ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 10, 2009

DOI: 10.1089/ars.2009.2615

NADPH Oxidases: Molecular Understanding Finally Reaching the Clinical Level?

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Abstract

NADPH oxidases (Nox) have been the subject of very intensive research over the past several years, which has led to in-depth understanding of the function of these enzymes in health and disease. Discovery of novel Nox enzymes and identification of a very wide range of tissue expression has increased our understanding of how NADPH oxidases may regulate so many distinct cellular functions and how the dysfunction of these enzymes may lead to disease. The present Forum issue summarizes the most novel aspects of NADPH oxidase biology, focusing on linking the molecular basis of NADPH oxidase function, compartmentalization, and differential expression patterns to diseases such as those of the pulmonary system, inflammation, central nervous system disorders, endothelial and vascular dysfunction, as well as disorders involving angiogenesis and stem cell and endothelial progenitor cell functions. Establishing these links may be the first step for future therapeutic use of NADPH oxidase inhibitors, which are discussed at length within this Forum issue. *Antioxid. Redox Signal.* 11, 2365–2370.

With many years of research and meticulous and innovative work performed in numerous laboratories around the world, our knowledge of NADPH oxidases (Nox) has vastly expanded. A decade ago, we knew of only one Nox (gp91^{phox}, now known as Nox2), the biochemistry and disease association of which had been well defined (1). From the discovery of the first Nox2 homologue, Nox1, in 1999 (4, 45), the Nox field has exploded. Scientists have now defined a family of Nox proteins (Nox1 through 5 and Duox1 and 2) with specific regulatory molecules, agonist sensitivity, downstream targets, subcellular localizations, tissue distribution, and disease associations (34). At the same time, we are still very limited in the development of new treatments for diverse NADPH oxidase–associated diseases (29).

The current Antioxidants & Redox Signaling Forum is devoted to the most recent developments in NADPH oxidase biology. Although much of this issue is focused on the molecular regulation of NADPH oxidase function and identification of downstream targets, we have not lost sight of the ultimate goal: establishing the link between NADPH oxidases and diseases. Enormous developments in this area have occurred over the past few years. Our understanding of the pathogenic role of NADPH oxidases has moved from solely immune functions and the cardiovascular diseases to other areas including pulmonary disease, central nervous system disorders, and progenitor cell dysfunction. Finally, more and more potential inhibitors are becoming available that can serve as discovery tools as well as future potentially therapeutic strategies (20).

The original NADPH oxidase (Nox2) was discovered as a major innate immune defense mechanism, as it is responsible for bacterial killing and the oxidative burst in phagocytes (1). Nox2 was characterized in great detail as a membrane-associated, multi-subunit enzyme composed of the membrane-bound catalytic subunit Nox2 and its binding partner p22^{phox}, as well as several cytoplasmic, regulatory subunits including p47^{phox}, p67^{phox}, p40^{phox}, and rac proteins (1). Genetic loss-of-function mutations in several of these NADPH oxidase subunits led to the development of chronic granulomatous disease resulting from phagocyte and granulocyte dysfunction (10). The focus on the phagocyte and granulocyte NADPH oxidase lasted for more than three decades.

Interest in NADPH oxidases then moved to the cardiovascular field with two discoveries. First, researchers found that superoxide is a key factor in the development of endothelial dysfunction through the destruction of endothelium-derived relaxing factor, now known to be nitric oxide (17). At first it was thought that this destructive superoxide came from phagocytes, (17) but the second important finding, that NADPH oxidases are functionally important in smooth muscle (15), challenged this idea. It soon became apparent that virtually all vascular cells (endothelium, smooth muscle cells,

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and adventitial cells), are able to produce superoxide by means of NADPH oxidases, which they readily express (14, 36, 41).

These findings caused a new awakening of interest in NADPH oxidases. The discovery of Nox2 homologues (Nox1, Nox3, Nox4, and Nox5, and Duox1 and Duox2), as well as homologues for some regulatory subunits, made it clear that NADPH oxidases are not exclusive in phagocytes and the vasculature but are expressed throughout different tissues (4, 28, 45). They have been shown to be functionally important in lung tissues, heart, the central nervous system, kidneys, gut, and bone cells—virtually everywhere—even in spermatozoa, where they are critical in the beginning of life (2). These homologues are also differentially regulated in various cell types and tissues, which may help to explain the multiplicity of functions that NADPH oxidases play in biology.

NADPH oxidases have been identified as key players in multiple diseases, sometimes with similar pathomechanisms, but at other times with completely distinct, and even opposite, mechanisms. This constantly increasing number of organs and diseases in which NADPH oxidases are involved, as well as the multiplicity of NADPH oxidase homologues, has made it quite difficult to unravel the exact mechanisms of NADPH oxidase participation in the physiology and pathology of various tissues. However, the use of molecular biology tools to ablate specifically individual homologues in individual tissues has brought us closer to such understanding. Indeed, these studies have helped us to understand how the individual NADPH oxidases regulate cell signaling, proliferation, transcription, and endoplasmic reticulum stress responses.

The current Forum issue consists of three original articles and nine reviews by many of the leaders in the NADPH oxidase field. The original articles focus on unraveling new aspects of Nox1 and Nox4 biology. The review articles have been carefully selected to highlight areas of recent novel discoveries and intense research in this field and cover a wide spectrum from the molecular regulation of NADPH oxidase activation, through the regulation of cellular functions by NADPH oxidases, to the importance of these enzymes in selected physiologies and pathologies.

In one of the original manuscripts, Basset *et al*. (5) describe a novel role for Nox1 in regulation of angiotensin II–induced calcium signaling. They show that Nox1-deficient mice have impaired surface expression of angiotensin type 1 receptors as a result of dysregulated caveolin phosphorylation. This observation is potentially clinically relevant, as angiotensin type 1 receptors are important in the development of hypertension and atherosclerosis (23). The other two original articles describe new mechanisms involved in the regulation of Nox4 expression in endothelial cells in response to cyclic strain (13) or serum (38). Because no published data are available concerning potential Nox4 regulatory subunits, it has been proposed that Nox4 regulation occurs mainly at the expression level (42), emphasizing the importance of studies of this type. Moreover, a healthy debate exists about whether Nox4 produces mainly hydrogen peroxide, in contrast to other Nox enzymes that generate superoxide (9, 30, 42). Goettsch *et al.* (13) question this idea, as they do not find increased hydrogen peroxide formation in endothelial cells overexpressing Nox4.

A new function of Nox4 is highlighted in the hybrid review by Santos *et al*. (40), which looks into the importance of NADPH oxidases during the unfolded protein response, at the crossroads of endoplasmic reticulum (ER) stress and mitochondrial dysfunction. The ER has evolved highly specific signaling pathways to ensure that its protein-folding capacity is not overwhelmed (25). These pathways, collectively termed the unfolded protein response (UPR), are required if the cell is to survive the ER stress. UPR is activated on accumulation of unfolded proteins in the ER lumen, thus reducing the amount of new protein in this compartment (25). The UPR is orchestrated by the coordinate transcriptional activation of multiple genes, decreasing translation initiation, and a concomitant shift in the mRNAs that are translated (25). The authors comprehensively summarize the literature regarding the complex redox-related mechanisms of adaptive and proapoptotic signaling, defined as the UPR induced by ER stress. In the context of current knowledge, they highlight the role of the Nox enzymes in the UPR (40). These mechanisms may be critical in the development of numerous diseases such as "conformational diseases" (Alzheimer or Parkinson disease), diabetes, vascular diseases, hyperhomocysteinemia, cancer, and viral diseases, all of which have been shown to be related to alterations of the UPR.

The review articles included in the first part of the Forum discuss the newest findings on the regulation of NADPH oxidases, as well as their downstream targets. Bokoch *et al*. (6) emphasize that despite a long history of research, the mechanisms that regulate this important family of enzymes are only beginning to be understood. They comprehensively review the literature regarding phosphorylation of both core and regulatory subunits through the actions of kinases such as protein kinase C, ERK1/2, p38, MAPK, Pak1, and Akt. The most well-studied phosphorylation-dependent mechanism of regulation is that of Nox2, which so far remains a paradigm for understanding the function and regulation of other NADPH oxidase enzymes. The question is, to what extent does this paradigm remain truly universal for novel homologues? Classically, the major regulation of Nox2 was found to occur through cytoplasmic subunits, in particular $p47^{phox}$ and $p67^{phox}$, which, on phosphorylation by protein kinase C, translocate to the membrane and lead to conformational changes that activate the enzyme (1). Variations on this theme are true for Nox1, which is negatively regulated by protein kinase A–mediated phosphorylation of the $p67^{phox}$ homologue NoxA1. This phosphorylation event creates a binding site for the scaffolding protein 14-3-3ζ, leading to its sequestration in the cytosol. Moreover, evidence is emerging that phosphorylation of Nox proteins themselves may be an important means of modulating enzyme activity. Some evidence for direct phosphorylation of Nox5 is discussed (6).

Our understanding of the potential importance of Nox5 in physiology and pathology, as well as insight into its mechanisms of regulation, has expanded over the past few years, as reviewed in depth by Fulton (12). Many differences exist between Nox5 and other Nox isoforms, including alternative splicing, transcriptional regulation, calcium dependence of the enzyme, and tissue distribution. Nox5 was initially identified in prostate cancer cells, testis, and immature lymphocytes, but recent evidence suggests that Nox5 is expressed and functional in vascular endothelial and smooth muscle cells and may play a role in cardiovascular pathologies. This may be particularly important in relation to its calcium dependence and the clinical use of calcium channel blockers (19). Recent discoveries implicating Nox5 in human clinical

pathologies such as cancer and coronary artery disease also are discussed in Fulton's review.

Recent work has also brought huge advances in our understanding of Duox oxidase biology, as discussed by Fischer (11). Initially described in the thyroid where they participate in thyroid hormone generation (8), Duoxes are also expressed in lung epithelia, where they produce H_2O_2 important for normal lung function, including acid and base secretion. Moreover, Duox-derived H_2O_2 is used by lactoperoxidase to generate bactericidal HOSCN, creating a novel system of host defense analogous to the Nox2 system. This is a critically important observation, because it can explain why patients with cystic fibrosis tend to exhibit chronic airway infection. These patients have a mutation in the CFTR channel that impairs its ability to conduct small anions such as SCN^- or HCO_3^-, thus interfering with the ability of lactoperoxidase to carry out Duox-assisted production of HOSCN and ultimately with bactericidal activity.

The last review focusing primarily on cellular regulatory aspects of NADPH oxidases discusses the downstream targets of NADPH oxidases, with particular emphasis on understanding the consequences of compartmentalization of Nox enzymes in the cell. The subcellular location of these enzymes is critical, as ROS produced by NADPH oxidases most likely act on adjacent targets (22, 46). Nox-derived ROS have been shown to participate in redox-sensitive signal transduction that is specific to particular Nox homologues. Chen *et al*. (7) discuss the mechanisms by which this specificity is conferred, focusing on subcellular localization, the chemical characteristics of the oxidants produced, and their specific reactivity with target proteins. They pay particular attention to protein tyrosine phosphatases, which have recently been shown to be a direct target of Nox4, kinases, small-molecular-weight G proteins, protein disulfide isomerases, and peroxiredoxins. They also discuss the role of Nox-derived ROS in the regulation of protein–protein interactions and the possibility that these molecules may actually participate in regulating the activity of other Nox enzymes.

The second group of review articles in this Forum is focused on the roles of NADPH oxidases in health and disease, which have been of particular interest during recent years as we move toward a translational medicine approach. The potential roles of Nox enzymes in the central nervous system (CNS) (43), pulmonary disease (16), and stem cell biology and angiogenesis (47) are highlighted in this series.

NADPH oxidases identified in CNS cells, such as microglia, astrocytes, and neurons, include Nox1, Nox2, Nox3, and Nox4. ROS produced by these enzymes play numerous functional roles, such as regulation of receptor function (*e.g.*, NMDA) or membrane potentials, and microglia function (43). Whereas Nox1 has been shown to participate in CNS-dependent hypertensive responses or pain sensitivity during inflammation, the discovery of expression of Nox3 in the CNS has drawn considerable attention, as we have limited knowledge regarding this homologue in general. Mutation of Nox3 in mice causes vestibular defects due to altered otoconia in the inner ear (3, 35). Other studies suggest involvement (maybe interaction) of NoxO1 and $p22^{phox}$ with Nox3 in this process (27, 32). Nox enzymes have been implicated in several CNS diseases (in particular, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and neurotoxic responses). For example, β-amyloid causes Nox-2–dependent ROS generation in microglia or astrocytes (31). Interestingly, Nox2 may also be related to cognitive-function regulation, as chronic granulomatous disease (CGD) patients or mouse models of CGD often have cognitive deficits (26, 37).

The involvement of Nox/Duox in pulmonary diseases, ranging from pulmonary hypertension through infectious lung injury–associated diseases, bronchial pathologies such as asthma or COPD, and lung cancer, is discussed next by Griffith *et al*. (16). This review of the clinical aspects of Nox regulation of pulmonary biology builds on the basic aspects of Duox function in the lung covered by Fischer *et al*. (11) and provides a much-needed context for often-conflicting *in vitro* results. The role of NADPH oxidase homologues in the lung has received surprisingly little attention so far, despite the fact that lung is the actual interface with molecular oxygen (33). Duox1, Duox2, Nox1, Nox2, and Nox4 are expressed in the lung, and recently some intriguing data confirm Nox3 expression, with implications for the pathogenesis of emphysema (11) and lung destruction during inflammatory responses mediated by TLR4 (49).

A large amount of new data on the role of stem/progenitor cells in health and disease has been produced in recent years, which has created enormous publicity related to the therapeutic potential of these cells. It is thus important to evaluate critically the role of NADPH oxidases in the regulation of progenitor cell function, especially in the context of angiogenesis and tissue repair.

In the next Forum review, Ushio-Fukai (47) summarizes the known work in this area, indicating that NADPH oxidase components, mainly those associated with Nox2 (expected, because they are closely related to bone marrow–derived cells), are expressed in stem cell/progenitor cells at various stages of development and regulate their proliferation, differentiation, and survival. Recently, human progenitor cells were found to express not only Nox2, but also other homologues, including Nox1, Nox4, and $p22^{phox}$, as well as a full complement of known cytoplasmic subunits and their homologues (39). The role of NADPH oxidases in progenitor cell function is ambiguous. When produced in highly controlled fashion from specific sources in specific cellular compartments, ROS regulate proper endothelial progenitor cell function, but produced in excess, or perhaps outside of normal control mechanisms, they lead to endothelial progenitor cell dysfunction, resulting in impaired mobilization and differentiation. This is particularly evident in models of disease such as diabetes or myocardial infarction. However, the differential roles of Noxes 1 through 5 in endothelial progenitor cell function, their regulation, and their molecular downstream targets remain unknown and warrant further studies.

The Forum concludes with an extremely valuable review of current NADPH oxidase inhibitors, contributed jointly by the research groups of some of the leaders in the Nox field: Drs. Lambeth, Krause, and Clark (24). These authors discuss the strengths and weaknesses of small-molecule Nox inhibitors, from the perspective of proper screening techniques, their usefulness as research tools, and their potential for therapeutic activity. They exclude peptide inhibitors because it is difficult to use peptides therapeutically. Their in-depth review of specific Nox inhibitors includes a consideration of aryliodonium compounds, thiol-modifying compounds, natural compounds from plants, endogenous compounds, synthetic compounds, apocynin, and Nox inhibitors developed by pharmaceutical

companies. The authors conclude that none of the currently available Nox inhibitors is ready for clinical use. This is unfortunate, because Nox inhibitors, by preventing the formation of ROS, have considerable theoretic advantages over antioxidants, which act only to moderate the effects of ROS that have already been produced. As we unravel novel aspects of Nox biology that bring us closer to understanding their specific participation in the pathogenesis of different diseases, development of specific Nox inhibitors that can be used clinically will become increasingly important.

Unanswered Questions

Despite the recent progress in NADPH oxidase research, many questions remain unanswered, and many avenues remain to be explored. Although we have learned much about individual Nox proteins in particular cells, we now must link these to specific cellular functions and pathologies. The jury is still out on which Nox proteins can actually produce (or at least release from their molecule) ROS other than superoxide. Moreover, many cells have been shown to express multiple Nox proteins at the same time. It is important to understand whether and how these interact. Is it possible that they regulate each other's expression and function? Although compartmentalization of Nox enzymes has now been addressed and characterized in some cells and tissues, the consequences of disturbance of this compartmentalization remain unclear, in part because of the need to understand further the downstream targets for each Nox homologue.

Progress in Nox biology has been slowed by a lack of high-quality research tools, including specific antibodies and animal models. Although the Nox2- and $p47^{phox}$-knockout animals have been used extensively, only recently have Nox1-knockout animals and tissue-specific Nox1 overexpressors become available. Nox4-knockout animals have been made by a number of groups, but no studies have yet been published describing their phenotype. A full complement of knockout and transgenic animals for each Nox family member (except Nox5, which is not present in rodents) will greatly enhance the pace of research in this field.

Relatively little is known about human population genetics of NADPH oxidases. Several studies have focused on the $p22^{phox}$ subunit encoding gene CYBA, some finding that specific polymorphisms are functional or related to clinical phenotypes, in particular cardiovascular disease (21, 48), but others finding no such association. However, these studies neither have been conclusive nor have they used modern comprehensive genetic approaches. The genetics of other NADPH oxidase subunits or homologues remains an unopened chapter, which is surprising, considering the explosion of population genetic studies over the past 10 years.

We also need to use more extensively translational approaches to Nox research to determine whether Nox enzymes are realistic therapeutic targets once specific small-molecule inhibitors have been developed. Although some initial efforts have been made in this area in characterizing NADPH oxidases in human vasculature (18, 44), other tissues and organ systems must be evaluated to confirm findings described in animal models and in tissue culture.

Finally, we are now at the point at which research must focus on finding specific inhibitors of NADPH oxidases. Researchers have been using a variety of molecules to inhibit NADPH oxidases, and it is very important to assess critically their value as tools of discovery. Moreover, before we start thinking of introducing them to the clinic as drugs, we must generate more specific inhibitors of individual oxidases. Although the vast spectrum of potential NADPH oxidase inhibitors is discussed in this Forum, we need a detailed characterization of their specificity.

Acknowledgments

Dr. T.J. Guzik is supported by EMBO. Dr. Kathy Griendling is funded by NIH grants HL38206, HL058863, and HL075209.

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Date of first submission to ARS Central, April 1, 2009; date of acceptance, April 9, 2009.

Abbreviations Used

CFTR = cystic fibrosis transmembrane conductance regulator
CNS = central nervous system
CYBA = cytochrome b_{245}, α polypeptide
Duox = dual oxidase
EPC = endothelial progenitor cell
ER = endoplasmic reticulum
MAPK = mitogen activated protein kinase
NADPH = nicotinamide adenine dinucleotide phosphate, reduced form
NMDA = *N*-methyl-D-aspartate
Nox = NADPH oxidase
Phox = phagocytic oxidase
PKC = protein kinase C
TLR = toll-like receptor
UPR = unfolded protein response