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Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses

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Summary

Phagocytes such as neutrophils and monocytes play an essential role in host defenses against microbial pathogens. Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, the hydroxyl radical, and hypochlorous acid, together with microbicidal peptides and proteases, constitute their antimicrobial arsenal. The enzyme responsible for superoxide anion production and, consequently, ROS generation, is called NADPH oxidase or respiratory burst oxidase. This multicomponent enzyme system is composed of cytosolic proteins (p47phox, p67phox, p40phox, and rac1/2) and membrane proteins (p22phox and gp91phox, which form cytochrome b₅₅₈) which assemble at membrane sites upon cell activation. The importance of this enzyme in host defenses is illustrated by a life-threatening genetic disorder called chronic granulomatous disease in which the phagocyte enzyme is dysfunctional, leading to life-threatening bacterial and fungal infections. Also, because ROS can damage surrounding tissues, their production, and thus NADPH oxidase activation, must be tightly regulated. This review describes the structure and activation of the neutrophil NADPH enzyme complex.

Key words: **NADPH oxidase • neutrophils • phagocyte • CGD**

Abbreviations: **phox** – phagocyte oxidase, **ROS** – reactive oxygen species, **CGD** – chronic granulomatous disease, **MPO** – myeloperoxidase, **SOD** – superoxide dismutase, **PMA** – phorbol myristate acetate, **FMLP** – formyl-methionyl-leucyl-phenylalanine.

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INTRODUCTION

Human polymorphonuclear neutrophils constitute one of the most powerful host defenses against bacteria, yeasts, and fungi. Schematically, the functional activities of neutrophils consist of oriented migration (chemotaxis), adherence to and engulfment of the pathogen, and release of toxic substances such as reactive oxygen species (ROS), proteolytic enzymes, and bactericidal proteins contained in granules⁷⁷. The migration of neutrophils towards the infection site is the first step necessary and essential to ensure bactericidal activity. This can be done in a way directed by chemoattractants, such as the C5a fraction of complement, formyl-methionyl-leucyl-phenylalanine (FMLP), interleukin 8 (IL-8), and leukotriene B4 (LTB4). Once arrived at the site of infection, neutrophils recognize and engulf the pathogen. This recognition and engulfment are mediated by opsonins, such as the immunoglobulins G and the C3b and C3bi fractions resulting from the activation of complement, and also by ligands preserved during the evolution of the pathogens which bind to receptors of the Toll-family (Toll-like receptors – TLR), as, for example, TLR2 and TLR4, which recognize zymosan particles from yeast and lipopolysaccharide (LPS) from bacteria, respectively^{2, 7}. Recognition is generally followed by engulfment of the particle, which is surrounded by a membrane envelope resulting in a vacuole called the phagosome. This encapsulation of the bacteria starts the activation process of the neutrophils which will lead to their death and destruction using proteases and ROS. ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^\bullet$), and hypochlorous acid (HOCl). They are produced by phagocytes in a powerful “oxidative burst”³, characterized by a rapid, cyanide-insensitive increase in oxygen uptake, an increase in glucose consumption, and abrupt ROS production.

The enzyme responsible for ROS production is called NADPH oxidase or respiratory burst oxidase⁴. The phagocyte NADPH oxidase plays a key role in host defenses against microbial pathogens by generating superoxide anions and other ROS³. The vital importance of this enzyme is illustrated by a human genetic disorder called chronic granulomatous disease (CGD), which is associated with life-threatening bacterial and fungal infections⁶³. However, excessive ROS release can also damage bystander host tissues, thereby amplifying inflammatory reactions⁵. The active NADPH oxidase is a multicomponent enzyme system located in the plasma membrane of activated cells¹¹. It consists of several components, namely cytochrome b_{558} (a heterodimer composed of gp91phox and p22phox; phox – phagocyte oxidase), p47phox, p67phox, p40phox, and

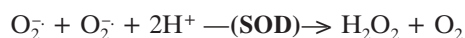
either Rac1 (in monocytes) or rac2 (in neutrophils). It is dormant in resting cells but becomes active when the cells are stimulated. In intact cells, NADPH oxidase activation is accompanied by phosphorylation of p47phox, p67phox, p40phox, and p22phox, along with several protein-protein interactions^{4, 11}. In addition to phagocytic cells (neutrophils, eosinophils, monocytes, and macrophages), NADPH oxidase has mainly been studied in B lymphocytes immortalized by Epstein-Barr virus (EBV)⁷⁴. EBV-transformed B lymphocytes are an excellent model system for studying NADPH oxidase because, contrary to neutrophils, they can be maintained in culture and transfected³⁷.

ROS PRODUCTION AND CHEMISTRY:

O_2^- produced by NADPH oxidase is the source of all ROS generated in the phagosome. It is produced by monovalent reduction of oxygen in the following reaction^{5, 42}:



O_2^- is transformed into H_2O_2 by spontaneous dismutation (at acid pH in the phagosome) or enzymatic dismutation (by superoxide dismutase (SOD) in the cytosol):



Interaction between H_2O_2 and O_2^- can, through the Haber-Weiss reaction in the presence of a transition metal (or the Fenton reaction in the presence of iron), give rise to the $OH^\bullet$:



Myeloperoxidase (MPO) released from azurophilic granules catalyses the transformation of H_2O_2 in the presence of a halogen (Cl^- , Br^- , I^-) into highly toxic molecules:



Other reactions between hypochlorous acid (OCl^-) and H_2O_2 can lead to the formation of singlet oxygen. Most of the OCl^- thus generated is converted into toxic chloramines:



THE COMPONENTS OF THE PHAGOCYTE NADPH OXIDASE

NADPH oxidase is composed of both membranous and cytosolic proteins (Fig. 1):

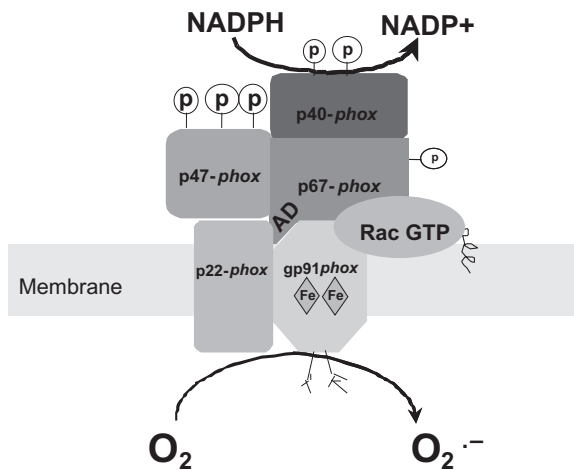


Figure 1. The NADPH oxidase complex. Active NADPH oxidase is composed of several cytosolic proteins (p67phox, p47phox, p40phox, rac2) and membrane-bound cytochrome b_{558} (composed of gp91phox and p22phox). Activated NADPH oxidase uses cytosolic NADPH to reduce oxygen.

Flavocytochrome b_{558}

This is the central membranous component of NADPH oxidase^{4, 11}. It is composed of a glycosylated 91-kDa protein subunit (gp91phox) and a non-glycosylated 22-kDa subunit (p22phox) in a 1:1 complex. It is known as cytochrome b_{558} for its spectral absorption at 558 nm, and as cytochrome b-245 for its midpoint reduction of -245 mV. Cytochrome b_{558} contains one FAD and two hemes and is the electron transfer chain of NADPH oxidase. It seems to be the central docking component for the cytosolic components, and particularly p47phox. Cytochrome b_{558} associates with the small G protein rap1A, but the role of this interaction is not known⁶¹.

The cytosolic components of phagocyte NADPH oxidase are p47phox, p67phox, p40phox, and a small G protein (rac1 or rac2). In resting cells, p47phox, p67phox, and p40phox interact to form a cytosolic complex⁵⁹. During activation, approximately 10% of these proteins migrate to the plasma membrane^{13, 33, 60}.

p47phox

This is a protein composed of 390 amino acids. Its COOH-terminal sequence is very basic and rich in serines and arginines. The amino acid sequence of p47phox also contains two SH3 domains, one PX domain, and at least one proline-rich region. p47phox binds to cytochrome b_{558} during activation^{19, 21}. It is the subunit responsible for transporting the cytosolic complex (p47phox-p67phox-p40phox) from the cytosol to the membrane during oxidase activation^{33, 60} and is the organizer of NADPH oxidase subunits.

This process cannot occur *in vivo* without extensive p47phox phosphorylation³⁷.

p67phox

The predicted 526-amino-acid sequence of p67phox also contains two SH3 domains, four tetratricopeptide-rich regions, and at least one proline-rich region⁴. p67phox associates tightly with the cytoskeleton^{28, 33, 78} and is phosphorylated during neutrophil stimulation²⁹, but to a lesser degree than p47phox. p67phox interacts with rac1/2 and with cytochrome b_{558} ¹⁴ and can regulate its catalytic activity via a sequence called the activation domain⁵⁵.

p40phox

This is a 339-amino-acid protein which was initially identified through its binding to p67phox^{73, 75}. It contains one SH3 domain and one PX domain. It is weakly phosphorylated during activation⁹. This component is not required for NADPH oxidase activation in a cell-free system and its role *in vivo* is controversial: both inhibitory and stimulatory effects have been observed^{49, 65, 72}.

Rac

In human neutrophils, p21-rac2 is the most abundant rac protein, but p21-rac1 (92% homologous with Rac2) is also present⁶⁰. Like cytochrome b_{558} , p47phox, and p67phox, Rac is essential for optimal NADPH oxidase activation in a cell-free system and in intact neutrophils^{1, 76}.

CHRONIC GRANULOMATOUS DISEASE

CGD is an inherited immune deficiency in which phagocytes are unable to produce ROS⁶³. CGD has an incidence of between 1 in 200,000 and 1 in 250,000 live births. Recurrent, often life-threatening bacterial and fungal infections usually start during childhood. Common infectious syndromes include pneumonia and lung abscesses, skin and soft tissue infections, lymphadenopathy, suppurative lymphadenitis, osteomyelitis, and hepatic abscesses. The most common pathogens encountered in CGD patients are Gram-positive bacteria (*Staphylococcus aureus*), Gram-negative bacteria (e.g. *Salmonella*, *Pseudomonas cepacia*, *Serratia marcescens*), and fungi (*Aspergillus*, *Candida albicans*). *Aspergillus* species can cause intractable pneumonia and sometimes septicemia in CGD patients and are a frequent cause of death. Histologically, CGD is characterized by a formation of large granulomas resulting from the fusion of macrophages that have phagocytosed bacteria but

are unable to destroy them because NADPH oxidase activity is lacking. The granulomas can obstruct vital structures such as the gastrointestinal and genitourinary tracts. CGD results from mutations in the NADPH oxidase component genes, namely the CYBB gene (Xp21), which encodes the gp91phox subunit, and the CYBA (16q24), NCF1 (7q11) and NCF2 (1q25) genes, which encode p22phox, p47phox, and p67phox, respectively. The most frequent form of CGD (approximately 70% of all cases) is the X-linked gp91phox-deficient form, followed by the autosomal form deficient in p47phox (approximately 25%). Less frequent forms are autosomal CGD deficient in p67phox (<5%) and p22phox (<5%). Only one case of rac2 gene mutation has been described so far⁷⁶. To date there are no reports of CGD caused by defects in the gene for a fifth oxidase subunit, p40phox.

ACTIVATION OF NADPH OXIDASE

Cell-free activation

NADPH oxidase can be activated *in vitro* by mixing neutrophil cytosol and plasma membranes (or the corresponding recombinant proteins) in the presence of Mg^{++} , guanosine triphosphate (GTP), and an activating anionic amphiphile such as arachidonate or sodium dodecyl sulfate¹⁰, which mimic phosphorylation by providing negative charges. This cell-free system can also be activated by protein kinase C (PKC)

instead of anionic amphiphile agents³². Use of the cell-free system has contributed to identifying the components of the enzyme. Using this system, Segal and colleagues showed that NADPH oxidase activity could be fully reconstituted by cytochrome b_{558} , p47phox, p67phox, and rac alone¹.

Activation in intact neutrophils

NADPH oxidase can be activated by both receptor-mediated and receptor-independent mechanisms⁸. NADPH oxidase activation in phagocytes can be induced by a large number of soluble and particulate factors such as opsonized bacteria, opsonized zymosan, latex particles, complement fragment C5a, formylated peptides such as FMLP, LTB₄, PAF, diacylglycerol (DAG), calcium ionophores (ionomycin, A23187), and PKC activators such as phorbol myristate acetate (PMA). LPS from Gram-negative bacteria induces ROS production in monocytes⁷¹, but induces priming of NADPH oxidase in neutrophils²⁰ through binding to TLR4. Other agents, such as angiotensin II, the main hormone of the renin-angiotensin system, stimulates superoxide production in neutrophils²⁷.

Two events accompany NADPH oxidase activation: 1) protein phosphorylation and 2) translocation of cytosolic components to the plasma membrane (Fig. 2).

In physiological conditions the oxidative burst is triggered by the binding of a stimulus to a specific recep-

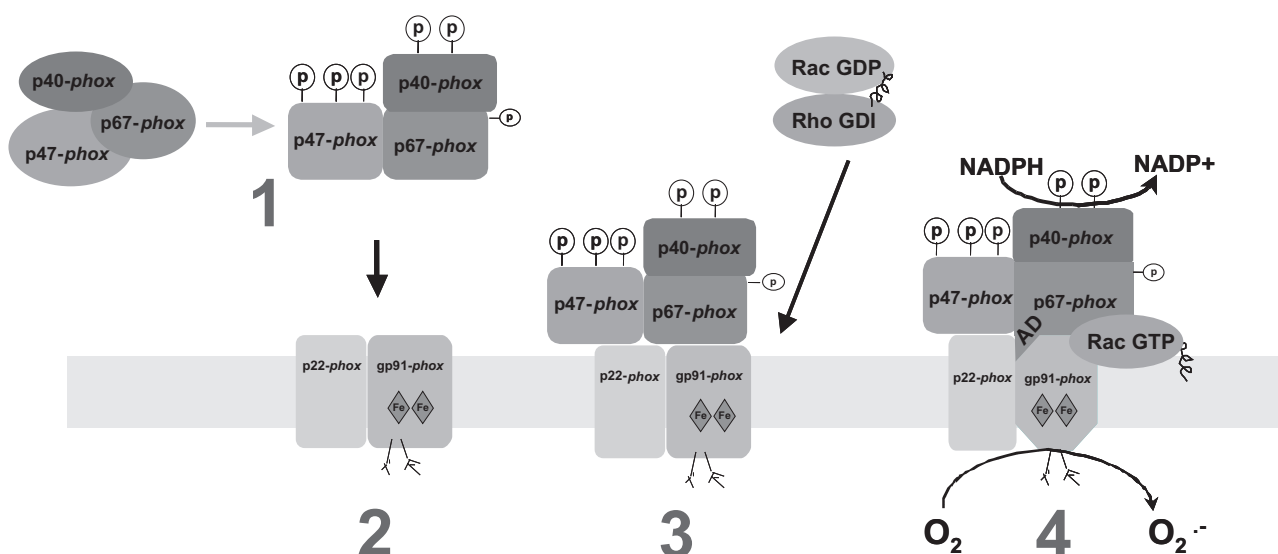


Figure 2. Activation of NADPH oxidase. In resting cells the components of NADPH oxidase are distributed between the cytosol and the membranes. **1** – Upon cell activation the cytosolic components p47phox, p67phox, and p40phox are phosphorylated, and **2** – migrate to the membranes where they associate with the membrane-bound components gp91phox and p22phox; **3** – at the same time, rac 2 exchanges its GDP by GTP, dissociates from its inhibitor rho-GDI, and migrates to the membrane; **4** – cytochrome b_{558} , which contains both flavin and heme groups, is then activated by p67phox via its activation domain (AD) and rac2, which promotes the interaction between p67phox and cytochrome b_{558} . Activated NADPH oxidase then uses cytosolic NADPH to reduce oxygen and to produce superoxide anions.

tor such as the FMLP or C5a receptor. The receptor transmits information through the cytoplasmic membrane via a family of heterotrimeric G proteins (proteins binding GTP). The G proteins then activate membrane enzymes such as phospholipase C (PLC), phospholipase A2 (PLA2), and phospholipase D (PLD), leading to the release of intracellular messengers⁸. For example, PLC cleaves a membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP2), into DAG and inositol-triphosphate (IP3). IP3 is involved in the release of calcium from intracellular pools, while DAG activates the PKC family. Activation of PLD results in phosphatidic acid production from phosphatidylcholine. Activation of PLA2 leads to the production of arachidonic acid, which can then be used as a substrate for leukotriene and prostaglandin synthesis. Neutrophil activation is accompanied by the activation of many protein kinases, such as protein tyrosine kinases, PKA, PKC, and MAP-kinase, which in turn phosphorylate many proteins with important cellular functions, such as NADPH oxidase components.

During neutrophil stimulation, p47phox is heavily phosphorylated. This phosphoprotein was first identified because it was missing in the neutrophils of some CGD-patients^{57, 68}. In resting cells, p47phox is a very basic, unphosphorylated protein. When cells are activated with PMA or FMLP, 8–9 phosphorylation states of p47phox are observed in the cytosol and membrane, the two most acidic forms being located in the membrane^{57, 64}. The stepwise phosphorylation mechanism points to sequential p47phox phosphorylation in the cytosol and membrane.

p47phox is phosphorylated on multiple sites in the carboxy-terminal portion of the protein, including serines 303 to 379^{30, 31}. Combined mutation of all these serines was used to show that p47phox phosphorylation is required for NADPH oxidase activation in intact cells³⁷. Individual mutation of each serine showed that only serine 379 was important for oxidase activation³⁷, but double mutations also showed that two pairs of phosphorylated serines (serines (303+304) and serines (359+370)) are necessary for NADPH oxidase activation^{45, 46}.

In human neutrophils, various protein kinases have been implicated in the regulation of NADPH oxidase activity, among which the PKC family appears to play a major role after FMLP or PMA activation^{16, 18}. Other kinases, such as extracellular signal-regulated protein kinases 1 and 2, may also participate in these phosphorylation reactions²². LPS and proinflammatory cytokines, such as GM-CSF and tumor necrosis factor α , which do not activate NADPH oxidase but

prime its activation by a secondary stimulus³⁴ such as FMLP and C5a, induce partial phosphorylation of p47phox on a specific peptide¹⁵ and upregulate NADPH oxidase assembly²⁰. In contrast to PMA and FMLP, GM-CSF-induced partial p47phox phosphorylation is not inhibited by the PKC inhibitor GF109203X, but is sensitive to protein tyrosine kinase and PI3K inhibitors. This suggests that p47phox phosphorylation could be controlled by several pathways and that many protein kinases participate in this process by targeting specific sites.

Other oxidase proteins, such as p67phox, p40phox, and p22phox, are also phosphorylated in human neutrophils. p67phox is phosphorylated on serine and threonine residues by PKC-dependent and PKC-independent pathways^{29, 39}. ERK1/2 may synergize to phosphorylate p67phox at specific sites¹⁷. One p67phox-phosphorylated site is threonine 233³⁹. p40phox is phosphorylated on serine 315 and threonine 154 by a PKC-dependent pathway⁹. p22phox is phosphorylated on threonine residues by a phosphatidic acid-activated kinase and PKC⁶².

The action of these phosphorylations is unknown, except in the case of p47phox¹⁹. Several reports suggest that p47phox phosphorylation induces conformational changes^{44, 58} which initiate assembly of the active enzyme via interaction of the SH3 domain with the proline-rich region of p22phox⁴⁴. Indeed, p47phox contains two SH3 domains, one of which interacts intramolecularly with a p47phox-polyproline sequence in the non-phosphorylated protein. This interaction switches to the p22phox-polyproline sequence when the enzyme is activated by phosphorylation. p47phox and p40phox contain a PX domain (a sequence of about 125 amino acids) that binds phosphoinositides and may help to assemble the oxidase complex at precise sites of ingested pathogens⁷⁹.

Inactivation of the NADPH oxidase

In contrast to PMA, FMLP and C5a, which are physiological activators of neutrophils, induce short activation of the NADPH oxidase. The fast inactivation could be due either to phosphorylation of one or more serines or to the dephosphorylation of the p47phox; implication of phosphatases PP1 and PP2A was suggested⁶. Antagonism between the regulatory small G proteins rac and cdc42 was also reported as a possible inhibitory mechanism of NADPH oxidase²⁴.

Although our understanding of the structure of NADPH oxidase has advanced substantially, the mechanisms of activation, assembly, and phosphorylation of the components are less well known.

Identification of the pathways, kinases and phosphatases involved in the regulation of NADPH oxidase could lead to novel therapeutic agents for inflammatory diseases involving abnormal neutrophil superoxide production.

THE NOX FAMILY AND OTHER EFFECTS OF ROS

ROS production by various non-phagocytic cells was described many years ago, although the origin and functions of these ROS were unclear. Recently, several homologues of the catalytic subunit (gp91phox) responsible for ROS production by phagocytes were described in human tissues such as lung, kidney, and colon and in various cellular types (epithelial cells, endothelial cells, vascular smooth muscle cells)⁵⁰. These homologues, currently seven in number, have been cloned and grouped under the acronym Nox, for NADPH oxidase (Nox-1 to Nox-5), or Duox, for dual oxidase (Duox1 and Duox2). One of the essential differences between phagocytic gp91phox (referred Nox-2) and other Nox species is that ROS production by Nox-2 occurs on the external face of the plasma membrane, releasing ROS into phagosomes or the extracellular medium, whereas ROS produced by other Nox species are detected in the intracellular medium and are produced in much smaller quantities. These new data suggest that ROS play a role in several cellular functions, such as local tissue-specific bactericidal activity and intracellular

signaling, stimulating major scientific and medical interest in the understanding of the mechanisms controlling their production^{25, 38, 48, 66}. For example, thyroid cells produce H₂O₂ used in the biosynthesis of hormones²⁶. Fibroblasts produce very small quantities of ROS in response to growth factors^{36, 47}. H₂O₂ activates the transcription factors AP-1 and NF- κ B which are involved in the regulation of cell division and protein synthesis in response to inflammatory mediators^{53, 67}. In response to LDL, endothelial cells can produce ROS in an NADPH oxidase-dependent manner⁵⁴, and vascular smooth muscle cells have NADPH oxidase that produces superoxide anions in response to angiotensin⁶⁹.

NADPH OXIDASE INHIBITORS

A number of NADPH oxidase inhibitors have been described. Endogenous biological molecules which inhibit NADPH oxidase activity or its activation are nitric oxide^{12, 40}, steroids⁵¹, adrenaline⁵⁶, IL-10³⁵, and IL-4⁸⁰. Numerous exogenous pharmacological inhibitors are known, the most used being diphenylene iodonium, which inhibits electron transport by gp91phox⁴³. Apocynin, a methoxy-substituted catechol, is a natural molecule which inhibits NADPH oxidase⁷⁰. Other molecules, such as phenylarsine oxide⁵², 4-(2-aminoethyl)-benzenesulfonyl fluoride²³, and N- α -tosyl phenylalanine chloromethyl ketone⁴¹, inhibit NADPH oxidase by inhibiting complex assembly.

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