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REVIEW

Nox enzymes in immune cells

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Abstract The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of phagocytes is a multi-component electron transferase that uses cytoplasmic NADPH to convert molecular oxygen to superoxide anion, consequently delivering reactive oxygen species to the site of invading microorganisms. Together with soluble factors and other phagocyte-derived agents, the resultant toxic species kill and degrade the ingested microbe. Flavocytochrome b_{558}, a heterodimeric protein composed of gp91^{phox} and p22^{phox}, is the membrane component of the NADPH oxidase and was previously thought to be uniquely expressed in phagocytes. Based on structural homology with gp91^{phox}, recent studies have defined a family of NADPH oxidase proteins (Nox) that is widely distributed throughout the plant and animal kingdoms and in many tissues in multicellular organisms. The goals of this review are to review features of the phagocyte NADPH oxidase that serve as a paradigm for exploiting oxidants for host defense, and to discuss contributions of other Nox proteins to innate immunity.

Keywords Nox · NADPH oxidase · Reactive oxygen species · Flavocytochrome b_{558} · gp91^{phox} · Phagocytes

Introduction

Initiating an issue of *Seminars in Immunopathology* devoted to "Nox Enzymes in immuno-inflammatory pathologies" with an overview of the contributions of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family of proteins to immune cell function and innate immunity seems only just, given that structural homology with gp91^{phox} of phagocytes defines membership in the newly recognized Nox protein family. Furthermore, not only does consideration of Nox proteins in immune cells recapitulate the history of the discovery of the protein family, but it also represents a logical format for the presentation of such a broad topic. NOX2, the 91-kDa membrane glycoprotein that is an essential component of the heterodimeric flavocytochrome b_{558} of the phagocyte NADPH oxidase, is the patriarch of the NOX protein family. It was the object of intense study long before its homologs were discovered or the broad distribution of NADPH oxidases throughout the animal and plant kingdoms was recognized. Nearly five decades of study of the phagocyte NADPH oxidase has yielded considerable insight into its composition, structural organization, regulation, and biochemistry, thereby providing a model for comparing and contrasting features of the other family members. To that end, one goal of this contribution will be to summarize what are currently understood to be key features of the phagocyte oxidase in order to serve as a focal point for comparison with critical elements highlighted in subsequent chapters that discuss specific NOX proteins in other settings.

The second explicit goal of this article is to summarize the contributions of NOX proteins to the immune system that are mediated by cells that are *not* granulocytes, thereby expanding the appreciation of how NADPH oxidases participate in host defense in ways outside the context of phagocyte biology. With the recognition that expression of at least one member of the NOX family has been reported for nearly every tissue examined [1], demonstrating how widely expressed these proteins are in nature, one would anticipate that some would be represented in nonphagocytic

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members of the immune system. Current understanding of the presence and function of NOX proteins within the context of normal immune function in nonphagocytes will be presented, discussing NOX5 and Duox in lymphoid cells and epithelial cells, respectively. Emphasis will be placed on how NOX5 and Duox differ from NOX2 within the framework of human host defense.

Biochemical properties of the phagocyte NADPH oxidase

The appreciation that human polymorphonuclear leukocytes (PMN) utilize reactive oxygen species to kill ingested microorganisms reflects in large part the convergence of two independent scientific pursuits that occurred simultaneously during the middle decades of the last century. One body of work pursued a better understanding of the immunologic defect(s) that cause chronic granulomatous disease (CGD), an inherited disorder typically affecting male children [2]. Although it is likely that some cases of CGD were included in earlier reports of patients with frequent infections, the first explicit definition of the clinical syndrome and its features was in 1959 [3]. The four boys described presented with hypergammaglobulinemia, recurrent pyogenic infections, and granulomatous lesions in skin, lymph nodes, and liver. The patients had normal numbers of circulating granulocytes in peripheral blood and exhibited an appropriate leukocytosis during infection. Although the isolated phagocytes ingested bacteria normally, investigators still suspected that an underlying immunologic defect was responsible for the secondary infectious complications that affected patients experienced.

At nearly the same time that these clinical observations were being made, other investigators recognized that homogenates of resting guinea pig PMN generated H_2O_2 from oxygen in an NADPH-dependent fashion [4]. Moreover, homogenates made from PMN that had ingested particles were more active than those derived from resting PMN, providing the first hint that the burst in respiration was agonist-dependent [5, 6]. As a result of the intense efforts of investigators in several different countries, biochemical features of the phagocyte "respiratory burst" were identified. The enzyme is NADPH-dependent; for the human PMN, the K_m is 33 μM NADPH and 930 μM for NADH [7]. Given that the intracellular concentrations of NADPH and NADH are both ~70 μM, the enzyme uses NADPH under normal physiologic conditions.

The relative greater affinity for NADPH is not species-specific, as it holds for the phagocyte oxidase in guinea pig granulocytes (46 μM vs 580 μM [8]) and in rat alveolar macrophages (5 μM vs 1,000 μM [9]), to provide only two examples. Oxidase activity is resistant to inhibition by azide [10–13] or by cyanide [10, 12, 14–17], a property that early on indicated that the respiratory burst was not due to a sudden increase in mitochondrial activity, as was first suspected.

As an appreciation of the biochemical events that accompanied phagocyte stimulation grew, evidence mounted that CGD neutrophils had profound abnormalities in selected responses [18, 19]. The link between the inherited defect in CGD PMN and the cyanide-resistant, NADPH-dependent, leukocyte oxidase was made [20] and this recognition has provided investigators in the field with a biologically relevant context in which to evaluate hypotheses and a clinically important therapeutic target for biochemical manipulation.

In addition to its dependence on flavin and NADPH and its resistance to mitochondrial inhibitors, the PMN oxidase has other defined biochemical features. Oxygen is the substrate for the phagocyte oxidase, with a K_m for oxygen of ~10 μM, equivalent to ~7 mmHg pO_2 [21]. Thus the oxidase can function at very low oxygen tensions present in tissue, but might be compromised in clinical settings associated with extensive tissue necrosis. Nearly all of the oxygen consumed by PMN during phagocytosis can be recovered as superoxide anion [22] and the enzyme turnover is robust, ~150–160 electrons $heme^{-1}$ s^{-1} [23–27]. When quantitating extracellular superoxide anion as the superoxide dismutase-inhibitable reduction of ferricytochrome *C* [13], human PMN release 7–10 nmol $min^{-1}10^{-6}$ PMN and 2–4 nmol $min^{-1}10^{-6}$ PMN after stimulation with 100 nmol of phorbol myristate acetate and opsonized zymosan (20:1 particle to PMN), respectively.

However, the rate, amount, and duration of superoxide generation vary as a function of the state of the PMN (primed vs resting, in suspension or adherent) as well as the agonist (reviewed in [28]). NADPH availability in the cytosol limits oxidase activity under normal conditions [29]. As one might anticipate, the electron transferase activity of the activated oxidase necessitates charge compensation across the plasma or phagosomal membrane [30, 31]. Most evidence supports a proton channel as the dominant mediator of charge compensation during oxidase activity [32–34], although the electrophysiology is not completely elucidated. Drs. Rada and Ligeti review the obligate coupling oxidase activity with other electophysiological events elsewhere in this issue (Rada and Ligeti).

Flavocytochrome b_{558}: the membrane component of the phagocyte NADPH oxidase

Flavocytochrome b_{558} is a heterodimeric integral membrane protein, comprised of a 91-kDa glycoprotein ($gp91^{phox}$) and a 22-kDa protein ($p22^{phox}$), that resides in the plasma membrane and membranes of secretory vesicles and of

specific granules of PMN and in the plasma membrane of other granulocytes (review [2]). Within the context of a discussion of the Nox protein family, the extent and duration of the controversy that surrounded the identification of flavocytochrome b_{558} as a component of the phagocyte oxidase seem both remarkable and ironic. First noted by several investigators in Japan [35–37], flavocytochrome b_{558} was rediscovered by the Segal lab [38] and noted to be absent from patients with the X-linked form of CGD [39, 40]. Eventually, the heavy subunit of flavocytochrome b_{558}, gp91^{phox}, was identified as the molecular defect in X-linked CGD (*CYBB*) [41, 42], whereas the gene encoding p22^{phox} (*CYBA*) was subsequently localized to chromosome 16 [43]. With the birth of the Nox protein family [44], gp91^{phox} was subsequently relegated to the designation Nox2, although both gp91^{phox} and Nox2 are used nearly interchangeably.

As Nox2 has not been crystallized, the model for its disposition in the membrane has been derived from integrating data from many different analytical approaches (see discussion in [45]). A model proposed by Jesaitis integrates much of the available data from diverse sources and incorporates the structural features identified most consistently [46]. In this model, there are six transmembrane helices, two heme groups, and an extended cytosolic domain containing both NADPH and FAD binding sites. The heme groups are coordinated with histidine residues in parallel helices that are perpendicular to the plane of the membrane and are stacked. Since they have different mid-point potentials, −225 and −265 mV [47], this arrangement provides the means for shuttling electrons across the membrane.

The amount of flavocytochrome b_{558} in membranes or detergent extractions of membranes can be quantitated using reduced-minus-oxidized difference spectroscopy, measuring from the peak at 558 nm to the trough at 540 nm and using $\Delta\varepsilon_{558-540}$ of 21.6 mM^{-1} cm^{-1} [48]. Taken together, evidence indicates that Nox2 is the catalytic core of the phagocyte oxidase, functioning as the electron transferase to reduce molecular oxygen, although the precise mechanism by which electrons are transported is incompletely understood. Multiple steps are involved in the two electron transfer from NADPH to FAD, to the inner heme, to the outer heme, and finally to oxygen, as recently summarized in detail [49]. The immediate product of the NADPH oxidase is superoxide anion, although it rapidly undergoes spontaneous dismutation (pH optimum of 4.8) to yield H_2O_2, which in turns supports the generation of an array of reactive species (*vide infra*).

The unheralded essential partner: p22^{phox}

In contrast to the electron transferase activity of Nox2, p22^{phox}, the partner of Nox2 in flavocytochrome b_{558}, has no demonstrated enzymatic activity. However, the stability of both Nox2 and p22^{phox} requires the formation of heterodimers, as the absence of either protein in myeloid cells results in the absence of both. Circulating PMN from patients with inherited defects or deficiency of Nox2 or p22^{phox} lack *both* proteins [2, 50, 51], thus demonstrating their interdependence and the importance of heterodimer formation. Studies of flavocytochrome b_{558} in human myeloid precursors demonstrate that solitary precursors of Nox2 or p22^{phox} in the endoplasmic reticulum (ER) undergo proteolytic degradation, mediated in part by the proteasome [52]. In Epstein Barr virus-transformed B lymphocytes from patients with X-linked CGD, p22^{phox} can be rescued from ER-associated degradation by transfection of normal Nox2 [53–55], and the reverse is also true, with normal p22^{phox} able to rescue Nox2 precursors [56].

The requirement of heterodimer formation for stability of Nox2 and p22^{phox} was exploited experimentally to identify the specific histidine residues that coordinate with the hemes in gp91^{phox} [57]. Furthermore, as heterodimer formation is also prerequisite to exit the ER efficiently [52, 58], p22^{phox} contributes to the subcellular localization of Nox2. It is important to recognize that ER quality control in myeloid cells may differ from that in other cell lines, as free gp91^{phox} and p22^{phox} each is more stable in cell lines such as COS7, epithelial cells, murine 3T3 fibroblasts, and CHO cells [58–60]. As demonstrated in Fig. 1, isolated membranes from CHO cells transfected with gp91^{phox} express predominantly the 65-kDa precursor of gp91^{phox} that resides in the ER [52, 58]. However, coexpression of *both* gp91^{phox} and p22^{phox} results in heterodimer formation and promotes maturation of the ER precursor into mature gp91^{phox}. Surface staining of transfectants with 7D5, a murine monoclonal antibody that recognizes an extracellular epitope on flavocytochrome b_{558} [61–63] demonstrates the augmented surface expression of flavocytochrome b_{558} when p22^{phox} is coexpressed with gp91^{phox} (Fig. 1)

p22^{phox} associates as well with Nox1, Nox3, and Nox4 [64–69]. In its association with Nox3, *p22^{phox}* apparently serves precisely the same role as it does for Nox2, extending the half-life of Nox3 and allowing export from the ER to the proper membrane destination. The critical importance of normal p22^{phox} for *both* Nox2 and Nox3 is demonstrated by the consequences of an inherited missense mutation in p22^{phox}, in which the histidine at codon 121 is replaced with tyrosine (Y121H). Mice with Y121H mutation have clinical disorders involving *both* their phagocytes and their vestibular system [219]. PMN from Y121H mice have the functional phenotype of CGD, with an absence of NADPH oxidase activity and increased susceptibility to infection. They also have a "head tilt" phenotype caused by the absence of otoconia in their inner ear, a manifestation of Nox3 dysfunction [70].

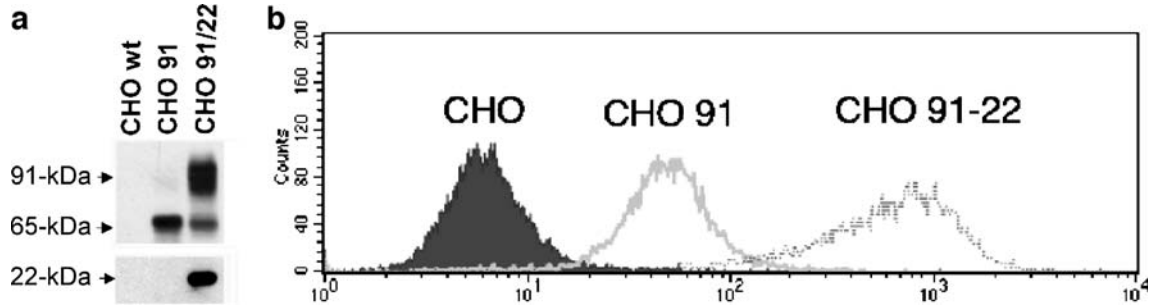

Fig. 1 Augmented surface expression of gp91^{phox} by coexpression of p22^{phox} in CHO cells. **A** Light membranes from wild type CHO cells (WT) and CHO cells stably transfected with gp91^{phox} (CHO 91) or with gp91^{phox} and p22^{phox} (CHO 91–21) were separated by SDS-PAGE and immunoblotted, using a murine monoclonal antibody directed against gp91^{phox} (54.1) and a commercial rabbit polyclonal antibody directed against p22^{phox}. In the transfectants expressing only gp91phox (CHO 91), the predominant species is the 65-kDa ER precursor, with very little mature gp91^{phox}. However, coexpression of p22^{phox} (CHO 91–22) significantly increases the maturation of the ER precursor and expression of gp91^{phox}. **B** The same cells as shown in panel A were analyzed by FACS for staining with 7D5, a murine monoclonal antibody directed against an extracellular epitope in flavocytochrome b_{558}. Although there is detectable surface expression of gp91^{phox} in CHO 91 cells, coexpression of p22^{phox} increases surface expression more than ten-fold. Both panels are previously published [56] and shown with the publisher's permission

It is likely that humans with inherited deficiency of p22^{phox} likewise have vestibular disease, but that visual compensation renders the symptoms subclinical. However, given this new discovery, it is important now to directly assess the contribution of p22^{phox} to normal Nox3 function in humans, using sensitive and specific assays of inner ear function in patients not previously exposed to vestibulotoxic drugs such as aminoglycosides.

Despite these insights, the mechanism underlying the contribution of p22^{phox} to Nox structure and function is unknown. The determinants of heterodimer formation are incompletely defined but under study [56].

Dependence of Nox2- *p22^{phox}* on cytosolic cofactors

Clinicians long recognized that only ~1/2 the patients with CGD displayed an X-linked pattern of inheritance, thereby hinting that the phagocyte NADPH oxidase was a multicomponent system. Thus, the molecular identification of flavocytochrome b_{558} as the membrane component of the oxidase and genetic localization of Nox2 to the X chromosome solved only part of the phagocyte oxidase puzzle. The development of the broken cell superoxide system demonstrated that one or more protein(s) in the cytosol of resting phagocytes was required to support Nox2-dependent superoxide production [71]. In the broken cell system, the light membranes and organelle-free cytosol from unstimulated phagocytes are combined in the presence of anionic amphiphiles and NADPH to generate superoxide. Subsequent studies from several laboratories identified three cytosolic proteins that are absolutely essential for Nox2 activity in human PMN: p47^{phox} [72, 73], p67^{phox} [72, 73], and the small molecular weight G protein Rac2 [74]. The inherited absence of Nox2, p22^{phox}, p47^{phox}, p67^{phox}, or Rac2 provides the molecular basis for all cases of CGD [75].

The significance of a 47-kDa phosphoprotein in stimulated PMN was first suggested by Segal, who noted that PMA stimulates the phosphorylation of several cytosolic proteins in human PMN, including one of ~44-kDa [76]. Phosphorylation of the 44-kDa protein was temporally linked to oxidase activation and these findings were confirmed by other investigators [77–81]. An activation-induced phosphoprotein of the same size was identified in the cytosol of PMN from other species [82–84], and it was suspected that the 44-kDa phosphoprotein might serve as the proximal electron-transferring molecule in the oxidase or some other component whose phosphorylation is a prerequisite for oxidase activity. The link between the 44-kDa phosphoprotein with a functional oxidase was extended by clinical data, as patients with autosomal CGD lacked it, whereas those with X-linked CGD had normal phosphorylation of the 44-kDa protein [76, 85–87].

Many of these predictions proved true, as the subsequent identification and molecular characterization of p47^{phox} [72, 73] revealed several functionally important structural features, including two src-homology (SH3) motifs, a proline-rich region (PRR), and a region with clustered arginine and serine residues, likely targets for serine phosphorylation. Of note, an N-terminal region of p47^{phox} proved to contain a novel phosphoinositide-binding motif [88] that was later found in a wide variety of proteins and subsequently designated as PX domain, to acknowledge its initial description in *phox* proteins p47^{phox} and later p40^{phox} [89–93]. In general, the PX, SH3, and PRR motifs mediate intermolecular associations, including protein–protein and protein-phospholipid interactions, and thus are essential

structural features in the multicomponent NADPH oxidase complex. Residing in the cytoplasm of resting PMN in a complex with $p67^{phox}$ and $p40^{phox}$, $p47^{phox}$ adopts a conformation that renders several of its interactive domains cryptic and unavailable to support interactions with their partners at the membrane.

For example, the PX domain of $p47^{phox}$ is destined to associate with the SH3 domain [94] in flavocytochrome b_{558} but is masked by intramolecular interactions when in the cytoplasm of resting PMN [92]. However, phosphorylation of the serine residues in the arginine-rich autoinhibitory region (AIR) relaxes the conformational constraints on $p47^{phox}$, exposing the PX and SH3 (Fig. 2). As a consequence, PMN stimulation and concomitant phosphorylation of $p47^{phox}$ trigger its translocation from cytoplasm to membrane [95–97], where it docks with flavocytochrome b_{558} [98]. This agonist-dependent translocation of $p47^{phox}$ as a prerequisite for oxidase assembly [99] and provides the phagocyte with a novel mechanism for regulating oxidant generation, based on the spatial segregation of critical components when the cell is in the resting state. In this way, $p47^{phox}$ serves to organize the assembly of the Nox2 oxidase, which serves as a general feature for the Nox protein family (*vide infra*).

In contrast to $p47^{phox}$, which lacks intrinsic enzymatic activity, $p67^{phox}$ regulates NADPH reduction of FAD and thus serves as an essential activating cofactor [100]. When PMN are stimulated, $p67^{phox}$ translocates to the membrane in association with $p47^{phox}$ and $p40^{phox}$. Whereas $p47^{phox}$ can translocate in the absence of $p67^{phox}$, $p67^{phox}$ fails to become membrane-associated in PMN that lack $p47^{phox}$ [99].

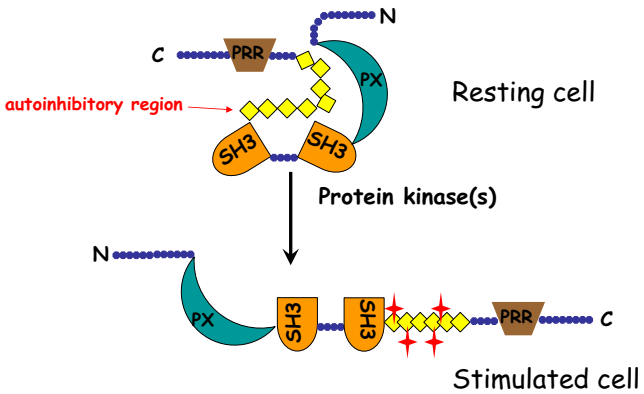

Fig. 2 Conformational changes in $p47^{phox}$ during neutrophil stimulation. As discussed in the text, $p47^{phox}$ possesses structural motifs that mediate intermolecular interactions, including a PX domain, two SH3 regions, and a PRR. In addition, there is an arginine- and serine-rich region known as the autoinhibitory region (AIR). However, intramolecular interactions in $p47^{phox}$ result in its adopting a conformation in the cytoplasm of resting neutrophils that render the potential intermolecular domains cryptic. Agonist-dependent phosphorylation of the AIR, indicated by red stars, alters the conformation of $p47^{phox}$, exposing interactive sites that can mediate association of $p47^{phox}$ with the membrane and with flavocytochrome b_{558} and thereby support assembly of a functioning oxidase

Rac2, the third essential cytosolic component of the human PMN NADPH oxidase, exists in the cytoplasm of resting cells in the GDP state bound to RhoGDI. PMN stimulation triggers phosphorylation of RhoGDI with subsequent dissociation from Rac-GTP [101] and translocation of Rac-GTP to the membrane independent of the $p47^{phox}$–$p67^{phox}$–$p40^{phox}$ complex [102]. At the membrane, Rac2 contributes to oxidase activity, interacting with $p67^{phox}$, Nox2, or both [103, 104]. Thus, agonist-dependent, post translational modifications mediate the translocation of both cytosolic complexes and thereby regulate oxidase activity by controlling oxidase assembly.

Whereas spatial segregation dominates regulation of Nox activity in PMN, there is evidence for limited transcriptional control, predominantly in monocytes or macrophages [105–107]. Regular prophylactic administration of interferon γ is considered the standard of care for patients with CGD because of its dramatic beneficial impact on the occurrence and severity of infections [108], although the underlying mechanisms are unknown. In the few patients with CGD due to splicing defects in the gene encoding $gp91^{phox}$, interferon γ treatment partially restores normal transcription of $gp91^{phox}$ in myeloid precursors [109, 110]. However, there is no detectable transcription of $gp91^{phox}$ in mature PMN and the findings on patients with defects in splicing *CYBB* do not apply in general to the positive clinical impact of γ interferon in the infectious complications of CGD. In contrast to the arrangement of Nox2 regulation in PMN, it is very likely that transcriptional control of oxidase activity will figure prominently in the context of the other Nox protein family members.

Immune consequences of activation of the Nox2 oxidase

Phagocyte oxidase activation accompanies phagocytosis and contributes to the complex biochemical environment in the phagosome that is inhospitable to ingested microbes. These oxygen-dependent events are only part of the general response of phagocytes, which collectively comprise the many events that culminate in killing and degrading the ingested potential pathogen [111]. As mentioned above, the proximal product of the Nox2 oxidase is superoxide anion that quickly dismutates to generate H_2O_2. Although H_2O_2 alone exerts modest antimicrobial action, it contributes to innate host defense by serving as an essential cofactor in the MPO– H_2O_2–chloride system in the normal PMN phagosome [112, 113]. In the presence of H_2O_2, MPO forms compound I and catalyzes the two electron oxidation of Cl^- to Cl^+ and the production of HOCl.

The HOCl produced can chlorinate a wide range of biological substrates [114–117], including both susceptible targets in microbes [118–121] that compromise the viability of microbes, as well as host proteins [122], some of which provide extended antimicrobial activity. For example, chloramines can be produced by the action of MPO-derived HOCl on amines [123–125], some of which decompose to produce bioreactive aldehydes [115, 126–128].

Within the context of a consideration of Nox2 and host defense, it is important to recognize that superoxide anion produced by the NADPH oxidase also has effects that are indirect yet integral for effective antimicrobial action. Evidence suggests that superoxide interacts directly with MPO [129, 130] and thereby extends the functional life of the HOCl-generating system with the phagosome. In addition, Nox2 has been implicated in the process of antigen presentation by dendritic cells [131], thereby expanding the role of Nox2 activity to proper engagement and functioning of adaptive immunity, a link that may likewise include B cells and normal humoral immunity [132]. A recent study of *Aspergillus* infection in $p47^{phox}$-deficient mice indicated that the absence of superoxide-dependent tryptophan catabolism results in immunologic dysregulation and a hyperinflammatory state [133]. The excessive IL-17 production from an expanded subpopulation of γδ-T cells in the infected $p47^{phox}$ knockout mice links Nox2-dependent superoxide production to a generalized immune response to fungi, and perhaps other microbes, that extends well beyond the phagocyte or its phagosome.

Nox2 vs other family members: compare and contrast

Several excellent, comprehensive reviews of the Nox family have been recently published [1, 134–136], providing the curious reader opportunities to examine in detail how closely the phagocyte oxidase paradigm summarized above predicts the attributes of the newly defined Nox2 homologs. However, to provide a context for appreciating the contributions to follow, several features merit highlighting.

As noted before, Nox1, Nox2, Nox3, and Nox4 pair with $p22^{phox}$ to form heterodimers in membranes. The other Nox protein family members, namely Nox5, Duox1, and Duox2, are not associated with $p22^{phox}$. Many non phagocytic cells with Nox family members depend as well on cytosolic components that are necessary for optimal activity. These structural homologs of $p47^{phox}$ and $p67^{phox}$ serve as *o*rganizing and *a*ctivating elements and are designated as NoxO1 and NoxA1, respectively, to highlight their function within the Nox complex. To date, only Nox1 and Nox3 employ NoxO1 and NoxA1 as cofactors, although human Nox3 functions independently of NoxA1, whereas murine Nox3 requires both cofactors for optimal activity. Although the basis for the species specificity for Nox3 dependence on particular cofactors is not known, the additional 21 amino acid insert in the C-terminal region of human NoxO1 may contribute.

The absence of the AIR from NoxO1 is one structural difference between $p47^{phox}$ and the NoxO1 homolog that has known functional consequences. As discussed earlier (Fig. 2), domains in $p47^{phox}$ that are destined for intramolecular associations in the assembled oxidase are cryptic in cytosolic $p47^{phox}$ in unstimulated cells. Agonist-dependent phosphorylation of several serines in AIR results in conformational changes that relieve constraints on interactive domains and allow assembly at the membrane. The absence of AIR from NoxO1 renders its interactive protein motifs always accessible and supports constitutive association of NoxO1 with the available docking sites at the membrane. In some situations, the active Nox complex may assemble not at the cell surface but on membrane-bound intracellular organelles, such as endosomes, and thus provide a source of oxidants that may support redox-dependent signal transduction [137].

The contribution of Rac to the organization and activity of the nonphagocyte Nox proteins also differs among the protein family members. Whereas Rac1 supports Nox1 [67, 138–142], there is evidence supporting a role for Rac in the activity of endogenous Nox 4 [143, 144], but not when expressed in transfected cells [69]. Both Nox5 and the Duoxes are independent of Rac1 [145, 146] and there are conflicting data with reference to Nox3, with evidence supporting [67] and not supporting [65, 138] a contribution of Rac1 to Nox3 activity. Analyses of the evolution of the Nox protein family strongly suggest that the EF hand-containing family members Nox5 and Duox are most ancient [147], with Nox3 most recent to have evolved, thus indicating that the earliest Nox proteins were independent of cytosolic factors entirely.

Nox 5 in host defense

As noted, Nox5 and Duox represent ancient members of the Nox protein family [147]. Nox5 functions independently of protein factors and requires only Ca^{+2} to trigger its NADPH-dependent activity [145]. Ca^{+2}-dependent activation reflects in part binding of calmodulin to Nox5, thereby inducing a conformational change and increased sensitivity to calcium [148]. Protein kinase C activation can stimulate Nox5 as well [149], although the kinetics of activation differ from those seen when a Ca^{+2} ionophore is used as an agonist [150]. Stimulation of Nox5-expressing COS7 cells with phorbol myristate acetate promotes H_2O_2 generation at

a relatively slow rate, in comparison to the response provoked by ionomycin. Nox5 activation under these conditions requires phosphorylation of threonine 494 and serine 498, residues in the C-terminal region of Nox5 between the putative NADPH and FAD binding sites [149, 150].

Although the specific kinase mediating phosphorylation in the COS7 system is not known, the PKC-β inhibitor LY379196 blocks both phosphorylation and activity of Nox5. As a consequence of its phosphorylation, Nox5 can be activated at lower concentrations of Ca^{+2}. Thus, Nox5 phosphorylation provides one mechanism to modulate sensitivity to physiologic agonists that regulate intracellular Ca^{+2}concentration.

Nox5 is highly expressed in testis, spleen, and lymph nodes [151], with lower levels of expression noted in endothelium, smooth muscle, pancreas, placenta, ovary, uterus, stomach, the HaCaT human keratinocyte cell line [152], and several fetal tissues [153, 154]. Expression in the B-cell and T cell-rich regions of spleen and lymph node seemed to foretell a role for Nox5 in some aspect of immune function, but Nox5 is absent from circulating lymphocytes [151]. In contrast to its absence from normal mature lymphocytes in peripheral blood, malignant B cells in hairy cell leukemia express Nox5 in plasma membrane and exhibit Ca^{+2}-dependent H_2O_2 production [155]. The mechanisms underlying functional expression of Nox5 in hairy cell leukemia B cells are not known. The capacity to express Nox5 is not shared by other B-cell malignancies, including chronic lymphocytic leukemia, marginal zone leukemia, and mantle cell leukemia, and is not induced by integrin-dependent adhesion to vitronectin or fibronectin.

Perhaps Nox5 in splenic lymphocytes contributes to normal differentiation and proliferation of *developing* lymphocytes, functions limited in normal circulating lymphocytes and potentially deranged in selected B cell malignancies such as hairy cell leukemia. Nox5-dependent H_2O_2 production regulates growth and apoptosis in the cultured malignant prostatic cell line DU 145 [156], thus supporting a potential role for Nox5 in events relevant to cell proliferation. However, at this time, no data link Nox5 function with host defense or immunological events.

Duox in mucosal immunity

Although non phagocyte Nox proteins have been implicated in many biological functions across a broad array of tissues and organ systems [1, 49, 136, 157–159], the immune system has been relatively underrepresented to date [157, 160, 161]. The localization of Nox1 to the gastrointestinal tract [44, 139, 162–164] promised to link local oxidant production in the gut with host defense, but a convincing demonstration for a role for Nox1 local host defense has not emerged. However, extensive evidence supports an important role for Duox and its homologs as ancient participants in innate immunity.

Before discussing the details of Duox contribution to host defense, two generalizations should be made regarding principles that apply to the system. First, H_2O_2 and derived reactive oxygen species (ROS) figure prominently not only in Nox2-dependent phagocyte function but also in host defense systems in a wide variety of multicellular organisms. In the plant kingdom, oxidants generated by respiratory burst oxidase homologs (Rboh) mediate a wide-variety of biological processes, including pathogen recognition, direct antimicrobial activity, growth and development, and wound healing [165–168]. Oxidants can act as virulence factors as well, as illustrated by their role in fungal pathogenicity of rice blast disease [169]. Invertebrates likewise rely on H_2O_2 and H_2O_2-dependent systems to defend themselves against infection [170–172].

Reactive oxygen species generation is linked to innate immunity in insects, as exemplified by the reduced survival of the mosquito *Anopheles gambiae* to bacterial challenge when fed antioxidants [173]. Second, ROS mediate their functional effects by creating post-translational changes in specific biological targets. Often the chemistry utilizes ROS as a substrate for generating the effective mediator, rather than serving directly to modify the target. The oxygen- and MPO-dependent antimicrobial system of human PMN serves as the paradigm, with MPO as the peroxidase, Nox2 as the H_2O_2-generator, and Cl^- or Br^- as the halogen to produce HOCl or HOBr, respectively [174]. Application of this model extends beyond immune cells, and the functional consequences of the structural changes produced by this system reflect both the chemical nature and the cell type or tissue specifically targeted (Table 1). For example, thyroid hormone biosynthesis by thyrocytes depends on H_2O_2 from Duox2 and thyroperoxidase (TPO) to iodinate thyroglobulin [175–178].

Defects or deficiencies in either Duox2 or TPO can cause congenital hypothyroidism [179–182]. Using similar

Table 1 Many of the Nox proteins modify target proteins in a peroxidase (PRX)-dependent fashion

Event	PRX	H_2O_2 source
PMN antimicrobial activity	MPO	Nox2
Eosinophil antimicrobial activity	EPO	Nox2
Thyroid hormone synthesis	TPO	Duox2
Sea urchin	OVO	Udx1
Airway mucosal defense	LPO	Duox1

Although the fundamental biochemistry is nearly identical with the different Nox proteins, the functional consequences are very diverse and reflect the specific biological context of the target

chemistry, ovoperoxidase catalyzes H_2O_2-dependent diotyrosine generation and cross-linking of the fertilization envelope of sea urchin eggs [183, 184], using H_2O_2 generated by the Duox homolog Udx1 [185]. Duox-derived H_2O_2 supports tyrosine cross-linking in the cuticle of *Caenorhabditis elegans* as well [186]. These functionally important structural variants on the phagocyte oxidase paradigm are likely the first of many tissue-specific adaptations of members of the Nox protein family.

Like Nox5, Duox 1 and 2 (referred below collectively as Duox) possess EF-hands, two compared to the four in Nox5, are regulated by calcium, and are independent of $p22^{phox}$, Rac, or cytosolic activating or organizing proteins [134, 187–189]. In contrast to Nox5, Duox has been implicated in serving an important function in innate immunity at mucosal surfaces, most notably in the gastrointestinal and pulmonary tracts. Duox 2 is distributed along the length of the gastrointestinal tract [190], from oral salivary glands to the rectum [188], with sites of increased expression in the apical membranes of enterocytes and in the brush border of cecum and sigmoid colon [190]. The antibacterial activity of saliva had long been known [191] and early studies identified lactoperoxidase (LPO) as the responsible peroxidase [192], although the H_2O_2 source was unknown. The LPO-dependent system (reviewed in [192]) includes H_2O_2, LPO, and the pseudohalide thiocyanate (SCN^-), which is widely distributed in mammalian tissues and body fluids. In adult humans, serum concentrations of SCN− are in the micromolar range (0.034–0.05 mM) with concurrent salivary concentrations much higher (0.8–1.5 mM [192]). Even before the source of H_2O_2 was identified, the biological properties of this system were characterized in detail [125, 192].

With the identification of Duox as the H_2O_2 source, the organization of the salivary gland antimicrobial system could be defined [188]. Although there was ample evidence that LPO-Duox H_2O_2–SCN system operates in vitro and generates antimicrobial activity that might contribute to mucosal innate immunity, the clearest link to the in vivo relevance for the Duox-based host defense in the gastrointestinal tract comes from studies in Drosophila. Selectively silencing expression of drosophila Duox (dDuox) in gut epithelial cells increases the mortality of adult flies ingesting food contaminated with Gram positive or Gram negative bacteria [193].

As in the gut, all elements of the LPO–H_2O_2–SCN antimicrobial system have been detected in airway epithelium [157, 187–189, 194–200], where it contributes to local host defense. LPO is largely secreted by bronchial submucosal glands in the airways of humans [201] and other animals [202, 203], and, under experimental conditions that exclude contamination by saliva, is present at 3–12 μg/ml [201]. Just as thiocyanate can be detected in saliva, lower airway secretions obtained from intubated patients contain amounts of SCN (*e.g.* ~0.5 mM [201]) sufficient to support the antimicrobial action of the LPO–H_2O_2–SCN system [195, 204–206]. Expressed in the apical membranes of airway epithelial cells, Duox2 provides the H_2O_2 to complete the system and there generates agent(s) with bacteriostatic and bactericidal activities [125, 207, 208]. It merits noting that the reactive species produced by the LPO–H_2O_2–SCN system that mediates antimicrobial activity is not precisely known, as the major product(s) have been difficult to identify. Depending on the analytical technique used, candidates include HOSCN [209, 210], CN^- [210], NCS-O-SCN^- [209], and $^-$OSC N [211]. Precise identification of the reactive product(s) that mediate cytotoxicity will facilitate unraveling the mechanisms underlying antimicrobial action of the LPO–H_2O_2–SCN system.

Regardless of the identity of the active species, the system spares airway epithelial cells while generating sufficiently reactive products to kill bacteria [199]. This relative sparing of mammalian cells during antimicrobial action contrasts with the collateral tissue toxicity often seen with HOCl or HOBr generated during inflammation [212–214]. Thus, the LPO–H_2O_2–SCN system is ideally suited for targeted host defense at mucosal surfaces. One can speculate as well that the low level of antimicrobial activity in this system serves the host well in rapidly clearing small inocula of airborne organisms that might gain access to the lower airway during respiration. If the number or virulence of aerosolized organisms exceeds the capacity of the LPO–H_2O_2–SCN system, other local factors, including soluble agents and alveolar macrophages, and recruited PMN are enlisted, resulting in a bona fide inflammatory response.

However, most low-level microbial challenges would be handled by the mucosal surface system below the threshold of airway inflammation. Ongoing studies have linked the LPO–H_2O_2 (Duox)–SCN antimicrobial system to the local host defense dysfunction seen in patients with cystic fibrosis [199]. Evidence linking thiocyanate transport to a functioning CFTR [197] provides a potential molecular mechanism underlying the depressed antimicrobial activity of epithelial airway fluid from cystic fibrosis patients.

In addition to supporting the generation of bioreactive agents that directly damage or kill microbes, Duox participates in innate host defense in the airway in other ways. Duox expression has been linked to regulation of mucous production in the airway. Production of mucins MUC1 and MUC5AC by stimulated airway epithelium involves expression of Duox1 [215–217], demonstrating a second contribution of Duox to maintaining the integrity of the airway and thereby contributing to host defense. Data also implicate Duox1 in augmenting endotoxin-elicited IL-8 production by airway epithelial cells [218], suggesting

that in situations where Duox-mediated direct antimicrobial action may be insufficient, the system can relay signal to recruit additional host defense participants into the airway.

Summary

The multicomponent NADPH-dependent oxidase system, once thought to be uniquely expressed in phagocytes and dedicated to support innate host defense, represents instead a specialized application of ROS biochemistry. The recognition of the Nox protein family, based on structural homology with gp91^{phox}, has extended the appreciation of the widespread role of ROS and their products in biology. This overview of the contributions of Nox proteins to immunity includes examples of how ROS *directly* mediate antimicrobial activity as well as evidence that ROS support signaling events that modulate the quantity and quality of the host response. The discovery that the phagocyte oxidase is but one member of a larger, ancient protein family provides new and exciting challenges, which promise to expand our understanding of many facets of plant and animal biology.

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