for therapeutic interventions (90) (Fig. 3). However, since every chronic inflammatory disease of the airways might be complicated by the recurrent bacterial or viral infections, understanding the disease pathogenesis and its different stages is crucial to face the challenges in the development of efficient but safe NOX modifiers.

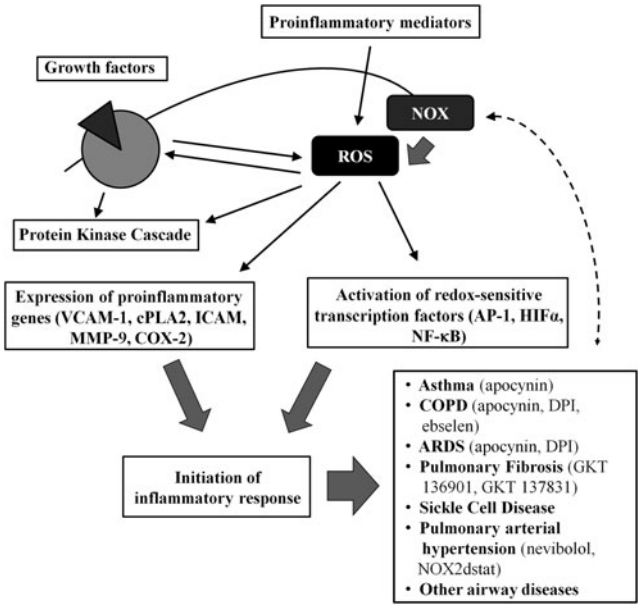

FIG. 3. NOX/ROS play a role in the development and regulation of airway diseases. Through activation of redox-sensitive transcription factors and pro-inflammatory gene expression, NOX-derived ROS initiate inflammatory responses in the airways. A correlation between ROS formation and protein kinase C (PKC) activation might play an important role in regulating inflammatory gene transcription. Uncontrolled inflammation, in turn, intensifies ROS generation; thus, NADPH oxidases, as ROS sources, seem to be pivotal therapeutic targets (90)—modified. Some of the currently studied NOX inhibitors have shown promising effects in human settings (*in vitro* or *in vivo*) or in animal models of airway diseases. AP-1, activator protein 1; HIF-1α, hypoxia-inducible factor 1-alpha; VCAM-1, vascular cell adhesion molecule-1; cPLA$_2$, cytosolic phospholipase A_2; ICAM-1, intercellular adhesion molecule-1, MMP-9, matrix metalloproteinase-9; COX-2, cyclooxygenase-2; COPD, chronic obstructive pulmonary disease; ARDS, acute respiratory distress syndrome. Refer to text for further details.

Asthma

Airway structural cells (epithelial cell, SMCs, resident macrophages, *etc.*) and infiltrating inflammatory cells (eosinophils, lymphocytes, and dendritic cells) are implicated in the pathogenesis of asthma at different stages of the disease. Respiratory epithelium plays a crucial role in inflammatory responses regulation—from response to commonly inhaled allergens to inhaled pathogens and allergic sensitization (61). Since airway and alveolar epithelial cells expressing NOX1–2 and DUOX 1–2 are able to generate ROS in response to stimulation, epithelial ROS production is considered important in asthma pathogenesis (157). Data presented by Schwarzer *et al.* (128) revealed the high expression of DUOX1/2 in whole lung and in the human tracheal epithelium where both DUOX1/2 were expressed at levels that were 1000 times more compared with other NOX isoforms and, thus, represent the major NOX isoforms in airway epithelial cells. Furthermore, it was demonstrated that the baseline DUOX1 expression was consistently higher than DUOX2. DUOX1 expression increased two to five-fold after treatment with IL-4 or IL-13, key cytokines in Th2-derived allergic inflammation (62). Therefore, in addition to its proposed role as a host defense enzyme within the airway lumen (107), it was further established that DUOX1 may be a mediator of redox signaling within the epithelium and might be a potential target of therapeutic modulation at the initial stages of allergic sensitization. Moreover, ROS may cause direct contraction of the airway smooth muscle, which might be enhanced when the epithelium is injured or removed. Excessive ROS production or an imperfect protective system also results in bronchial hyper-responsiveness, which is characteristic in asthma (23, 90). NOX4 is overexpressed in the airway SMCs in patients with asthma (145). Airway smooth muscle from these patients exhibited increased agonist-induced contraction, and this was abrogated by NOX4 small interfering RNA knockdown and less specific NOX pharmacological inhibitors, diphenylene iodonium (DPI) and apocynin (4-hydroxy-3 methoxyacetophenone) (145). In this sense, NOX4 might serve as a potential target to prevent bronchial hyper-responsiveness. Apocynin has already been shown to prevent ozone-induced asthma exacerbation in asthmatics (117), confirming ROS implication in airway hypersensitivity. Analysis of several biomolecular oxidation markers such as bromotyrosine, isoprostanes, nitrates, and nitrites revealed their presence in the airways or lung tissue of asthmatics often in correlation with the extent of ongoing inflammation and with the severity of clinical symptoms (127, 167). Isoprostane F2α-III, a biomarker of lung oxidative stress *in vivo,* is a potent constrictor of human airways, and hydrogen peroxide constricts airway smooth muscle *in vitro* (90). We and others have shown that these markers are useful for noninvasive monitoring to the extent of oxidative stress in the airways. We have also shown that apocynin significantly decreases selected ROS and reactive nitrogen species (RNS) in mild asthmatics (141).

COPD and Pulmonary Emphysema

In COPD, oxidants present in cigarette smoke can stimulate alveolar macrophages to produce ROS and to release mediators attracting inflammatory cells to the lungs. Oxidants can also directly damage epithelial and endothelial cells, contributing to the pathogenesis of COPD. Oxidative stress also contributes to a proteinase-antiproteinase and an antioxidant-oxidant imbalance (151, 153). Primary macrophages isolated from mice overexpressing antioxidant enzyme-extracellular superoxide dismutase or from NOX2-deficient mice showed reduced oxidative stress in response to cigarette smoke treatment (151). Similarly, p47phox-null mice have reduced inflammation and inflammatory cytokines levels in the lung lavage specimens (87). However, there are also contradictory reports showing increased cigarette-smoke induced lung inflammation and emphysema in NOX2 and p47phox-deficient mice, apart from the overall decrease in ROS production (176). The same authors, though, pointed out the differences in the responses to cigarette smoke in different strains of mice (175), underlining the importance of the more definitive human studies. There are a few studies showing the contribution for p22phox gene (official gene name designated as *CYBA* (human) or *Cyba* (mouse)—here, we used *p22phox* throughout for clarity) to the respiratory system disorders. Associations of its genotypes with COPD have been analyzed (161), indicating a positive association of the *p22phox* polymorphisms with COPD, claiming that *p22phox* is an important oxidative stress candidate. There are limited reports with regard to the function and expression of p22phox in the lungs, but there are data from other systems. For example, cigarette smoke was shown to increase p22phox in the brain of Lewis rats, along with the enhanced expression of NOX4 and DUOX1 (78).

Emphysema is a complication and an end stage of COPD, with no clear pathogenesis. Unexpectedly, recent data point at the role of the NOX3 isoform in this process. TLR4 deficiency caused up-regulation of NOX3 in the lungs of aging mice, resulting in increased oxidant generation, enhanced elastolytic activity, and development of emphysema. The treatment of $TLR4^{-/-}$ mice or endothelial cells with chemical NADPH inhibitors (apocynin and DPI) or NOX3 siRNA reversed the observed phenotype (180). This study raises a hope for targeting NOX3 as a modulating strategy in this otherwise-irreversible condition, though further studies are needed to evaluate these findings.

Smoking cessation is one of the most important of first-line therapies in COPD. However, it is known that once activated, oxidative stress is not easily quenched in the airways of COPD patients, even after quitting smoking (94). In this sense, an effective NOX modulator might be a beneficial add-on therapy to prevent the deleterious effects of prolonged oxidative stress in the airways. We have shown that treatment with nebulized apocynin significantly decreases concentrations of hydrogen peroxide and nitrite in exhaled breath condensate in patients with COPD (140).

Acute Respiratory Distress Syndrome

Acute lung injury or acute respiratory distress syndrome (ALI/ARDS) is a condition with substantial morbidity and mortality that is diagnosed both clinically and radiologically based on the presence of noncardiogenic pulmonary edema and respiratory failure in critically ill patients (74). Lung infection, aspiration, sepsis, trauma, or other insults lead to the disruption of the lung endothelial and epithelial barriers, inflammation, and pulmonary edema (74). The role of NOX

enzymes in ARDS has been reviewed elsewhere (16, 58). NOX1, NOX2, and NOX4 in different cells in the airways are involved in the pathogenesis of ARDS (16). NOX1 in alveolar epithelial cells plays a crucial role in the mediation of hyperoxic lung damage in mice (16). Both NOX1 and NOX4 might be involved in epithelial cell death and, in addition, NOX4 might be involved in fibroblast proliferation and fibrotic responses (15). Hyperoxia, a model condition of mechanical ventilation-induced lung injury, increases nonmitochondrial ROS production in human pulmonary endothelial cells (111). From all four NOX isoforms expressed in pulmonary endothelial cells (pulmonary artery and microvascular endothelium), NOX4 seems to be the most abundantly present and its expression is further enhanced by hyperoxia (113). NOX2 is probably most important in ARDS-associated inflammatory responses (83). NOX2-deficient mice have reduced but not totally abrogated hyperoxia-induced pulmonary edema and neutrophil influx to the lungs, suggesting the possible redundancy between NOX2 and other NOX isoforms (113). Nitrogen oxides and H_2O_2 have been proposed for a noninvasive monitoring of the course of lung injury in animals (34). In terms of possible treatment of ALI/ARDS, less specific strategies of ROS scavenging or more than one NOX isoform inhibition have proved their partial potency in animal models. N-acytylcysteine reduced the apoptosis of epithelial cells and neutrophil lung infiltration in rats exposed to mechanical ventilation (147). Apocynin significantly reduced ROS generation and the extent of septic lung injury in guinea pigs (166).

Pulmonary Fibrosis

Pulmonary fibrosis is a lung-specific response to known (*e.g.,* drugs, autoimmune diseases, environmental cues, and radiotherapy) or unknown (idiopathic) factors (80). Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease associated with aging that is characterized by the radiological and histopathological pattern of interstitial pneumonia. Recent data indicate that fibrotic response might be driven by abnormally activated alveolar epithelial cells, leading to the activation of myofibroblasts and the deposition of extracellular matrix (81). Sustained exposure to ROS from exogenous or endogenous sources is able to cause a direct injury to the alveolar epithelium, which may lead to fibrosis. Cui *et al.* demonstrated that oxidative stress contributes to the induction and persistence of transforming growth factor (TGF)-β1-induced pulmonary fibrosis (32). Increasing scientific evidence points to NOX4 as the key source of ROS in the pathogenesis of IPF (15, 65). The severity of fibrosis has been correlated with increased levels of H_2O_2 in IPF patients' exhaled breath condensate. Phagocytic NOX-derived ROS signaling has been demonstrated to play a key role in promoting tumor necrosis factor (TNF)-induced, NF-κB-dependent acute inflammatory responses and tissue injury specifically in the lungs (179). Insulin growth factor 1 (IGF-1) has been recently implicated in the pathogenesis of lung injury and fibrosis, with proven potency of IFG-1 receptor blockade in the murine bleomycin-induced lung injury (24). Although there are no data directly linking IGF-1 with NOX enzymes in the airways, there are some from other systems. Edderkaoui *et al.* recently described a mechanism in which IGF-1 activates NADPH oxidase through transcriptional up-regulation of p22phox in pancreatic cancer cells (43). The authors demonstrated that growth factors can stimulate Akt, mediating the activation of NF-κB transcription factor which up-regulates p22 phox expression (14, 67) (Fig. 4).

Recently, developed selective inhibitors of NOX1 and NOX4, derivatives of pyrazolo-pyrido-diazepine dione (*e.g.,* Genkyotex compounds GKT 136901 and GKT137831), showed significant potency in *in vitro* assays and are currently expected for further clinical trials in pulmonary fibrosis (49, 88).

Cystic Fibrosis

CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. It results in the dysfunction or total lack of CFTR in the plasma membrane and abnormal Na^+ and Cl^- ion transport in various tissues, including lungs, sweat glands, pancreas, gastrointestinal tract, liver, and male reproductive system (124). In the course of the disease bacterial colonization in the lungs, recurrent airway infections and progressive airway obstruction are mainly responsible for mortality (124). Normal CFTR is expressed in the apical membrane of airway epithelial cells. Since DUOX1–2 expression and function has been strongly indicated in airway defense (107), a reduction in ciliated cells in a de-differentiated phenotype is expected to deprive the epithelium of an important defense mechanism (47). Currently, there are a few mechanistic hypotheses that require normal CFTR function for the DUOX/LPO system in the airways to work properly (50). Moskwa *et al.* showed that healthy airway epithelial cells are the source of a strong bactericidal agent $OSCN^-$, which requires DUOX function along with CFTR-dependent SCN^- secretion and LPO activity (107). In CF, $OSCN^-$ generation is diminished due to a CFTR-dependent defect in SCN^- secretion, which leads to a collapse of the oxidative antimicrobial mechanism (107). Shao and Nadel proved that DUOX1 plays a crucial role in mucin expression in the airway epithelial cells *via* PKCδ/PKCθ-DUOX1-ROS-TACE-pro-ligand-EGF receptor cascade (tumor necrosis factor-α-converting enzyme [TACE], Fig. 5). Furthermore, a signaling pathway mediating mucin production involving PKCδ/θ-DUOX1-ROS-TACE-pro-TGF-α-EGFR-mitogen-activated protein kinase has been identified in airway epithelial cells (133). Another study suggests that the bacterium *Pseudomonas aeruginosa*, a most common pathogen in CF, uses ROS to up-regulate mucin expression through a protein kinase C (PKC)-NADPH oxidase signaling pathway in human airway epithelial cells (173). Moreover, data provided by Sham *et al.* emphasize a pivotal role of DUOX1 in epidermal growth factor receptor

FIG. 4. Mechanism of anti-apoptotic effect of growth factors mediated by p22phox component in cancer cells. Akt mediates insulin growth factor 1 (IGF-1)-induced nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) activation through which the GFs stimulate p22phox expression. Anti-apoptotic effect of Akt is mediated, at least in part, through up-regulation of p22phox (43). IGF-I pathway has been recently implicated in the pathogenesis of lung injury and fibrosis (24).

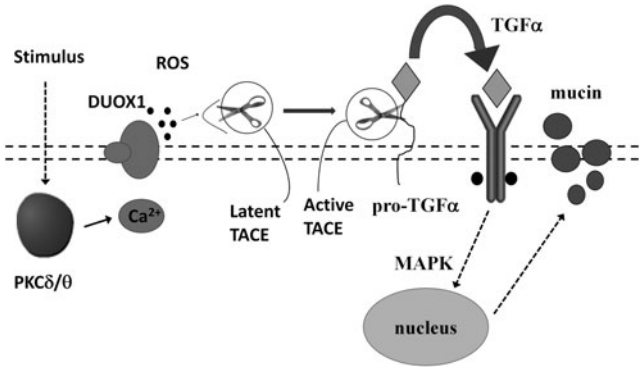

FIG. 5. Mechanism of DUOX1- and ROS-dependent mucin production involving PKC*δ/θ* in airway epithelial cells. Schematic model of Shao and Nadel (modified) (133). PKC isoforms are activated by stimulus (*e.g.,* phorbol myristate acetate [PMA]). PKC then stimulates DUOX. In turn, ROS generated by DUOX activate TACE, which cleaves pro-TGF into TGF. TGF activates EGFR and initiates MAPK, leading to mucin gene expression. TACE, tumor necrosis factor-α-converting enzyme; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; DUOX, dual oxidase.

(EGFR) transactivation in airway epithelial cells and indicate Src and ADAM metallopeptidase domain 17 (ADAM17, tumor necrosis factor-α-converting enzyme [TACE]) as targets for redox regulation in response to DUOX1 activation (131).

Taken together, in the early phases of CF, an aberrant epithelial DUOX1–2 function and the decreasing number of H_2O_2-producing ciliated cells (50) lead to the inefficient oxidative innate immunity response. This results in chronic bacterial colonization and neutrophil infiltration. In advanced phases of chronic infection of CF lungs, neutrophil-derived ROS are predominant (47), and now, they are a source of deleterious oxidative stress, tissue injury, and airway narrowing. Therefore, a profound understanding of the role of NADPH oxidases in the initiation, course, and chronicity of CF is needed to predict the usefulness, timing, and efficiency of specific NOX inhibitors.

Sickle Cell Disease

Lung disease is a major cause of morbidity and death in SCD. Vendramini and colleagues investigated the prevalence of airway hyper-responsiveness in adult sickle cell patients, as it has been noted in children (159). Their study suggests that there is a high prevalence of airway hyperresponsiveness in adult patients with SCD without a history of reactive airway disease. In patients suffering from SCD, increased phosphorylation of p47phox in monocytes was observed after phorbol 12-myristate acetate (PMA) stimulation, compared with normal control subjects. This suggests that NADPH oxidase in these cells is preactivated and capable of more extensive further activation (99). Increased expression of NOX2 and higher phosphorylation of p47phox, occurring in monocytes, according to authors is presumably mediated by interferon gamma (IFN-γ) and other inflammatory mediators, which are present in augmented amounts in SCD patients (99). In the recent study, George *et al.* demonstrated that a significant part of ROS production in sickle cells is mediated enzymatically by NADPH oxidase, which is regulated by PKC, Rac GTPase. Moreover, they also showed evidence that NADPH oxidase activity in red blood cells can be induced by plasma inflammatory cytokines. These findings suggest a novel pathogenic mechanism in SCD, namely that systemic inflammation and enzymatically derived ROS within the sickle erythrocyte act in a positive-feedback loop to contribute to acute and chronic organ damage of SCD (53).

Bacterial and Viral Lung Infections

NOX-dependent ROS production has long been known as one of the most important mechanisms of the antibacterial innate host defense. This knowledge has been derived from the studies on chronic granulomatous disease (CGD), a primary immunodeficiency, resulting from the genetic defects in NADPH oxidase components (37). CGD phagocytes are not able to neutralize bacterial and fungal pathogens, which leads to recurrent life-threatening infections. Mutations in the genes encoding NOX2 and p47phox are responsible for ~90% of CGD cases (35, 70, 170). One of the major, very susceptible sites of infection are the lungs. The majority of CGD patients suffer from respiratory disease, including pneumonia, lung abscess, and pulmonary fibrosis. Apart from NOX2 and p47phox, p67phox subunit also contributes to CGD pathogenesis. In p67-phox-deficient CGD mutants, the NOX2 complex is in an inactive state. The addition of recombinant p67-phox drives the transition from the inactive to the active state (160). A recent study by Honda *et al.* analyzed the potential of recombinant proteins to compensate for defective components—p67phox and p47phox deficiency in CGD neutrophils. It appeared that the delivered p67phox and p47phox subunits localize in the cytoplasm and move to the membrane on stimulation. Moreover, they elicit minimal nonspecific activation in neutrophils (68). This unique ''gain-of-function'' NOX-modifying therapeutic strategy might be crucial for CGD patients. On the other hand, bacterial and fungal infections are common complications of every chronic inflammatory airway disease. Therefore, it is crucial to understand CGD pathogenesis and the importance of ROS-generating mechanisms in host defense, in an attempt to develop a potent and safe NOX inhibition strategy in chronic airway diseases.

Apart from the various bacteria, viruses are very common airway pathogens that may lead to primary severe lung infections or exacerbation of chronic lung diseases. Excessive ROS production has been shown to exert deleterious effects in influenza A-induced lung injury (71). NOX2-deficient or p47phox-deficient mice have decreased ROS production and inflammatory lung infiltration, reduced edema and lung injury, as well as diminished airway epithelial cell apoptosis in response to influenza A infection (71, 163, 164). Moreover, viral clearance rate and lung function is significantly improved in these mice (136, 163). On the other hand, NOX2 has also been identified as an essential modulator of antiviral responses in airway epithelial cells (139). Soucy-Faulkner *et al.* showed that NOX2-derived ROS are critical in the activation of IRF3 transcription factor and the expression of downstream antiviral genes—*IFIT1* and *IFNβ* (139). Moreover, it has been shown that NOX1 acts as a protective agent, suppressing influenza A-induced lung inflammation and

oxidative stress (130). However, rhinovirus-induced barrier dysfunction in polarized airway epithelial cells is mediated by the activation of Rac1 and NOX1 activity (28), suggesting possible virus-specific NOX responses in different cells. Therefore, a specific therapeutic approach against NOX isoforms and subunits serves as an interesting possibility to prevent noxious effects of viral infections. Nevertheless, further studies and cautious, possibly virus-specific, cell-targeted approaches are needed to develop truly specific and safe agents.

Molecular mechanisms of NOX and subunit regulation in airway inflammation

Although the developmental pathways and transcription factors triggering the expression of different NADPH oxidase components in various tissues have been, at least, partly established, the putative regulatory mechanisms that counterbalance these factors are still unclear (155). NOX2 has been, so far, the best studied isoform in the airways and elsewhere. NOX5 expression in the lungs is uncertain, thus next, we describe pathways of NOX1–4 regulation. A deeper understanding of the molecular regulatory mechanisms of NOX isoforms and subunits would help predict the desired and unwanted effects of NOX modifiers.

Transcriptional regulation of expression for the NOX2 system

While alternative mRNA splicing has been shown to influence the activity of several NOX-family proteins, NOX2 splice variants that would be functionally relevant have not been previously demonstrated. Harrison *et al.* recently provided evidence that NOX2 undergoes alternative mRNA splicing and yields a 30 kDa protein, NOX2β, regulating NADPH oxidase activity in mice and human macrophages (63) (Fig. 6). This variant lacks several regions binding prosthetic groups involved in the electron transfer from NADPH to molecular oxygen (56). Possibly, these splice variants represent novel regulatory protein groups that are incorporated into their respective NADPH oxidase complexes to enhance the catalytic activity of the full-length NOX proteins.

Several transcription factors regulate *NOX2* gene expression (official gene name designated as *CYBB* [human] or *Cybb* [mouse]—here, we used *NOX2* throughout for clarity) through *cis*-elements in a 450-base pair sequence in the 5′-flanking region of the gene. Yang *et al.* presented data showing the eosinophil-specific regulation of the *NOX2* gene by transcription factors GATA-1 and GATA-2 through the GATA-binding site (174). It has long been considered that the expression mechanism of *NOX2* in eosinophils is the same as that in other phagocytes. A restricted expression of *NOX2* in eosinophils from X-linked CGD patients implied a certain eosinophil-specific expression mechanism of the gene. Although an eosinophil-specific GATA-3 suppression mechanism was suggested by Sadat *et al.* (125), no positive regulatory mechanisms of the gene have, however, been shown in eosinophils. Eklund *et al.* (44) have proposed a cooperative activation of the *NOX2* gene by PU.1 and interferon regulatory factor-1 in myeloid cell lines, and Suzuki *et al.* have shown that PU.1 is an essential activator for the expression of the *NOX2* gene in human neutrophils, monocytes, and B-lymphocytes (146).

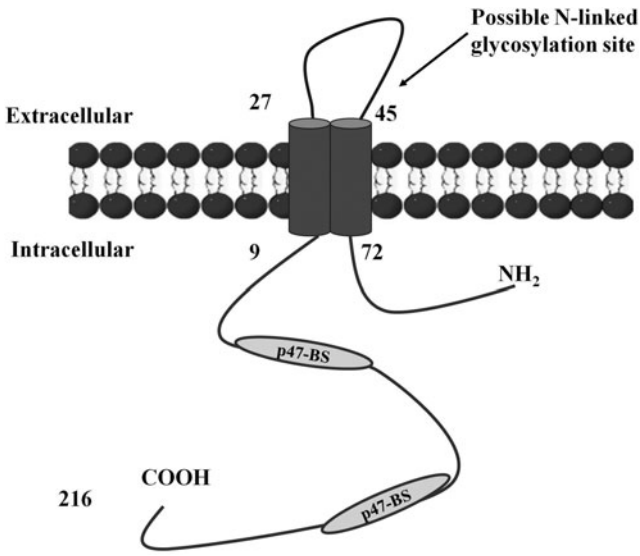

FIG. 6. The scheme of NOX2β protein. NOX2β is a novel splicing variant of NOX2 isoform. p47-BS indicates the conserved binding sites of p47phox (63). The NOX2β variant lacks several regions binding prosthetic groups. These are four conserved histidine residues that are postulated to bind two Fe-heme complexes and which are involved in the electron transfer from NADPH to molecular oxygen.

In isolated rat lungs, NOX2 expression was increased by an ethanol-stimulated increase in renin-angiotensin system) activity (118). These findings extend previous work linking ethanol ingestion to oxidative stress in the lung and susceptibility to lung injury (59), but the precise mechanism remains unknown. Interestingly, lung p22phox or p47phox expression levels remained unchanged.

The transcriptional regulation of the *p22phox* gene is a mechanism that controls NADPH oxidase activity (162). According to Gauss *et al.*, *p22phox* gene is regulated by NF-κB and AP-1 (52). The authors provide evidence that in human aortic SMCs, the inhibition of the AP-1 pathway reduces the activity and expression of NADPH oxidase and that AP-1 physically interacts with *p22phox* gene promoter. Since AP-1 is a redox-sensitive transcription factor, it might be presumed that there is a positive feedback mechanism in which ROS, generated by the NADPH oxidase, is important for the persistent superoxide generation. This hypothesis is supported by data obtained from endothelial cells, with regard to the redox regulation of the NADPH oxidase subunit p22phox in endothelial cells (38).

The activation of NOX2 is strictly dependent on the p47phox subunit. Using p47phox-and NOX2-deficient mice that lack NADPH oxidase function, Segal *et al.* showed that NOX limits inflammation by attenuating the pro-inflammatory transcription factor NF-κB and by activating nuclear factor erythroid 2-related factor (Nrf2), a key redox-sensitive anti-inflammatory transcription factor. The studies demonstrate the pharmacological activation of Nrf2 as a potential therapeutic strategy in CGD. This work identifies NADPH oxidase as a critical regulator of innate immunity and provides a novel understanding of the mechanisms that regulate lung inflammation (129).

Direct regulation of activity for the NOX2 system

The process of regulation of NADPH oxidase complex is complicated by various issues, which are necessary in its activation (Table 1). Moreover, the strict control is needed to deliver the activation at the appropriate time and place. Thus, several signaling pathways have been established that regulate the NADPH oxidase downstream of cell surface receptors. Phosphatidylinositol 3-kinase (PI3K)-dependent pathways, the blockade of which severely limits activation of the oxidase to several stimuli, play a pivotal role. The precise roles of the phosphoinositide products of PI3K activity in regulating NADPH oxidase assembly and activation are still unclear; however, emerging data suggest that they might play an important role *via* the regulation of guanine nucleotide exchange in the Rac component. There is also strong evidence that the PI3K products—PtdIns(3, 4)P2 and PtdIns3P can bind directly to the phox homology (PX) domains, which are located in the p47phox and p40phox, respectively (64). Based on available studies, Yamamori *et al.* presented a model of signal transduction pathways leading to p47phox phosphorylation and NADPH oxidase by the activation of PKCs (Fig. 7) (172).

According to Gao *et al.*, superoxide production is required for the induction of sepsis-induced lung microvascular injury in the murine model. The authors suggest that NADPH oxidase-derived $O_2^{\bullet -}$ generation has an important bactericidal role, such that an impairment in bacterial clearance in NADPH oxidase-defective mice results in increased chemokine generation and lung tissue PMN infiltration (51).

Translocation of Rac-GTPase to the plasma membrane, independent of p47phox and p67phox, is essential for the assembly and activation of NADPH oxidase (116). Current studies support a complex formation between p67phox and Rac in the membrane for optimal electron transport through NOX2 in $O_2^{\bullet -}$ production. However, molecular mechanisms regulating the activation of Rac GTPases in phagocytosis and ROS generation remain to be clearly defined (Fig. 8). Several NOX isoforms have been implicated in Rac-dependent ROS generation and are regulated *via* Rac (69, 106). However, it remains unclear as to whether Rac1 interacts directly or requires other components—p47phox or NOXA1—to regulate NOX isoforms. Dang *et al.* demonstrated that p67phox is constitutively phosphorylated in resting human neutrophils and furthermore, that the state of p67phox phosphorylation is controlled by an upstream tyrosine kinase, MEK1/2 and the serine/threonine phosphatases PP1/2A. Dysregulation of the balance between these two activities could play a role in ROS production by human neutrophils at inflammatory sites (33). How this constitutive phosphorylation of p67phox may regulate NADPH oxidase activation remains unknown, but it is speculated that it could have a role in maintaining the oxidase inactive in the cytosol or it could prepare p67phox for more efficient activation of the enzyme (33). The phosphorylation, according to the authors, has no modifying effects on the binding of p67phox to the p40phox and p47phox complex. Unlike p47phox or p67phox, the p40phox component is an alternative for NOX2 activity. It is absent from CGD patients who lack p67phox, suggesting that the protein is stable only on binding to p67phox (103). However, available data suggest that p40phox is specific for NOX2 though as NOX1 and NOX3 could potentially be activated by the p47phox/p67phox complex, a role of p40phox in this scenario cannot be excluded (8). Using ionizing radiation Ostuni *et al.* demonstrated that ROS might stimulate NOX activity (110). The study shows that p67phox integrity is crucial for the oxidase activity and that irradiation of p67phox drastically decreases its interaction with arachidonic acid (AA) and destabilizes the p47phox–p67phox complex (93, 110). The precise role played by AA still remains unclear, though Souabni *et al.* have shown that *trans*-arachidonic acid (*trans*-AA), in contrast to *cis*-AA, cannot activate the NADPH oxidase but is able to inhibit *cis*-AA-activated generation of $O_2^{\bullet -}$ through a direct interaction with p67phox and with the membrane fraction, probably indirectly *via* modification of the membrane physical properties (138).

NOX1, NOX3, and NOX4 Modulation Mechanisms

Although each NOX family member is typified by six transmembrane domains along with a cytoplasmic domain that binds NADPH and flavin adenine dinucleotide (FAD), each isoform is distinguished by the specific catalytic subunit, interacting proteins, and subcellular localization.

NOX1

The considerable constitutive activation of NOX1 in the absence of cell stimulation is explained partly by the absence

Table 1. Summary of Mechanisms Regulating Activity and Expression of NOX and DUOX Enzymes

NOX/DUOX	*Regulatory subunits*	*Other regulatory issues*	*Requiring of p22phox*
NOX1	NOXO1, NOXA1, Rac1	STAT1, INF-γ, LPS, angiotensin II, urokinase plasminogen activator, platelet-derived growth factor, prostaglandin F2, phorbol ester, activated K-Ras	Yes
NOX2	p47phox, p67phox, p40phox, Rac2/1		Yes
NOX3	NOXO1, NOXA1		Yes
NOX4	Constitutively active	Nrf2, MAPK, MEK1-ERK1/2	Yes
NOX5	Ca^{2+} and phosphorylation		No
DUOX1	Ca^{2+}	IL-4, IL-13	No
DUOX2	Ca^{2+}	IL-1α, IL-1β, IFN-γ	No

DUOX, dual oxidase; ERK1/2, extracellular signal-mediated protein kinases 1 and 2; IFN-γ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; MEK1, dual threonine and tyrosine recognition kinase; Nrf2, nuclear factor erythroid 2-related factor.

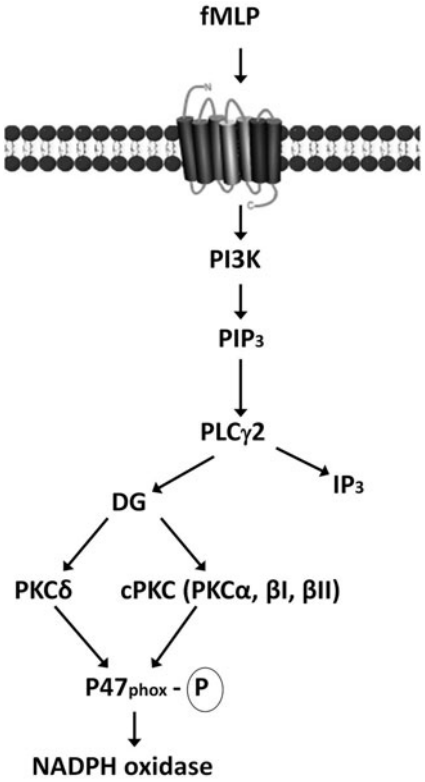

FIG. 7. NADPH oxidase activation *via* PI3K. Ligand binding to fMLP receptors triggers phosphatidylinositol 3-kinase (PI3K) activation and subsequent phosphatidylinositol 3,4,5-triphosphate (PIP3) production. PIP3 induces protein-dependent kinase (PDK) activation, and PDK, in turn, phosphorylates and activates Akt. Phospholipase Cc2 (PLCc2) is directly activated by PIP3 and leads to the activation of diacylglycerol (DG)-dependent protein kinases C, cPKC, and PKCd. The activation of these PKCs results in p47phox phosphorylation and the subsequent NADPH oxidase activation (172). fMLP, formyl-methionyl-leucyl-phenylalanine.

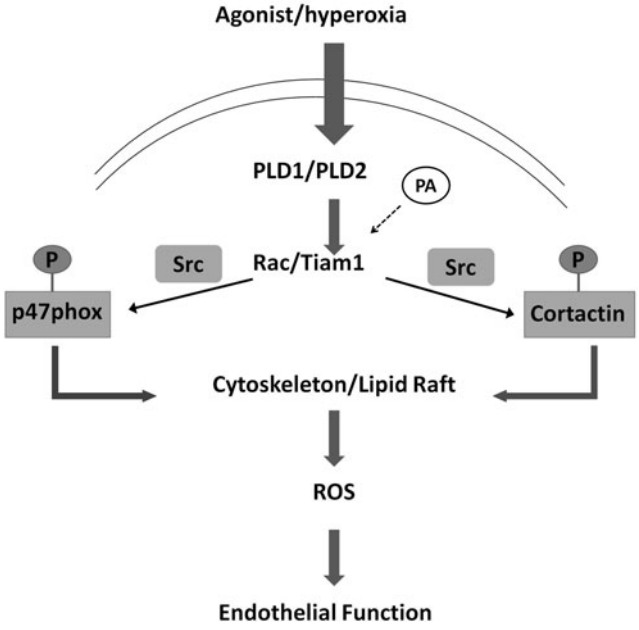

FIG. 8. Rac1-dependent NADPH oxidase activation and ROS generation. Schematic illustration of the mechanism dependent on Rac1 subunit, leading to phosphorylation/translocation of p47phox and activation of endothelial NADPH oxidase (116)—modified. Stimulation of cells results in phospholipase D1 and D2 (PLD1 and PLD2) activation and activation of Rac1/Tiam1 (which are regulated by phosphatidic acid [PA]). This leads to Src-dependent phosphorylation of p47phox and cortactin. Subsequently, assembly of the NADPH oxidase complex occurs for ROS generation. Tiam, T-cell lymphoma invasion and metastasis 1.

of regulatory phosphorylation sites on NOXO1 and the ability of NOXO1 to localize to the resting cell membrane *via* its PX domain (20). NOXO1 is a p47phox homolog, which, in contrast to p47phox, has a total of four different splice variants (19). The major difference between p47phox and NOXO1 is the presence of an autoinhibitory domain in p47phox that does not occur in NOXO1. This domain prevents association with p22phox (42).

NOX1 transcription is activated by inflammatory mediators such as INF-γ or lipopolysaccharide, which also induce the expression of NOXO1. NOX1 is up-regulated by growth factors and growth-related agonists; for example, angiotensin II, urokinase plasminogen activator, platelet-derived growth factor, prostaglandin F2, phorbol ester, and mutationally activated K-Ras in various tissues (25, 86, 89, 98).

Unlike the NOX2, the NOX1 system seems to have relatively few regulatory elements. Only guanine nucleotide exchange on Rac1 is convincingly documented as a regulatory trigger, although NOX1 activation by Rac1 is apparent only when cellular levels of NOXO1 and NOXA1 are low (18).

In a human pulmonary epithelial cell line, increased NOX1 expression is caused by hypoxia, which, in addition, activates HIF-1α-dependent pathways. It is not yet established whether HIF1α itself induces NOX1 expression (55, 89).

NOX3

NOX3 is the closest homologue of NOX2 (58% identity) in the NOX protein family (17). NOX3 is unique in its ability to be activated by NOXO1 alone in the absence of activator subunits. Cheng *et al.* characterized the requirements of NOX3 for regulatory subunits, including p47phox, p67phox, NOXO1, and NOXA1. The research team suggested that NOX3 shows unusual flexibility in its ability to be activated by both phox and NOX regulatory proteins, although they concluded that NOX3 regulation does not require an intact activation domain of p67phox, indicating that other regions of p67phox are important for regulating NOX3 activity. NOX3 appeared to be maximally activated by NOXO1 alone, and with no requirement of NOXA1 (21).

Using human kidney cells and Chinese-hamster ovary cells, Nakano *et al.* indicated that p22phox is physically associated with both NOX3 and NOXO1. The plasma membrane localization of NOX3 but not of NOXO1 requires p22phox. Moreover, the glycosylation and maturation of NOX3 requires p22phox expression, suggesting that p22phox is required for the proper biosynthesis and function of NOX3 (108).

It is, though, important that subunit regulation differs depending on species: Human NOXO1 dramatically stimulates NOX3 activity in the absence of NOXA1 or p67phox (21). However, mouse NOX3 activity requires both NOXO1 and NOXA1 (7).

NOX4

NOX4 isoform of NADPH oxidase strongly contributes to lung pathologies, especially to pulmonary fibrosis. Transcriptional regulator of antioxidant genes, Nrf2, has been demonstrated to regulate NOX4 expression that was confirmed in human lung endothelium in response to hyperoxia and in the mouse lung. Lack or diminished levels of hyperoxia induce NOX4 expression in human lung endothelial cells or mouse lung with reduced levels of Nrf2. Moreover, Nrf2 expression knockdown with siRNA attenuates the expression of NOX4 (114, 115).

Recent studies implicate a role for the NOX4 in tissue repair functions of myofibroblasts and fibrogenesis. NOX4 is also up-regulated in type II epithelial cells in IPF patients and participates in TGF-β-induced epithelial cell apoptosis, an early crucial step in the pathogenesis of IPF. The data indicate that NOX4 is up-regulated in human IPF as well as in mice lungs with bleomycin- or hapten-induced injury (65).

TGF-β1 has been shown to induce NOX4 expression in human lung mesenchymal cells through SMAD-3 protein. This pro-fibrogenic mediator specifically induces mRNA/protein expression and enzymatic activation of the *NOX4* isoform in differentiated myofibroblasts. NOX4-dependent H_2O_2 generation seems to be required for myofibroblast differentiation, synthesis of ECM proteins, and contractility mediated by TGF-β1. Moreover, in murine models of lung injury, genetic or pharmacologic targeting of NOX4 attenuated fibrogenesis (3, 65, 76).

Inhibitors of NOX/NADPH Oxidases

NADPH oxidases inhibitors may be grouped into two main categories: small molecules (nonpeptide, chemical inhibitors) and peptide inhibitors synthesized to target specific NADPH oxidase complex subunits. So far, most of them have been used in *in vitro* and animal model-based experiments. However, among them, there are some with proven safety and efficacy in small clinical studies in human airway diseases (*e.g.,* apocynin). Others are currently used in clinics in the nonairway-related disorders, but their newly discovered NOX inhibitory potential may lead to broadening their spectrum of use (*e.g.,* nebivolol). Recent discoveries have questioned the selectivity of most widely used small-molecule inhibitors, while the newer peptide based are facing problems with the targeted delivery, stability, and potential immunogenicity. Nevertheless, significant progress has been made and high-throughput screening technologies may result in the development of the selective, stable, nontoxic, and efficient NOX modifiers.

Small-Molecule Inhibitors

One of the first identified NADPH oxidase inhibitors, still widely used in *in vitro* experiments, is DPI, which inhibits NOX activity (92, 119). DPI suppressed ROS production by NOX4 and significantly decreased the radiation-induced expression of α-smooth muscle actin and fibronectin in human lung fibroblasts (112). Moreover, it attenuated the pulmonary injury induced by PMA in guinea pigs (165). However, it is also known that DPI is a nonspecific blocker of many flavoprotein-dependent enzymes, including complex I of the mitochondrial electron transport chain, nitric oxide synthases, and cytochrome P450 (132).

Phenylarsine oxide (PAO), a sulfhydryl reagent for vicinal or proximal thiol groups, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) inhibit NADPH oxidase through preventing assembly of the complex (36, 39). However, these agents are also known as inhibitors of other enzymes. PAO is a phosphotyrosine phosphatase inhibitor, and AEBSF is a serine protease inhibitor. An intraperitoneal injection of PAO to rats reduced ROS production by rat phagocytes and neutrophil infiltration into the lung after the inhalation of lipopolysaccharide (123). According to Weissmann *et al.*, AEBSF attenuated alveolar macrophage oxidative burst elicited by PMA in rabbit system, in a dose-dependent manner (168). AEBSF inhibits binding of the cytoplasmatic subunit p47phox to the NOX2/p22phox complex, but still, little is known about its usefulness in airway tissues (36).

Accumulating evidence confirm a connection between the actin cytoskeleton as well as actin-binding proteins and NOX activation (phagocytic and nonphagocytic) (104). The activation of NADPH oxidase by actin-destabilizing agents (*e.g.,* cytochalasin D) may involve multiple signaling pathways that are associated with actin cytoskeletal reorganization (154). Coronin and cortactin—actin-binding proteins are known to interact with NADPH oxidase components and are engaged in the regulation of oxidase-dependent ROS production. A role for cortactin in hyperoxia-induced translocation of p47phox and the activation of NADPH oxidase and the generation of $ROS/O_2^{\bullet -}$ has been demonstrated in human lung endothelium (152, 154).

A broad spectrum of targets of the agents described earlier significantly decrease their potential in clinical settings, raising the possibility of adverse effects and unexpected toxicity. Therefore, in recent years, substantial effort has been invested into (i) performing small but well-designed and controlled clinical trials with existing, nonspecific NOX modifiers in clearly defined patient populations; (ii) redefining currently used drugs, but with newly described characteristics into a new disease spectrum; and (iii) high-throughput screening approaches to find new selective NOX inhibitors. These three strategies have led to the development of new agents and the re-introduction of older agents as potential new treatment options.

One such example is apocynin, a molecule structurally related to vanillin, believed to interact with p47phox (171). In a rat model, the administration of apocynin attenuated the inflammatory response, lung permeability, and ischemia reperfusion in the isolated, perfused lung (22). Nevertheless, apocynin can also inhibit NOX1 and it can scavenge ROS, which makes it a nonspecific NOX inhibitor (46, 66). Moreover, there are studies suggesting that apocynin is not an NOX inhibitor but an antioxidant, or that it increases ROS generation by NADPH oxidase instead of decreasing it (66, 158). However, *in vivo*, a metabolite of this compound may act as a true NOX inhibitor, and it may show some specificity for NOX2. We and others have shown that nebulized apocynin is safe (142), prevented ozone-induced exacerbations of asthma, and decreased selected ROS and RNS in mild

asthmatics and patients with COPD (117, 140, 141). Therefore, even the nonselective ROS-scavenging and NOX-modifying strategy might be beneficial in inflammatory airway disorders and deserves further clinical studies in clearly defined populations and with the reasonable caution of the possible adverse effects and cytotoxicity.

Nebivolol, a β1 receptor blocker, is currently used in the treatment of hypertension and left ventricular failure (1). Recently, it was shown to also act as an NOX inhibitor. It decreases oxidase expression by the inhibition of membrane association, an interaction of p67phox and Rac. Moreover, nebivolol inhibits NOX1-dependent superoxide production (102, 105). Therefore, as a drug of known pharmacological characteristics, it raises a hope for broadening its use in pulmonary diseases; for example, in idiopathic pulmonary hypertension (101).

Data provided by Smith *et al.* indicate that ebselen and its analogs represent a class of compounds which inhibit ROS generation by interrupting the assembly of NOX2-activating regulatory subunits (135). Ebselen and analogs potently inhibit NOX1 and NOX2 activity, and they were less effective against other isoforms. Ebselen also blocked the translocation of p47phox to neutrophil membranes. Furthermore, previous investigations in challenged guinea pigs suggest that ebselen significantly inhibits late airway response and suppresses airway inflammation (177). Ebselen also inhibits cigarette-smoke-induced lung inflammation in mice (40). It has been tested in humans in stroke (57) and bipolar disorders (134) clinical trials.

Finally, discovered by application of a high-throughput screening technology, derivatives of pyrazolo-pyrido-diazepine dione (*e.g.,* Genkyotex compounds GKT 136901 and GKT137831) appear highly promising therapeutics for the treatment of IPF and other nonpulmonary diseases, as they target NOX4 and NOX1 isoforms (6, 49, 88). These compounds, especially one called 7c, are highly potent in *in vitro* assays on human lung fibroblast differentiation and in murine models of bleomycin-induced pulmonary fibrosis. They demonstrate a superior efficiency to Pirfenidone, which is the only drug accepted for the treatment of IPF in Japan and Europe these days (49, 88).The specificity of these compounds was confirmed in *in vitro* pharmacological profile, showing concomitant benefits such as good oral bioavailability and high plasma concentrations *in vivo* (6, 49, 88).

High-throughput screening technology has also led to the development of fulvene-5 and its derivatives, potent and efficacious inhibitors of NOX2 and NOX4 (11), as well as ML171, a specific and selective NOX1 inhibitor (54). Fulvene-5 has been successfully applied *in vivo* to block the growth of endothelial tumors of mice (11). However neither of them has yet been used in the context of lung diseases.

Peptide-Based Inhibitors

Peptide-based inhibitors seem to be promising for providing NOX isoform-specific inhibitors. NOX2, p47phox, p67phox, and Rac are likely targets for peptides directed against their unique sequences (46).

Peptides derived from NOX2 have been used to inhibit NADPH oxidase. A peptide mimicking one of NOX2/p47phox interaction sequences that corresponds to amino acids 77–93 has a potent inhibitory effect on human NADPH oxidase. By binding to p47phox, NOX2ds-tat (designated also as gp91ds-tat) inhibits the activity of not only NOX2, but quite probably also NOX1, although this hypothesis has been recently challenged (31) NOX2ds-tat was described as the strongest in inhibiting superoxide generation in the vascular system, where NOX1 and NOX4 play a key role (26). This chimeric peptide that consists of a *tat* site derived from the tat peptide of the HIV is cell permeable. It also contains a fragment of NOX2, which has previously been shown to prevent the interaction of p47phox with the NOX subunits in cell-free preparations (72, 121, 150). In many studies, NOX2ds-tat has been used as a specific NADPH oxidase inhibitor (48, 84, 109), often in two forms—scrambled (tat scrambled) and unscrambled (tat unscrambled) (144, 150, 178). In human leukocytes, unscrambled but not scrambled NOX2ds-tat significantly reversed TNF-induced core 2 β-1,6-N-acetylglucosaminyltransferase (C2GNT) activity, demonstrating a novel signaling cross-talk between C2GNT, NADPH oxidase, and specific PKCβ1/2) (150). Furst *et al.* demonstrated that NOX2ds-tat reduced superoxide production even below control levels after an increase by atrial natriuretic peptide in the endothelium of intact rat lung vessels (48). On the other hand, Krotz *et al.* suggest that although NOX2ds-tat was able to reduce significantly basal superoxide release, it did not affect $O_2^{\bullet -}$ formation after an incubation with mycophenolate acid (MPA) anymore, suggesting that MPA had been blocked before this basal activity of endothelial NADPH oxidase in human umbilical vein endothelial cells (84). Furthermore, a study conducted by Norton *et al.* using NOX2ds-tat enabled to effectively and selectively inhibit NOX2 in isolated rat lungs (109). The data of the successful use of NOX2ds-tat in experimental vascular disease models suggest its potential in PAH, which, in general, lacks treatment options.

A sequence corresponding to amino-acid residues 323–332 inhibits the phosphorylation of p47phox, and its translocation, therefore, inhibits NADPH oxidase activation in a cell-free system (169). This peptide may compete either for p47phox phosphorylation or for an interaction with the NOX2/p22phox complex or p67phox, by acting as a pseudosubstrate for protein kinases. Another synthetic peptide, corresponding to residues 314–331, also inhibits p47phox phosphorylation and translocation in human neutrophils and inhibits PKC-mediated phosphorylation of p47phox (10).

Peptide-based inhibitors appear to be the most promising candidates for therapy. Nevertheless, the main problems with inhibitory peptides are their cell delivery and their stability in live organisms. Apart from limited bioavailability, peptides are challenged due to their gut degradation, associated toxicity, and inability to cross the plasma membrane of living cells. However, in terms of many lung diseases (*e.g.,* asthma, COPD), inhaled aerosol administration route of the drug is the preferable one. Therefore, this drawback in other disorders may, in fact, facilitate the development of efficacious airway peptide-based therapy (4). Nanoparticles and polymeric microparticles also hold promise for more efficient and targeted drug delivery, which has been an extensive field of research in recent years (85).

Perspectives and Conclusions

Available NOX inhibitors do not specifically target pathologic signaling, leaving physiologic signaling intact. The

perfect NOX inhibitors ought to be both—isoform specific and cell specific, and preferentially targeting pathological modes of NOX signaling. This level of specificity could potentially be achieved by direct interfering with the interface between a particular NOX isoform and the proteins with which it should interact to become active under pathological conditions (143). Such a profound understanding of NOX enzymes and their downstream targets regulation obviously requires further studies. High-throughput scanning strategies and the development of more sophisticated delivery techniques are strong indices of upcoming progress in this field. Airway diseases, with the preferred inhaled or nebulized mode of drug administration and with the existing ways of noninvasive monitoring of oxidative stress, seem to be a perfect setting for NOX-modifying therapy. Knowing the exact pathophysiology and variable courses of the diseases, nonspecific NOX inhibitors might be useful in the therapy. Simultaneously, though, adverse events remain a serious question. Bacterial and viral infections are common complications of chronic respiratory diseases. Therefore, probable clinical side effects of NOX inhibitors should emerge from careful studies of animals and humans carrying genetic mutations in NOX genes (73, 137).

To date, no therapeutically viable molecule exists but recent studies using current inhibitors have enhanced our knowledge on the role of NADPH oxidase and its possible modification strategy in airway diseases. The group of NADPH oxidase modifiers, particularly inhibitors, is growing each year; there are more data explaining mechanisms of their activity, as well as new smaller and larger clinical trials delineating their safety and potency. This situation certainly leads to the introduction of specific and effective NOX modulators, but still, we are a step away of optimal therapy.

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Address correspondence to:
Prof. Rafal Pawliczak
Department of Immunopathology
Faculty of Biomedical Sciences
and Postgraduate Training
Medical University of Lodz
7/9 Zeligowskiego St.
Lodz 90-752
Poland

E-mail: rafal.pawliczak@csk.umed.lodz.pl

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Abbreviations Used

AA = arachidonic acid
ADAM17 (TACE) = ADAM metallopeptidase domain 17 (tumor necrosis factor-α-converting enzyme)
AEBSF = 4-(2-aminoethyl)-benzenesulfonyl fluoride
ALI = acute lung injury
AP-1 = activator protein 1
ARDS = acute respiratory distress syndrome
CF = cystic fibrosis
CFTR = cystic fibrosis transmembrane conductance regulator
CGD = chronic granulomatous disease
cis-AA = *cis*-arachidonic acid
COPD = chronic obstructive pulmonary disease
COX-2 = cyclooxygenase-2
$cPLA_2$ = cytosolic phospholipase A_2
DPI = diphenylene iodonium
DUOX = dual oxidase
EGFR = epidermal growth factor receptor
fMLP = formyl-methionyl-leucyl-phenylalanine
HIF-1α = hypoxia-inducible factor 1-alpha
ICAM-1 = intercellular adhesion molecule-1
IFN-γ = interferon gamma
IGF-1 = insulin growth factor 1
IPF = idiopathic pulmonary fibrosis
LPO = lactoperoxidase
MAPK = mitogen-activated protein kinases
MMP-9 = matrix metalloproteinase-9
MPA = mycophenolate acid
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2 = nuclear factor erythroid 2-related factor
PAH = pulmonary arterial hypertension
PAO = phenylarsine oxide
PDK = protein-dependent kinase
PI3K = phosphatidylinositol 3-kinase
PIP3 = phosphatidylinositol 3,4,5-triphosphate
PKC = protein kinase C
PLCc2 = phospholipase Cc2
PX = phox homology
RNS = reactive nitrogen species
ROS = reactive oxygen species
SCD = sickle cell disease
SMC = smooth muscle cell
TGF = transforming growth factor
Tiam = T-cell lymphoma invasion and metastasis 1
TNF = tumor necrosis factor
trans-AA = *trans*-arachidonic acid
VCAM-1 = vascular cell adhesion molecule-1