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Forum Review

The Role of Endogenous NADPH Oxidases in Airway and Pulmonary Vascular Smooth Muscle Function

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ABSTRACT

Reactive oxygen species generated from NADPH oxidase(s) in airway smooth muscle cells and pulmonary artery smooth muscle cells are important signaling intermediates. Nox4 appears to be the predominant gp91 homologue in these cells. However, expression of NADPH oxidase components is dependent on phenotype, and different homologues may be expressed during different functional states of the cell. NADPH oxidase(s) appear to be important not only for mitogenesis by these cells, but also for O_2 sensing. The regulation of NADPH oxidase(s) in airway and pulmonary artery smooth muscle cells has important implications for the pathobiochemistry of asthma and pulmonary vascular diseases. *Antioxid. Redox Signal.* 5, 751–758.

INTRODUCTION

THIS REVIEW focuses on work from our laboratory on the role of oxygen metabolites in airway wall and pulmonary vascular remodeling associated with asthma and pulmonary hypertension. Free molecular oxygen (O_2) appeared on the earth's surface some 2 billion years ago as a result of photosynthetic microorganisms acquiring the ability to split water (34). It is the most abundant element in the earth's crust and the second most abundant element in the biosphere. O_2 is an unusual molecule in that it has two unpaired electrons with parallel spins. It is therefore a biradical. To overcome spin restriction, O_2 prefers to accept electrons one at a time, and the sequential addition of electrons leads to the formation of reactive oxygen species (ROS). As a consequence of metabolism, all aerobic organisms are subject to a certain level of physiological oxidative stress as ROS are produced continuously in numerous biological processes.

A considerable body of literature describes roles for ROS in the pathogenesis of a large number of pulmonary diseases, including asthma, chronic obstructive pulmonary disease, acute respiratory distress syndrome, and interstitial pulmonary fibrosis. Initially, the involvement of ROS in disease pathogenesis was conceptualized as the chemistry of "scorched earth," in which critical cell proteins, lipids, or sugars were indiscriminately oxidized and rendered metabolically inactive for their roles in normal cell function (35) with resultant injury and cell death. Now, evidence is accumulating that small amounts of ROS generated in select cell compartments act as signaling molecules, controlling gene expression (78). The first signaling components to be identified as ROS-sensitive were transcription factors. Nuclear factor-κB (NF-κB), a critical transcription factor for the expression of inflammatory mediators, is sequestered in the cytosol in a complex with its inhibitor IκB. ROS induce activation of NF-κB, although the mechanisms are not clearly understood (40). ROS also regulate activator protein-1, a transcription factor complex formed by homo- or heterodimerization of members of the Jun and Fos families of proteins (65). Mechanisms for this activation include the reversible *S*-glutathiolation of a single conserved cysteine residue (1), and redox regulation by thioredoxin and the nuclear protein Ref1 (80). ROS also activate both of the important mitogenic signal transduction cascades in smooth muscle (58), the mitogen-activated protein kinase (2, 3) and

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phosphatidylinositol 3-kinase (74) pathways. Furthermore, ROS activate p38 (76) and c-Jun NH_2-terminal kinases (50, 61, 74), stimulate receptor tyrosine kinase (RTK)-linked growth factor tyrosine phosphorylation (29) by modification of critical receptor sulfhydryls (19, 79) and/or oxidation of critical cysteine residues at the active site of opposing phosphatases (68, 72, 75), and activate p21 Ras (49). These effects have been suggested as important in the airway wall and pulmonary vascular remodeling (2, 3). Interestingly, RTK-dependent growth factors themselves can generate ROS. Platelet-derived growth factor (PDGF) (69), epidermal growth factor (9), insulin (46, 47), and transforming growth factor-β (71) have each been shown to stimulate production of superoxide anion (O_2^-) (14) and hydrogen peroxide (H_2O_2) (39, 64, 71) in nonphagocytic cells.

To understand better the role of ROS in airway wall and pulmonary vessel remodeling, several key questions need to be addressed. These include: what are the endogenous sources of ROS in airway and pulmonary artery smooth muscle cells?; how is their activity regulated?; and what are their physiologic and pathophysiologic roles? In this review, we present information from our laboratory addressing these questions. The results from our studies emphasize an important role for novel NADPH oxidases as sources of ROS that function as signaling molecules and mitogens. Additionally, we describe a possible role for these oxidases in hypoxia sensing.

AN NADPH OXIDASE INDUCES PROLIFERATION OF AIRWAY SMOOTH MUSCLE CELLS

Our initial studies (15) explored the role and source of ROS in mitogenic signaling of airway smooth muscle cells (AWSMC). We hypothesized that oxidant signaling might be especially important in the asthmatic airway, where smooth muscle proliferation in chronic severe asthma contributes to development of fixed airway obstruction. We explored the role of ROS in mediating AWSMC proliferation. We demonstrated that antioxidant treatment of AWSMC dramatically reduced cell proliferation in response to mitogenic stimulation with serum or PDGF (Fig. 1A). Antioxidants also inhibited expression of c-Fos, the product of an early response gene up-regulated by mitogens in response to cooperative protein kinase C and Ras/Raf stimulation of members of the mitogen-activated protein kinase superfamily of serine-threonine kinases. Finally, serum stimulation of AWSMC promoted a significant increase in O_2^- release by myocytes into culture medium. Taken together, these results provided evidence that ROS generated directly in response to mitogenic stimulation of AWSMC may be physiologically important in signaling subsequent events leading to proliferation.

We pursued the studies further to identify the enzymatic source and chemical identity of the ROS proximate in signaling (16). The endogenous sources that contribute to the generation of ROS in the lung are poorly characterized, but include the NADPH or NADH oxidoreductases, the mitochondrial electron transport chain, arachidonic acid-metabolizing enzymes, and the molybdenum hydroxylases. In AWSMC, increased su-

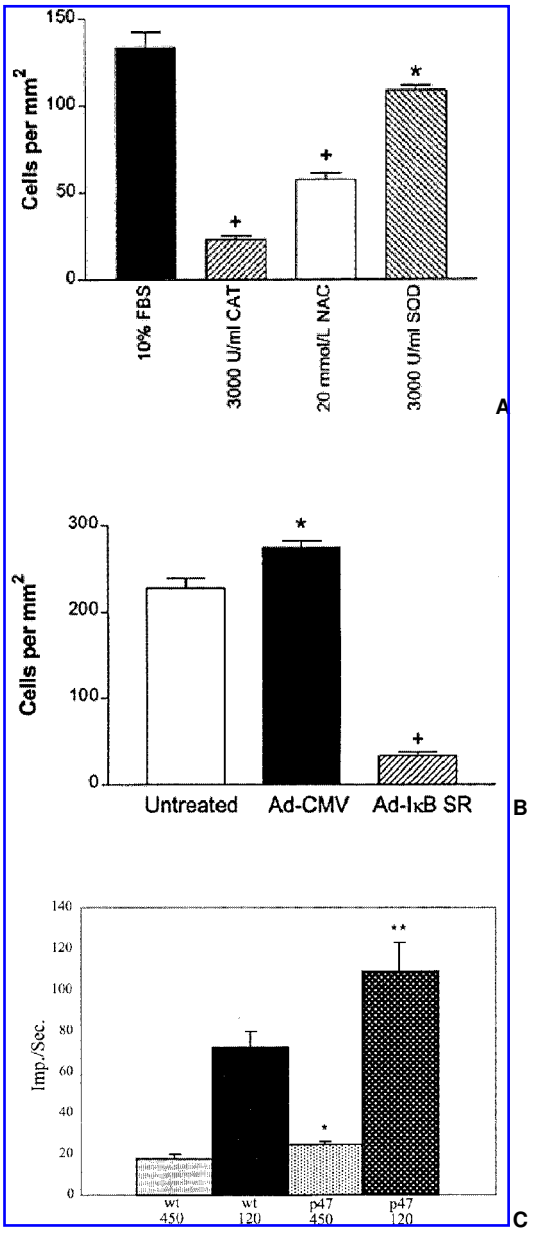

FIG. 1. (**A**) Antioxidants and flavoprotein inhibitors reduce growth of human airway smooth muscle. Cells stimulated with 10% fetal bovine serum (FBS) were seeded at a density of 50,000 cells/well in 24-well plates and grown in the presence of the antioxidants catalase (CAT; 3,000 U/ml), *N*-acetylcysteine (NAC; 20 mmol/L), or superoxide dismutase (SOD; 3,000 U/ml). After 48 h, cells were fixed in ice-cold buffered formalin 5% in Dulbecco's modified phosphate-buffered saline), permeabilized with two 30-min treatments of ice-cold methanol, stained with Wright's modified Giemsa, and counterstained with eosin. (**B**) Ad-CMV-treated (control) and AdIκBαSR-treated (NF-κB inhibited) cells 24 h after transduction. NF-κB inhibition by Ad-IκBαSR significantly reduces human airway smooth muscle proliferation. $*p < 0.05$ vs. untreated cells; $+p < 0.001$ vs. AdCMV or untreated cells. (**C**) Chemoreceptor activity averaged during 100 s at 450 and 120 Torr Po_2 in wild-type (wt) ($n = 7$) and $p47^{phox}$-null mutant mice ($n = 8$). $*p = 0.017$ for differences at 450 Torr; $**p = 0.026$ for differences at 120 Torr.

peroxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction, serum-induced cell proliferation, and c-Fos expression were prevented by pretreatment of cells with diphenylene iodonium (DPI), a flavoprotein oxidase inhibitor. This suggested that ROS-induced proliferation of AWSMC is due to a flavoprotein-dependent enzyme. In additional studies, catalase, but not SOD, prevented AWSMC proliferation, suggesting that the proximate diffusible oxidant species important for signaling was H_2O_2. Enhancement of PDGF-stimulated cell proliferation by SOD supported this supposition. A central role for H_2O_2 was not unexpected because enzymatic (via SOD) or nonenzymatic dismutation of O_2^- rapidly follows its generation to form H_2O_2, and compared with O_2^-, H_2O_2 is highly diffusible and therefore would be a better candidate to serve a signaling role.

We sought to characterize the NADPH oxidase in cultured human AWSMC and to understand better its contribution to signaling of growth and proliferation. The best studied NADPH oxidase is the leukocyte enzyme responsible for the microbicidal respiratory burst (8). This enzyme consists of two membrane proteins, gp91 (now officially Nox2) and p22, that bind a flavin adenine nucleotide (FAD) and form a unique cytochrome with a redox midpoint potential of −245 mV and a reduced minus oxidized difference spectrum of 558. In neutrophils, the NADPH oxidase has the unusual characteristic of using an electron from cytosolic NADPH to reduce extracellular O_2 to O_2^-.

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$$

At least two and possibly three cytosolic peptides (p47, p67, and p40) are also essential, and several other cytosolic components participate, including the small GTPases, Rac 1 or Rac 2 (84). The gp91 subunit appears to contain all factors necessary for transporting electrons from NADPH via FAD and then heme to molecular O_2. Current models postulate that the cytosolic components combine to cause a conformational change in the cytochrome that facilitates NADPH binding, and/or electron transfer between NADPH and FAD, and/or from FAD to heme. Genetic defects affecting the oxidase components cause chronic granulomatous disease, a disorder characterized by recurrent life-threatening infections.

Recent evidence indicates that low-activity NADPH oxidases with homology to the phagocyte oxidase are present in nonphagocytic cells and are important in cell signaling. This suggestion came initially from studies in plants. Over the past several years, a number of homologues of gp91 (10 homologues identified to date), p22, and rac have been identified that activate Ca^{2+} channels in plants, functioning, in part, as a mechanism that controls cellular expansion in the root elongation zone by the establishment of high concentrations of Ca^{2+} (25). Moreover, antibodies raised against human p22, p47, and p67 cross-react with appropriately sized polypeptides in plant extracts (22, 70, 81). More recently, NADPH oxidase(s) that generate ROS and act as signaling intermediates have been described in the endothelium and vascular smooth muscle (32, 51, 53, 54, 56, 73). This oxidase(s) appears to play an important role in the pathogenesis of hypertension, atherosclerosis, and myocardial hypertrophy/hyperplasia (33). O_2^- generated by a vascular NADPH oxidase neutralizes endothelial production of vasodilatory nitric oxide (13). Initial work on the molecular composition of the nonphagocytic NADPH oxidase proceeded on the assumption that the enzyme was structurally similar to the neutrophil oxidase. Immunohistochemical studies suggested expression of gp91 in vascular smooth muscle cells (51), but this was not a consistent finding (31, 73). Endothelial cells were reported to express low levels of all components of the phagocyte oxidase, including gp91 (43), and the endothelium of perfused lungs from wild-type but not gp91 null mice produced ROS during ischemia–reperfusion or K^+-induced depolarization (6). However, mesenchymal cells, such as human fibroblasts (41, 52) and glomerular mesangial cells (42), were demonstrated to contain p47, p67, and p22 and detectable levels of low potential cytochrome *b*, but lacked gp91 mRNA. As the substrate recognition site, the FAD-binding site, and the heme-binding site are contained in gp91, it is likely that this subunit contains the catalytic components of the enzyme. Within the past 3 years, six new homologues of gp91 were identified, and a new nomenclature for this protein family, the Nox family, was proposed (48). Unlike gp91, the recently described homologues are not expressed in phagocytes. Their biochemical properties and physiological functions are largely unknown. In brief, Noxl, was cloned from human colon and rat aortic smooth muscle cells (67). It is predicted to encode a ~65-kDa protein that is 58% identical to gp91. Noxl generates O_2^- when expressed in NIH 3T3 cells, produces cell transformation, and induces marked tumorigenicity. Suppression of native Nox1 expression in vascular smooth muscle cells inhibits cell division, supporting a role in cell proliferation. An alternatively spliced shorter form of Noxl serves as a H^+ channel (10). The function of the Nox3 homologue is not known, but may be related to development because it is expressed in fetal tissues (18). The Nox4 homologue is prominently expressed in adult kidney (27, 66). Nox5 is expressed in a variety of fetal tissues, and in adult spleen, testis, lymph nodes, and uterus (11, 18). Its function is also not known. Two additional gp91 homologues, duoxl and duox2, have NH_2-terminal peroxidase domains and are likely involved in thyroid hormone synthesis (20, 24). Thus, a number of NADPH flavoprotein-dependent oxidases have been recently described that are tissue- and cell-compartment specific.

In cultured human AWSMC, we demonstrated clear expression of p22 mRNA that, when sequenced, was identical to the sequence reported for the leukocyte analogue (16). In addition, immunoassays showed conspicuous evidence of p22 protein in human AWSMC. When antisense oligonucleotides for p22 were transfected into AWSMC, growth was significantly impaired. Thus, the p22 NADPH oxidase subunit is expressed in human AWSMC and is critically important for their proliferation. The identity of the critical gp91 homologue is less certain. Transcripts for gp91 were either absent or present in very low amounts. Moreover, genetic deletion of gp91 did not impair the proliferation of murine AWSMC in response to serum stimulation (15). It has been suggested that vascular smooth muscle cells express Nox1 as the partner for p22 in forming the membrane components of cytochrome b558 (67). In AWSMC, we detected no mRNA for Nox1. Recent studies in the laboratory suggest that Nox4 is the predominant homologue in human AWSMC, although additional confirmatory studies are needed. Reports indicate that human aortic smooth

muscle cells have the p47 cytosolic component of the leukocyte oxidase (38, 59). Also, overexpression of a dysfunctional NH_2-terminal fragment of p67 disrupted growth factor-stimulated cyclin D1 promoter activity in bovine AWSMC (57), suggesting that this cytosolic component is present in bovine airway smooth muscle. We detected PCR product for p67 in human AWSMC, but only after 36 cycles of amplification, and were unable to demonstrate any product for p47. Thus, the two cytosolic components of the leukocyte oxidase are either absent or expressed at low levels. Either circumstance might account for the low rate of ROS production in human AWSMC compared with the oxidase of leukocytes (45).

In our studies of human AWSMC, redox regulation of NF-κB appeared to be a major pathway mediating the influence of NADPH oxidase activity on cell proliferation. Inhibition of NADPH oxidase activity with the flavoenzyme inhibitor DPI prevented serum-induced activation of NF-κB, and overexpression of a superrepressor form of the NF-κB inhibitor IκB significantly reduced human airway smooth muscle growth (Fig. 1B). These findings suggest that an NADPH oxidase containing p22 regulates growth factor-responsive human AWSMC proliferation, and that the oxidase signals in part through activation of the prototypical redox-regulated transcription factor NF-κB.

Given the important contribution of the putative NADPH oxidase to regulation of airway smooth muscle proliferation, much additional work is needed to characterize fully the components of this oxidase and understand better its regulation. It is unclear whether the regulatory cytosolic component p47 is actually missing or simply expressed at such low levels as to be undetectable by our PCR methods. It has been postulated that the growth regulatory NADPH oxidase in AWSMC and other mesenchymal tissues is the more primitive form of a signaling enzyme system that has subsequently evolved into a host defense function in leukocytes (8). Consistent with this hypothesis is the demonstration that purified cytochrome b558 alone can be activated by an anionic amphiphile to catalyze O_2^- production at a low steady rate when provided an environment containing oxygen, reduced flavins, and Rac1, and at a much higher rate simply with addition of p67 (45). Thus, not all the specialized leukocyte cytosolic regulatory components are necessary for basic enzymatic oxidase function at the low rate of O_2^- generation observed in human AWSMC.

EXPRESSION OF NADPH OXIDASE IN PULMONARY ARTERY SMOOTH CELLS IS DEPENDENT ON CELLULAR PHENOTYPE

Recently, we have extended these studies to investigate the expression and role of NADPH oxidase in human pulmonary artery smooth muscle cells (PASMC) focusing on the presence and activity of NADPH oxidase as a function of cell passage and phenotype. With extended time in culture and cell passage, PASMC modulated their phenotype from a contractile to a proliferative form. Measurement of ROS generation in PASMC using lucigenin (10 μ*M*) and either NADH or NADPH (100 μ*M*) as donor showed a 10–100-fold higher level of chemiluminescence production/10^6 cells in first passage (primary) compared with subcultured cells. Assessment of the NADPH oxidase components as a function of time in culture showed that p22 was expressed throughout the culture period. Conversely, gp91, as in the case of human AWSMC, showed variability, as it was present in first passage cells of a few, but not most, samples. One possibility is the presence of low numbers of macrophages in some preparations as suggested by Sorescu and colleagues in coronary arteries (66). No expression of gp91 was detected in samples (n = 9) after first passage. Nox4 showed strong expression in primary cultures and reduced, but detectable, levels throughout the eight passages. Expression of mRNA transcripts of the other gp91 homologues was not detected. Expression of p67 and p47 transcripts was either low or absent. A recent study by Banfi and colleagues (12) identified novel p47 and p67 homologues in colon epithelium. Using the published primers and PCR conditions, we did not detect the p47 or p67 homologues in PASMC. Thus, the two key cytosolic components of the phagocyte oxidase are either absent or expressed at low levels. As with AWSMC, either circumstance might account for the low rate of ROS production in PASMC compared with the oxidase of phagocytes. These results emphasize the profound influence of cell passage and culture conditions on expression of NADPH oxidase in nonphagocytic cells. Differences in cell or tissue type, cell passage numbers, or culture conditions may account for the disparate reports in the literature related to characterization of the nonphagocytic NADPH oxidase. The results suggest that in PASMC Nox4 is the predominant gp91 homologue.

A ROLE FOR NADPH OXIDASE IN OXYGEN SENSING

In addition to cellular proliferation, ROS have been proposed to serve a role in O_2 sensing by cells. According to the hypothesis of Acker (4), O_2 sensing involves the generation of ROS in proportion to local tissue P_{O_2} by a membrane-bound NADPH oxidase, thereby altering the intracellular redox balance. Thus, as the intracellular environment becomes reduced, mechanisms intended to alleviate tissue hypoxia are initiated. O_2-sensing mechanisms used by PASMC (77), type I cells of the carotid body (5), erythropoietin-producing cells of the kidney (17) and cells of the neuroepithelial bodies (NEB) (83) [presumed airway chemoreceptors (26)] have been proposed to function in this fashion.

Recent studies utilizing gp91-null mutant mice suggest that the phagocytic-like NADPH oxidase may function as an O_2 sensor in a tissue-dependent fashion, although the results are disparate. O_2 sensing is unaltered in pulmonary arterial tissue (7) and carotid body type I cells (36, 62) from gp91-null mutant mice. In contrast, O_2-sensitive K^+ currents in NEB cells from gp91-null mutant mice are not inhibited by hypoxia or DPI (26). NEB are thought to be particularly relevant to O_2 sensing in the newborn (28), and, consistent with this notion, neonatal gp91-null mutant mice have a lower hypoxic ventilatory response than wild-type mice (44). Thus, the role of a phagocyte-like NADPH oxidase in O_2 sensing by critical peripheral sensors remains unclear. Furthermore, the possible involvement of NADPH oxidase components other than gp91 in O_2 sensing has not been evaluated.

We have conducted experiments to examine further the hypothesis that an NADPH oxidase is the O_2 sensor responsible for carotid body-mediated increases in ventilation and renal erythropoietin gene expression in response to hypoxia (63). Whole body plethysmography was used to study unanesthetized, unrestrained mice. When exposed to an acute hypoxic stimulus, gp91-null mutant and wild-type mice increased their minute ventilation by similar amounts (20%). In contrast, p47-null mutant mice demonstrated a doubling in minute ventilation in response to hypoxia. This significantly exceeded that of their wild-type counterparts (20%). *In vitro* recordings of carotid sinus nerve activity demonstrated that resting (basal) neural activity was marginally elevated in p47-null mutant mice, but markedly increased by hypoxia (Fig. 1C). Supporting data from preliminary experiments indicate that low O_2 elicits enhanced depression of K^+ currents and elevated Ca^{2+} responses in type I cells from p47 gene-deleted animals.

The differing effects of gp91- versus p47-gene deletion on carotid body function suggest that a homologue of the NADPH oxidase of phagocytes participates in chemotransduction. However, the data presently available do not clarify the precise role of the enzyme in the transduction cascade. The original hypothesis of NADPH oxidase involvement in O_2 sensing postulates that hypoxia would decrease ROS production in type I cells (4), but the relationship between pO_2 and ROS levels in type I cells has not been firmly established. In other cells and tissues, hypoxia can increase or decrease ROS production either in mitochondria or via NADPH oxidase (55, 76). In addition, the target of ROS in type I cells is an unknown and critical factor in determining the effect of NADPH oxidase on cell activity. Recent studies have indicated that voltage-sensitive K^+ channels in type I cells are modulated by hypoxia via a mechanism independent of soluble factors such as ROS (60). Thus, ROS do not appear to be necessary for cell activation. On the other hand, if hypoxia enhances NADPH oxidase activity, elevated ROS levels may increase the open probability of K^+ channels, thus facilitating cell repolarization. Such a scheme is consistent with elevated carotid sinus nerve activity in p47 gene-deleted animals. Clarification of these issues must await future measurements of the effect of hypoxia on NADPH oxidase activity, and evaluation of the interaction of ROS with the chemotransduction machinery in type I cells.

In summary, ROS generated from NADPH oxidase(s) in AWSMC and PASMC are important signaling intermediates. Nox4 appears to be the predominant gp91 homologue in these cells. However, expression of NADPH oxidase components is dependent on phenotype, and different homologues may be expressed during different functional states of the cell. NADPH oxidase(s) appear to be important not only for mitogenesis by these cells, but also for O_2 sensing. The regulation of NADPH oxidase(s) in AWSMC and PASMC has important implications for the pathobiochemistry of asthma and pulmonary vascular diseases.

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ABBREVIATIONS

AWSMC, airway smooth muscle cells; DPI, diphenylene iodonium; FAD, flavin adenine nucleotide; H_2O_2, hydrogen peroxide; NEB, neuroepithelial bodies; NF-κB, nuclear factor-κB; O_2^-, superoxide anion; PASMC, pulmonary artery smooth muscle cells; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SOD, superoxide dismutase.

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