



The phagocyte NOX2 NADPH oxidase in microbial killing and cell signaling

William M Nauseef

The phagocyte NADPH oxidase possesses a transmembrane electron transferase comprised of gp91phox (aka NOX2) and p22phox and two multicomponent cytosolic complexes, which in stimulated phagocytes translocate to assemble a functional enzyme complex at plasma or phagosomal membranes. The NOX2-centered NADPH oxidase shuttles electrons from cytoplasmic NADPH to molecular oxygen in phagosomes or the extracellular space to produce oxidants that support optimal antimicrobial activity by phagocytes. Additionally, NOX2-generated oxidants have been implicated in both autocrine and paracrine signaling in a variety of biological contexts. However, when interpreting experimental results, investigators must recognize the complexity inherent in the biochemistry of oxidant-mediated attack of microbial targets and the technical limitations of the probes currently used to detect intracellular oxidants.

Address

Inflammation Program, Department of Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, 501 EMRB, 431 Newton Road, Iowa City, IA 52242-1101, United States

Corresponding author: Nauseef, William M (william-nauseef@uiowa.edu)

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Introduction

For decades, the NADPH oxidase was considered an oxidant-generating system uniquely associated with phagocytes and dedicated exclusively to microbicidal action. However, the discovery of homologues of gp91phox in non-phagocytic cells [1,2] catalyzed an appreciation that nearly all cells in the plant and animal kingdoms possess at least one NADPH oxidase [3]. The family of NADPH oxidase (NOX) proteins includes NOX1, NOX2 (aka gp91phox), NOX3, NOX4, NOX5, DUOX1, and DUOX2. Although some proteins, such as NOX2 and DUOX, contribute to host defense against infection, most serve critical functions related to signaling in many different contexts, ranging from biosynthesis of otoconia and

normal vestibular function in the inner ear [4] to physiologic functions in the cardiovascular system [5]. In contrast to activities of the non-phagocyte NOX proteins, NOX2 in the phagocyte NADPH oxidase constitutes a high turnover enzyme complex that in human neutrophils generates ~ 10 nmol superoxide anion/min/ 10^6 cells, most of which targets ingested microbes. However, H₂O₂ from the NADPH oxidase acts in both an autocrine and paracrine manner to promote signaling, directly or indirectly, in a wide variety of biological settings [5–7]

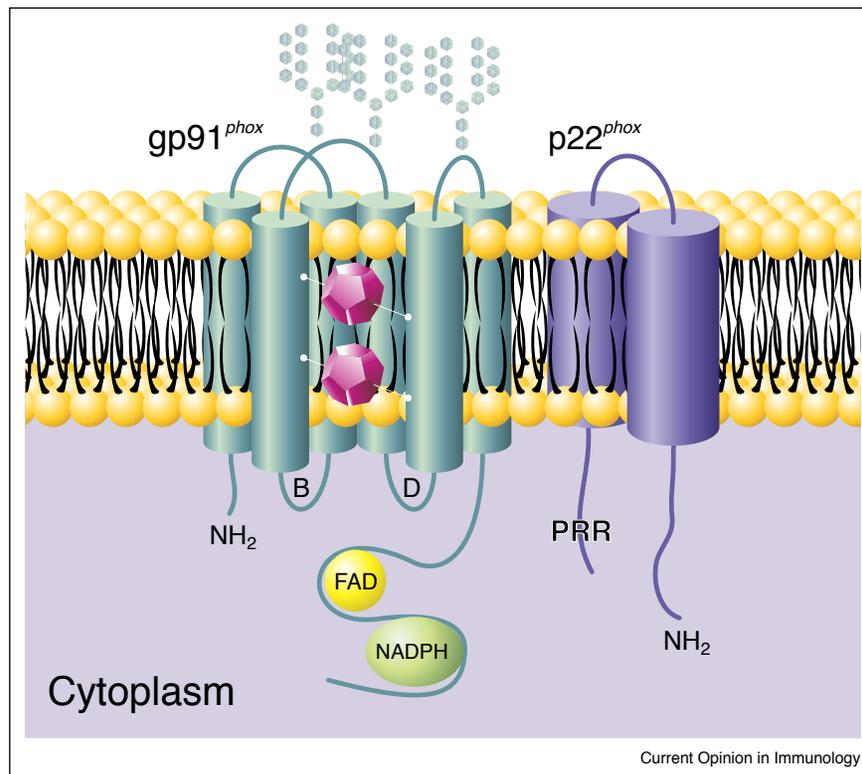
The phagocyte NADPH oxidase is a multicomponent enzyme complex

Unassembled and inactive in resting phagocytes, the NADPH oxidase complex includes at least five components distributed in membranes and in cytoplasm (reviewed in Ref. [8]). Flavocytochrome b₅₅₈, the heterodimeric integral membrane protein composed of gp91phox and p22phox, serves as the primary electron transferase and exists in the plasma membrane and membranes of secretory vesicles and secondary granules (Figure 1). Two protein complexes in the cytoplasm of resting phagocytes are essential for optimal oxidase activity. A ternary complex of p47phox, p67phox, and p40phox exists in a 1:1:1 stoichiometry, stabilized by multiple intramolecular and intermolecular interactions between complementary binding domains within individual components (reviewed in Ref. [8]). Although strong biochemical data generated over several decades suggested the presence of a multicomponent complex that translocates *en masse* to target membranes [9–12], Ziegler *et al.* have recently integrated analytical data from several sources with crystal structures of the isolated subunits and functional domains to construct a 3D model of this complex [13*].

Additional proteins copurify with the NADPH oxidase components under a variety of experimental conditions (reviewed in Ref. [8]), although the functional importance of many is not yet established. Zhou *et al.* recently reported that protein arginine deaminase (PAD) 4 associates with p47phox and p67phox [14*], adding to the complex and incompletely understood relationship between the NADPH oxidase and neutrophil extracellular trap (NET) formation. Oxidant-dependent changes in the cytoskeletal network reflect finely tuned coordination of glutathionylation and deglutathionylation of actin and tubulin and likely figure in NET formation [15*].

When phagocytes are activated during phagocytosis or exposure to soluble agonists, phosphorylation of p47phox

Figure 1



Flavocytochrome b_{558} .

Flavocytochrome b_{558} , composed of $gp91^{phox}$ (aka NOX2) and $p22^{phox}$, acts as the redox center of the phagocyte NADPH oxidase. The current model for human $gp91^{phox}$ includes six transmembrane helices and both amino (NH₂) and carboxy termini on the cytoplasmic face of the membrane. Residing between two intramembrane helices, two inequivalent heme groups (purple polygons) transport electrons across the membrane from cytosolic NADPH. There are three N-linked carbohydrate chains extracellularly and two cytoplasmic loops (B and D). The carboxy terminal region includes both FAD and NADPH binding sites. The current model for human $p22^{phox}$ has both amino and carboxy termini on the cytoplasmic side of the membrane and lacks glycosylation. The carboxy region of $p22^{phox}$ contains a proline-rich region (PRR) that associates with $p47^{phox}$ during oxidase assembly. Figure from Ref. [8] and used with permission.

on specific serine residues prompts conformational changes that expose otherwise cryptic domains in $p47^{phox}$ and $p40^{phox}$ that bind to specific phosphoinositides in the plasma and phagosomal membranes, respectively, associate with flavocytochrome b_{558} , and drive superoxide anion generation (Figure 2). Concomitantly, a second complex, composed of Rac2 in neutrophils (Rac1 in macrophages) and the GDP dissociation inhibitor for Rho, RhoGDI, independently translocates to membranes to promote oxidase activation and activity.

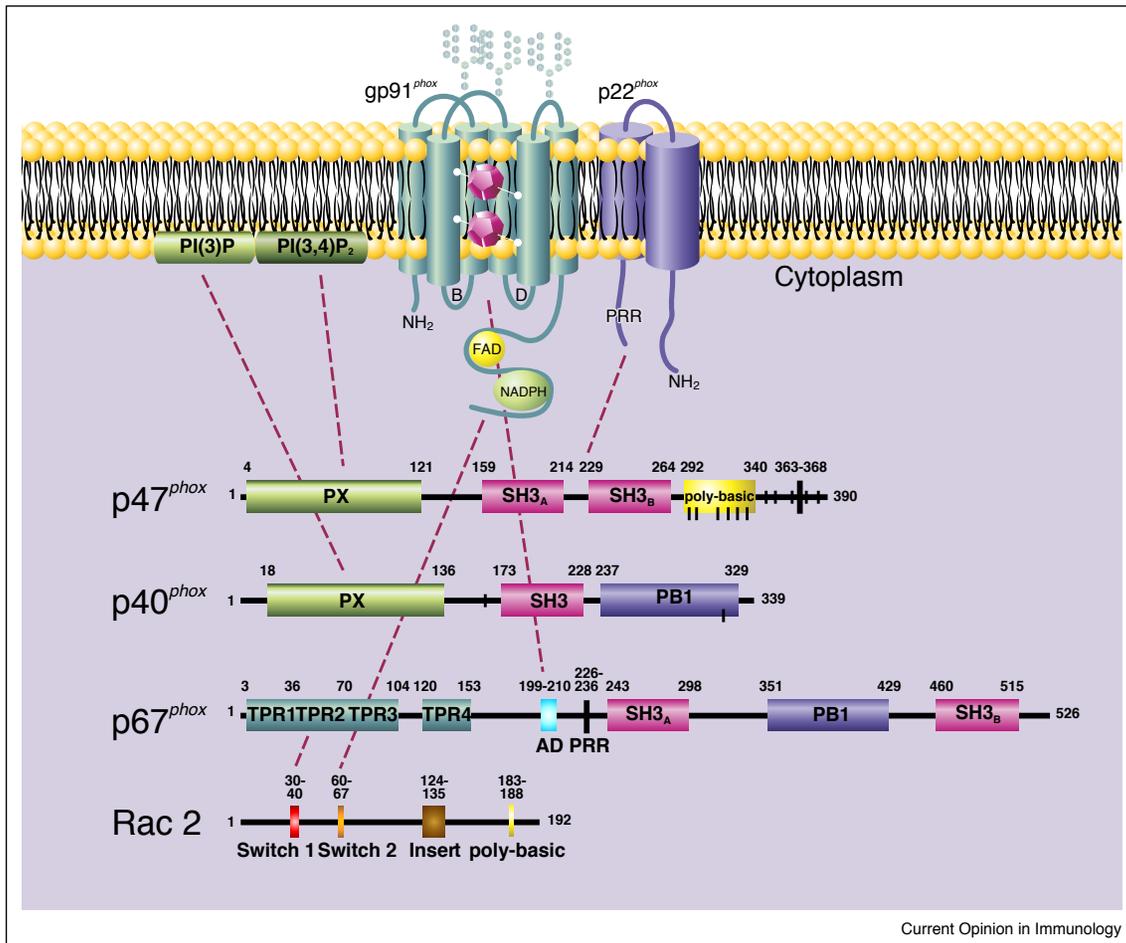
Activity of the phagocyte NADPH oxidase is not binary, that is, either off or on. Immunological modifiers, such as TNF α or GM-CSF, or microbial components, such as lipopolysaccharides, can enhance responsiveness of the phagocyte NADPH oxidase, a process known as priming. Intricate mechanisms underlying priming of the oxidase include phosphorylation of serine 345 of $p47^{phox}$ and participation of the proline isomerase Pin1 (reviewed in Ref. [16]). A reversible process, priming provides a

mechanism to modulate NADPH oxidase responsiveness to meet the demands during or after an inflammatory response.

Lessons in NADPH oxidase biology from chronic granulomatous disease

Explication of the molecular basis of chronic granulomatous disease (CGD), the clinical syndrome of enhanced susceptibility to specific microbial infections [17] and exuberant sterile granulomatous inflammation has informed much of our current understanding of the composition and activity of the phagocyte NADPH oxidase [18,19]. Mutations in the genes encoding any one of the five known components of the NADPH oxidase complex results in CGD, although phenotypes vary somewhat as a function of residual oxidase activity [20] and the specific oxidase component affected. Mutations in $p40^{phox}$ compromise oxidase assembly only on phagosomal membranes; oxidase translocation to plasma membrane is normal [21]. The similar but different

Figure 2



Oxidase assembly.

Multiple intermolecular interactions among the individual components and between the PX domains of p47^{phox} and p40^{phox} with PI(3,4)P₂ in plasma membrane and PI(3)P in phagosomal membranes, respectively, mediate assembly of the NADPH oxidase in stimulated neutrophils. Shown are some of the interactions, including those between proline rich regions (PRR) and src homology domains (SH3), activation domain of p67^{phox} (AD), and tetratricopeptide repeats (TPR) on p67^{phox} with their binding partners (reviewed in detail in Ref. [8]) that mediate assembly. For ease of illustration, interactions of the cytoplasmic complexes present in resting neutrophils are omitted. Figure from Ref. [8] and used with permission.

phosphoinositide recognition and binding by the PX domains of p47^{phox} and p40^{phox} serve to discriminate between plasma and phagosomal membranes, respectively [22,23]. Furthermore, the clinical phenotype of CGD due to p40^{phox} deficiency or dysfunction differs significantly from that of classic CGD, as recently demonstrated in a report of 24 individuals from 12 families [24^{**}]. In contrast to patients with other genotypes of CGD, p40^{phox}-deficient individuals do not experience invasive infections but rather suffer from exaggerated inflammation, such as lupus-like skin lesions and inflammatory bowel disease-like colitis, and superficial infections [24^{**}].

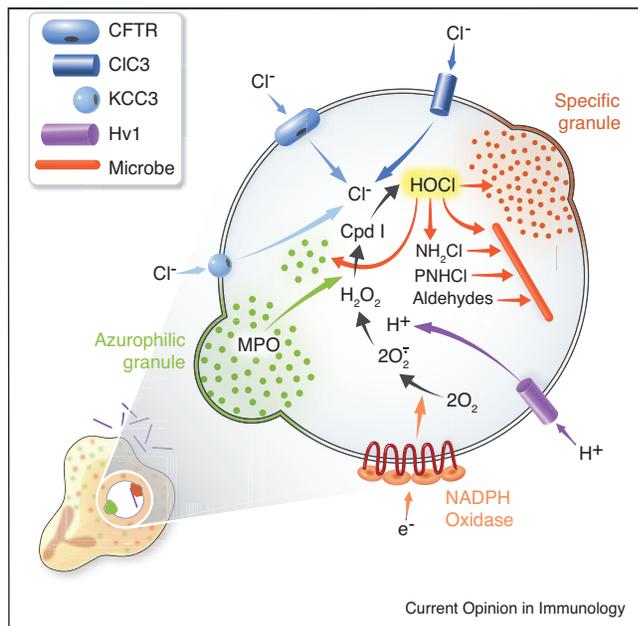
In addition to defective NADPH oxidase components resulting in CGD, inherited abnormalities in a

transmembrane protein essential for flavocytochrome b₅₅₈ expression [25^{**}] have been implicated as a cause of CGD. Two independent reports describe patients with the CGD phenotype and normal NADPH components but with loss of function mutations in *CYBC1* [26^{**},27^{**}], the gene encoding Eros (essential for reactive oxidant species) [25^{**}]. The contrasting clinical phenotypes of p40^{phox} deficiency with that of other genotypes of CGD and the discovery of a *CYBC1* mutation as a cause of CGD demonstrate that much about the composition and regulation of the phagocyte NADPH oxidase remains to be elucidated.

Dynamic events within neutrophil phagosomes

Once assembled and activated on phagosomal membranes, the NADPH oxidase mediates rapid transfer of electrons

Figure 3



MPO-dependent events in human neutrophil phagosomes. During phagocytosis, human neutrophils assemble and activate the NADPH oxidase and recruit granules to fuse with nascent phagosomes. Electrons transferred into the phagosome by the NADPH oxidase reduce molecular oxygen to superoxide anion ($O_2^{\bullet -}$) and H_2O_2 . The voltage-gated proton channel Hv1 delivers protons into the phagosome to compensate the charge generated by electron transfer from cytoplasm and thus sustain oxidase activity. The cystic fibrosis transmembrane conductance regulator (CFTR), with some contribution from chloride channel 3 (CIC3) and the potassium-chloride cotransporter (KCC3), provides chloride (Cl^-) from the neutrophil cytoplasm. MPO, delivered by fusion of azurophilic granules, and H_2O_2 , generated by the oxidase, react to yield Compound I (Cpd I) that in turn catalyzes the oxidation of Cl^- to produce hypochlorous acid (HOCl) or bleach. HOCl reacts with targets both from host and microbe to generate a variety of reactive products, including monochloramines (NH_2Cl) and protein chloramines (PNHCl), which can then decompose to form aldehydes. Collectively, the granule proteins, acting in both their native and oxidant-modified forms, along with HOCl and its derivatives act synergistically to attack microbial targets. Figure from Ref. [40] and used with permission.

from cytoplasmic NADPH into the lumen of phagosomes (Figure 3). Redistribution of electrons creates a charge inequity across the phagosomal membrane and drives membrane depolarization unless corrected. The failure to compensate charge created by electron transfer would terminate NADPH oxidase activity within ~ 100 ms (reviewed in Ref. [28]). A longstanding controversy as to the direct role of the NADPH oxidase in charge compensation resolved upon discovery of the presence of the voltage-gated proton channel Hv1 in phagocytes [29]. Activated in parallel with the NADPH oxidase, each molecule of Hv1 compensates the electrons transferred by 100 molecules of the NADPH oxidase, thereby efficiently correcting charge inequity. Murine neutrophils that lack Hv1 activate NADPH oxidase activity normally

but fail to sustain superoxide generation because of the inability to compensate charge and offset membrane depolarization [29]. Unrelated to properties of the oxidase but germane to neutrophil adhesion and chemotaxis, Hv1-dependent charge compensation support the calcium influx required for calcium-dependent cell responses [30].

The immediate product of the NADPH oxidase, superoxide anion ($O_2^{\bullet -}$), has a pK_a of 4.8 in equilibrium with its protonated form, the hydroperoxyl radical ($OH_2^{\bullet -}$) [31]. Although it may directly contribute to antimicrobial action (reviewed in Ref. [32]), superoxide anion figures predominantly serves as a precursor to H_2O_2 , either by spontaneous dismutation (i.e. $O_2^{\bullet -} + OH_2^{\bullet -} \rightarrow H_2O_2 + O_2$) or by an enzyme-catalyzed reaction. Dismutation occurs most readily near pH 4.8, when concentrations of superoxide and hydroperoxyl radical are the same. Given that the pH of neutrophil phagosomes is neutral or higher [33,34], equilibrium favors the hydroperoxyl radical and little spontaneous dismutation occurs.

Although the superoxide dismutase (SOD) endogenously expressed in neutrophil cytoplasm does not access the phagosomal lumen, recent evidence that extracellular SOD internalized from plasma resides in secretory vesicles [35] provides a potential mechanism for catalyzed generation of H_2O_2 if secretory vesicles fuse with nascent phagosomes during phagocytosis. However, the ferric form of myeloperoxidase, aka Compound III, rapidly reacts with $O_2^{\bullet -}$ to generate $H_2O_2 + O_2$ [36,37] and may be the predominant mechanism for H_2O_2 production from the phagocyte NADPH oxidase, although many variables influence the overall biochemistry (reviewed in Ref. [32]).

Oxidants in antimicrobial activity within phagosomes

The infectious morbidity and mortality in patients with CGD demonstrate that oxidants from the NADPH oxidase support optimal antimicrobial activity against many, but not all, ingested microorganisms [17]. For example, both normal and CGD neutrophils kill *Escherichia coli* equally well, but CGD neutrophils have nearly no microbicidal activity against *Staphylococcus aureus*.

Given the small volume of phagosomes, high levels of oxidants and granule proteins can be achieved, some, such as myeloperoxidase, reaching millimolar concentration [32,38]. Consequently, most phagocyte-mediated killing occurs in phagosomes and relatively little in the extracellular space. The overriding theme for intraphagosomal antimicrobial activity is synergy: synergy between oxidants and granule proteins as well as among individual granule proteins [38,39]. The collaborative interactions among phagosome contents culminate in a potent system effective against the varied microbial

threats a host may encounter in a lifetime. However, microbes do not passively experience hostile attack in phagosomes, and several organisms apply specific tactics to evade, endure, or neutralize agents of host defense and thus succeed as pathogens. Elsewhere in this issue, Criss *et al.* describe in detail some of these remarkable microbial adaptations to the harsh conditions within phagosomes.

Neutrophils and macrophages differ with respect to both NADPH oxidase activity (neutrophils \gg macrophages), phagosomal pH (neutral in neutrophils, acidic in macrophages) and granule constituents, which together influence the overall capacity for and nature of intraphagosomal antimicrobial activity. For example, whereas most of the H_2O_2 generated in neutrophil phagosomes will be consumed by the azurophilic granule protein myeloperoxidase (MPO) to generate HOCl [40] (see below), the absence of MPO from macrophages will leave their phagosomes without HOCl but with more H_2O_2 . Furthermore, macrophages in some settings supplement intraphagosomal NOX2-dependent H_2O_2 with oxidants derived from mitochondria. Ingestion of *S. aureus* elicits oxidant stress in macrophages and generation of mitochondria-derived vesicles that deliver H_2O_2 to phagosomes and thereby augment H_2O_2 -dependent action [41**]. In light of the many substantive differences in antimicrobial agents in neutrophils and macrophages and for ease of presentation, the discussion that follows uses human neutrophils to illustrate the principles at play in phagosomes.

In the presence of H_2O_2 , generated *de novo* by the NADPH oxidase, MPO catalyzes the two electron oxidation of chloride to produce HOCl, which is far more potent than is H_2O_2 both as an oxidant and as an antimicrobial agent [42]. Furthermore, HOCl reacts with both host and microbial proteins in phagosomes to yield chloramines and aldehydes whose reactivity extends the spectrum and duration of antimicrobial action (Figure 3). HOCl can inactivate susceptible granule proteins, as occurs with elastase, and thereby eliminate their contribution to antimicrobial milieu. The number and complexity of interactions that can occur among both host and microbial targets are difficult to replicate with individual components in reductionist experimental systems *in vitro*. Consequently, identification of a precise and single mechanism for killing ingested organisms presents a daunting challenge to those who study host defense.

Both host-based and microbe-based variables compound the challenge to our understanding of phagocyte antimicrobial action. Within a given neutrophil, phagosomes are remarkably heterogeneous with respect to HOCl production [43**]. This heterogeneity likely reflects variability both in the extent of granule fusion with and oxidase assembly on any given phagosome. Equally variable are

the microbial substrates present in a particular phagosome. The biochemical composition and structural organization of microbes differ; for example contrast the cell wall of *S. aureus* with the endotoxin on the surface of *E. coli* or the waxy mycolic acids on *Mycobacterium tuberculosis*. Many of these features vary with the growth phase of the organism, and some bacteria actively secrete biomolecules under stress conditions, thereby providing competing substrates for interaction with host defense molecules. In light of all the variables of host and microbial origin that influence events in phagosomes, the articulation of a single mechanism by which all ingested microbes are killed becomes a formidable challenge. A detailed description of events under limited and explicitly defined experimental conditions may be the most that can be expected.

The challenges facing the phagocyte NADPH oxidase driving signal transduction

Oxidants participate in a broad spectrum of biological phenomena other than those directed to killing microbes, ranging from mediating tissue damage in a variety of clinical settings to regulating complex physiologic responses [44–46]. However, the attribution of oxidants as the causative agents of observed cellular events frequently rests on weak data or unlikely chemistry [47]. Concerned about the lack of rigor in studies of redox events in experimental systems, experts in oxidant biology have provided investigators with guidelines to optimize evidence before assigning a given oxidant as cause of a particular biological phenomenon [47,48]. These guidelines apply as well to the study of NOX2-dependent oxidants in leukocyte signaling. It is especially important to know the chemistry and to recognize the limitations of detection systems being used to assess oxidant production in experimental systems. Redox-responsive fluorescent probes such as dichlorofluorescein (DCFH) are widely employed to assess intracellular oxidant generation but are problematic as detectors of H_2O_2 [49*]. H_2O_2 does not react directly with DCFH but requires a peroxidase or transition metal as a catalyst [50]. In addition, cytoplasmic proteins can directly oxidize DCFH independent of reactive oxygen species and thereby foster incorrect interpretations of results. For example, DCFH fluorescence in dying neuronal cells was interpreted as evidence that reactive oxygen species prompts apoptosis [51] until subsequent work demonstrated that mitochondrial cytochrome C released during apoptosis directly oxidizes DCFH [52]. Detection of DCFH fluorescence in cells reflects changes in the intracellular redox state but should not be attributed to the generation of specific oxidants. In addition to technical issues, certain conceptual hurdles to linking NOX2-generated oxidants directly to intracellular signaling need to be recognized.

Situated in lipid-enriched domains in the plasma membrane, the NOX2-centered NADPH oxidase operates as a

nodal point for intercellular and intracellular signaling [53], but several hurdles need to be surmounted to effect results. First, oxidants need to reach the relevant targets. Superoxide anion is short-lived and rapidly converted to H_2O_2 , which has been established as a signaling molecule in a wide variety of biological systems [44,54]. The topology of the NADPH oxidase in phagocytes results in generation of superoxide anion and its downstream products either in the extracellular space (across the plasma membrane) or in the phagosomal lumen (across the phagosomal membrane) (Figure 1). Uncharged and unreactive at neutral pH, NOX2-generated H_2O_2 must traverse the plasma or phagosomal membrane to access cytoplasmic targets in order to drive intracellular signaling cascades. Facilitated transport of H_2O_2 occurs through aquaporins (AQPs), integral membrane proteins that bidirectionally transport H_2O and H_2O_2 across membranes [44,55]. Isoforms of AQPs are expressed in plasma membranes and membranes of intracellular organelles in cells throughout the plant and animal kingdoms and support paracrine as well as autocrine H_2O_2 -dependent signaling. Varied distribution of particular isotopes in specific cellular locations allows for fine tuning of H_2O_2 flux and thereby modulation of oxidant-dependent cellular responses [56].

The second major challenge for H_2O_2 is to react with intracellular targets that initiate signaling cascades. A potent oxidant, H_2O_2 reacts directly with thiols but only slowly with cysteine residues in many proteins because of its high activation energy [54,57]. In contrast, proteins that support the catabolism of H_2O_2 , most notably catalases, peroxiredoxins (Prxs), and glutathione peroxidases (Gpxs), readily react with H_2O_2 and thereby protect critical and vulnerable intracellular proteins from detrimental oxidation. For example, the rate constant of the reaction of H_2O_2 with Prx 2 ($10^8 M^{-1} s^{-1}$) is more than 10^6 -fold greater than that with the signaling cascade protein tyrosine phosphatase 1B ($20 M^{-1} s^{-1}$), a difference that underscores the challenge for signaling molecules to react with H_2O_2 entering the cytoplasm [44]. Compounding the disadvantage of relatively low reactivity with H_2O_2 , redox-sensitive signaling molecules exist in much lower abundance in cytoplasm than do other potential targets. In fact, excluding organelle-compartmentalized catalase and heme-peroxidases, reduced Gpxs and Prxs predominate as cytosolic substrates for H_2O_2 [44].

Taken together, the greater abundance and reactivity of Prxs favor their reaction with and consumption of nearly all the H_2O_2 that enters cytoplasm [58*]. Two mechanisms, not mutually exclusive, have been proposed to explain how H_2O_2 overcomes its competitive disadvantages compared with Prxs and succeeds in supporting the oxidation of target proteins in signaling cascades [59]. Agonist-triggered phosphorylation can inactivate Prx and thereby allow for accumulation of H_2O_2 locally in sufficient concentrations to oxidize target cysteines in

signaling molecules. In this way, localized H_2O_2 supports signaling without promoting more widespread and deleterious oxidation. Such may be the case for B cell receptor (BCR)-dependent and T cell receptor (TCR)-dependent stimulation, whereby membrane-associated Prx1 is transiently phosphorylated and inactivated, thereby allowing local accumulation of H_2O_2 intracellularly [60]. Alternatively, oxidation of target cysteines can be indirect, mediated by oxidized Prx, which relays the oxidizing reactivity of H_2O_2 to downstream targets. In this scheme, Prx acts as both sensor of H_2O_2 and transducer of oxidation, thereby providing the cell with an efficient mechanism for transmitting signals with spatiotemporal precision.

NOX2-generated H_2O_2 in signaling

Just as the early studies of CGD proved seminal in the discovery of the NADPH oxidase in normal neutrophils [18], the hyperinflammation characteristic of this disease has revealed previously unrecognized links between the phagocyte oxidase and noninfectious inflammatory diseases. Studies of murine models of arthritis and autoimmune diseases have implicated the lack of a functional NADPH oxidase in the etiology of the increased inflammatory phenotypes associated with inflammatory arthritis, systemic lupus erythematosus (SLE), atopic dermatitis, and inflammatory bowel disease (reviewed in Refs. [7,61*]). The increased inflammation seen in the setting of NOX2 deficiency reflects both augmented proinflammatory activity and also dysregulation of responses that normally terminate inflammation. NADPH oxidase activity influences cell death pathways in neutrophils [62], and efferocytosis, the process by which macrophages engulf apoptotic and spent cells in inflammatory sites, is impaired in the absence of the phagocyte NADPH oxidase. Murine peritoneal exudate macrophages that lack *gp91phox* fail to remove apoptotic cells [63**].

There are intriguing differences in the phenotypes observed in murine models and in humans with CGD. Polymorphisms in *NCF1* (gene encoding p47*phox*) are associated with autoimmune chronic inflammatory diseases in rats and mice. In contrast, the association of *NCF1* polymorphisms with a predisposition to inflammatory human diseases has been difficult to make [64], and data from genome-wide association studies link mutations in *NCF2* (gene encoding p67*phox*) and *NCF4* (gene encoding p40*phox*) with autoimmune diseases such as SLE [65] and Crohns disease [66]. Furthermore, the phenotypes in murine models reflect in large part the genetic context in which mutations in NADPH oxidase are expressed. For example, p67*phox*-deficient NZM2328 mice develop accelerated SLE kidney disease, whereas *NCF2* null mice in a B6 background exhibit the expected CGD phenotype but no increased susceptibility to SLE disease [67]. Thus, excessive oxidants promote autoimmune events in hosts genetically predisposed to such diseases and not in all subjects.

Whereas most patients with CGD do not have autoimmune diseases, female carriers of X-linked CGD experience more discoid lupus erythematosus, granulomatous colitis, SLE, and immune-mediated thyroid disease than does the control population [68]. The latter data represent another puzzling observation: the presence of *some* cells without normal NADPH oxidase activity predisposes affected humans to autoimmune disorders but the proclivity of exaggerated inflammation is independent of the percentage of impaired cells [68]. Reconstitution of NOX2 activity in macrophages and dendritic cells rescues *Ncf1*-deficient mice from the exuberant tissue inflammation elicited by intradermal injection of β 1,3-glucan, a sterile agonist of microbial origin [69]. Although the responsible mechanisms are not understood, such observations underscore the idea that the phagocyte NADPH oxidase links innate and adaptive arms of the immune system [70] but in ways that are incompletely understood.

Complementing observations derived from animal models of NADPH oxidase-deficiency, *in vitro* studies likewise support the notion that NOX2-generated oxidants contribute in some way to cell signaling, with both neutrophils and macrophages as the likely sources of H₂O₂. NOX2 activation by human neutrophils augments NOX2 activity in a positive feedback loop, whereby H₂O₂ drives uptake of extracellular calcium and subsequent activation of c-Abl tyrosine kinase and protein kinase C δ [71]. Neutrophil NOX2 activity also acts in a paracrine fashion, contributing to myelopoiesis by progenitors and tissue repair in murine bone marrow after ischemia [72]. Maintenance of the integrity of immune receptor regulation depends on the presence of a functional NADPH oxidase. Although the underlying mechanisms have not been elucidated, CGD neutrophils display dysregulation of several receptors important for optimal innate immunity, including TLR5, TLR9, CD11b, CD18, CD35, and CXCR1 [73].

NOX2 activity and redox signaling play a central role in macrophage programing and contributes to changes in macrophage phenotype over the course of an inflammatory response [46]. Murine resident peritoneal macrophages normally express AQP3 in their plasma membrane. AQP3-deficient mice exhibit decreased survival in an experimental peritonitis model, with isolated macrophages defective in phagocytosis and other critical cell functions [74], presumably reflecting defective H₂O₂ influx during activation in the absence of AQP3 [74]. NOX2-generated H₂O₂ transported via AQP8 supports tyrosine phosphorylation triggered by BCR for optimal activation and differentiation of murine B cells [75]. H₂O₂ from dendritic cells or macrophages, operating as antigen-presenting cells, may oxidize susceptible cysteine residues near the TCR and thereby alter signaling [76], although the specific targets of oxidation have not been identified. Human B cells lacking either *gp91phox* or

p40phox exhibit defective MHC II- presentation of exogenous antigens [77], whereas TLR7 and TLR9 in *p40phox*-deficient or *p47phox*-deficient human B cells are upregulated and support augmented cytokine secretion and p38 MAPK activation when stimulated [78]. In the absence of *p47phox*, mice experience greater alveolar inflammation and damage after intratracheal instillation of lipopolysaccharide (LPS), secondary to increased LPS-induced NF- κ B activity in the absence of NADPH oxidase activity [79].

The presence of NOX2 influences the activity of the NLRP3 inflammasome, but the nature of that influence depends on the agonist, cell, and species studied. Murine macrophages lacking *gp91phox* respond to *M. tuberculosis* with exaggerated caspase-1 activity and IL-1 β secretion [80]. A study of human neutrophils (albeit not populations of ultrapure neutrophils) demonstrated that the NADPH oxidase is not required for caspase-1 activity but is necessary for optimal secretion of mature IL-1 β in response to LPS priming and stimulation with ATP [81]. However, uric acid treatment of CGD monocytes increases both caspase-1 activity and IL-1 β secretion compared to responses of normal monocytes stimulated in parallel [82]. How these apparently disparate findings fit into a coherent model of the role of NOX2 in cell signaling remains to be determined [83].

Conclusions and perspectives

From this brief overview of contributions of the phagocyte NADPH oxidase to two physiologic functions of phagocytes, namely supporting antimicrobial action against ingested microbes and initiating signaling cascades, several ‘take home’ points emerge. In both activities, the outcomes are clear: a significant fraction of ingested microbes are killed and in certain circumstances cellular responses to agonists occur in a NOX2-dependent fashion. However, there are impressive gaps in our current knowledge that leave the underlying mechanisms responsible for antimicrobial action and for cell signaling incompletely elucidated.

In the case of microbial killing within phagocytes, the complex biochemistry, reactive interactions among individual components, both host and microbial, and dynamic responses to the assault by ingested organisms render reductionist approaches to the study of neutrophil-mediated antimicrobial mechanisms in phagosomes as crude approximations of reality. The inherent complexity of events within phagosomes exhibits features of an emergent system, whereby the overall effect of the complete system *differs qualitatively from* the sum of activities of its individual components. Depending on the specific contributions from both host and microbe and the synergies ongoing among components that may exist in a given phagosome, the overall impact of antimicrobial action in human neutrophils may be greater or lesser than the sum

of the individual activities of oxidants, granule proteins or other elements. This perspective fails to provide a unifying theory of neutrophil antimicrobial action and leaves instead, in my opinion with resignation that neutrophils ‘operate as they do, in a way wholly above our weak understanding’ [84]. Going forward, investigators should be circumspect about conclusions drawn from studies of a particular phagocyte–microbe interaction. For certain, rigorous analysis of how, for example, murine peritoneal macrophages kill a particular fungus will yield accurate and informative insights into the mechanisms of those events in those well-defined conditions. However, those mechanisms may not apply to other phagocytes from different species challenged with unrelated microbes (e.g. human neutrophils fed *Neisseria gonorrhoeae*).

With respect to elucidation of NOX2-mediated oxidant signaling, technical limitations, most glaringly the lack of sensitive and specific probes to report unambiguously intracellular oxidants, slow meaningful progress. In light of these limitations, care should be taken not to extrapolate observations. NOX2-dependent oxidant production may alter the redox state of target cells in ways that profoundly alter phenotype but may not be directly the consequence of H₂O₂ attack on a specific constituent in a signaling pathway. For example, the absence of superoxide dismutase in murine macrophages results in increased DCF fluorescence in response to ATP and reduced caspase-1-dependent cytokine production [85]. The overall redox potential of the deficient mice is reduced, with subsequent glutathionylation of caspase-1 and decrease in activity, without oxidant-mediated changes in a specific signaling target. Investigations of NOX2-dependent oxidant signaling need to incorporate into their consideration the complex biochemistry that regulates cellular redox status [86].

Gaps in our current knowledge about the consequences of NOX2-dependent oxidant production provide fantastic opportunities for exploration of phagocyte biology and beyond.

Conflict of interest statement

Nothing declared.

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- of special interest
- of outstanding interest

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