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# NADPH Oxidase

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## Abbreviations

| | |
|---|---|
| AngII | angiotensin II |
| CGD | chronic granulomatous disease |
| FAD | flavin adenine dinucleotide |
| GDP | guanosine diphosphate |
| GTP | guanosine triphosphate |
| Mox-1 | mitogenic oxidase |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| Nox | NADPH oxidase |
| ROS | reactive oxygen species |
| SH3 | Src homology 3 |
| SOD | superoxide dismutase |
| VSMC | vascular smooth muscle cells |

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## Abstract

The phagocyte NADPH oxidase, also known as the respiratory burst oxidase, is a multicomponent enzyme that catalyzes the reduction of molecular oxygen ($O_2$) to superoxide ($O_2^{\bullet -}$). It is best known for its role in host defense against invading microorganisms. NADPH serves as the electron-donor for this one-electron reduction through the transmembrane protein cytochrome $b_{558}$, a heterodimeric complex of gp91phox and p22phox protein subunits. Upon activation, cytosolic proteins are translocated to the plasma membrane to form an activated oxidase. The electron transfer from NADPH to $O_2$ is through the electron carriers, flavin adenine dinucleotide (FAD) and two heme groups, located within the gp91phox subunit. Mutations in the various subunits results in a dysfunctional oxidase, which leads to the human genetic disorder chronic granulomatous disease (CGD). In addition to the well-known phagocyte NADPH oxidase, recent studies have identified a role for a non-phagocyte NADPH oxidase in other human diseases, including hypertension.

## Introduction

The NADPH oxidase in phagocytic cells including neutrophils and macrophages is a multicomponent complex that transfers electrons from NADPH to molecular oxygen ($O_2$) producing superoxide radical ($O_2^{\bullet-}$) (Reaction 1) [1]. Superoxide is scavenged rapidly by

$$NADPH + 2\,O_2 \rightarrow 2\,O_2^{\bullet-} + NADP^+ + H^+ \quad (1)$$

superoxide dismutase (SOD) to yield hydrogen peroxide ($H_2O_2$) and oxygen. Subsequently, $O_2^{\bullet-}$ and $H_2O_2$ can react with iron to produce the highly reactive hydroxyl radical ($HO^{\bullet}$) *via* the Haber-Weiss reaction. These reactive oxygen species (ROS) play a critical role in the killing of microorganisms during phagocytosis. Therefore, the presence and activity of the NADPH oxidase plays a crucial role in innate host defense. The catalytic component of the NADPH oxidase is a membrane bound flavocytochrome *b,* referred to as flavocytochrome $b_{558}$ because of the heme absorbance peak at 558 nm in the reduced state [2]. In addition to the membrane component, NADPH oxidase activity is dependent on the translocation of cytosolic subunits p47phox, p67phox, and GTP-binding protein Rac to the membrane [2]. This review summarizes the components and activation of the phagocyte NADPH oxidase, while addressing the role of the enzyme in human disease. In addition, this article will touch on the recently described non-phagocytic NADPH oxidase in hypertension.

## Phagocyte NADPH Oxidase

The catalytic core of the NADPH oxidase, flavocytochrome $b_{558}$, is a low-redox-potential protein that is formed by non-covalently linked subunits, p22phox and gp91phox, in a molar ratio of 1:1 [2]. The flavocytochrome is anchored in the membrane by a series of hydrophobic transmembrane segments. It is postulated that the gp91phox subunit is the true catalytic component as recent studies using spectral and potential measurements made on membranes of

monkey kidney cells (COS 7 cells) demonstrated that gp91phox contains two hemes with distinct mid-potentials of –225 mV and –265 mV [3]. These low midpoint redox potentials permit the direct reduction of $O_2$ to $O_2^{\bullet-}$ by the reduced cytochrome. In addition to the hemes, gp91phox contains a functional FAD prosthetic group. This was confirmed by demonstrating that purified flavocytochrome $b_{558}$ has little NADPH oxidase activity because most of the bound FAD is lost and oxidase activity is recovered upon addition of FAD to the purified complex [4]. Photolabeling and photoactivatable studies using azido derivatives of FAD and NADPH have demonstrated that the FAD- and NADPH-binding sites are localized on the carboxyl terminal of gp91phox within the cytoplasm [5]. The hemes are liganded by two pairs of histidine residues located in the N-terminal half of the molecule, more specifically in the transmembrane helices III and V of gp91phox (Figure 1) [2]. One of the hemes is positioned close to the cytosolic side of the membrane, in the proximity of the FAD-binding domain; however, the second heme is found on the opposite side of the membrane. Therefore, it is hypothesized that NADPH transfers electrons to FAD, which subsequently passes the electrons to the heme group near the cytoplasm. The electrons are then passed to the second heme and finally to $O_2$ to produce $O_2^{\bullet-}$ in the extracellular matrix or within the phagocytic vacuole (Figure 2) [6].

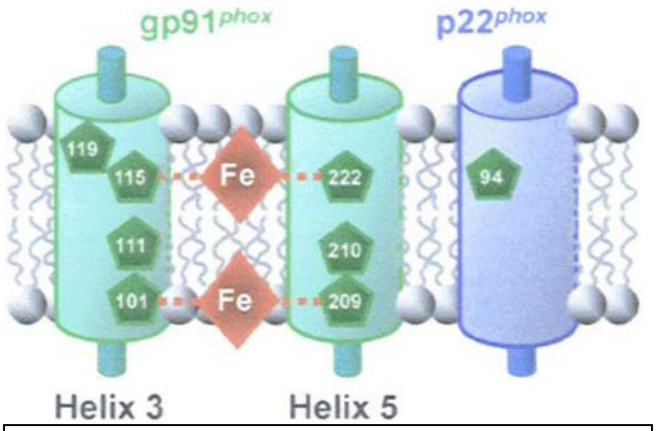
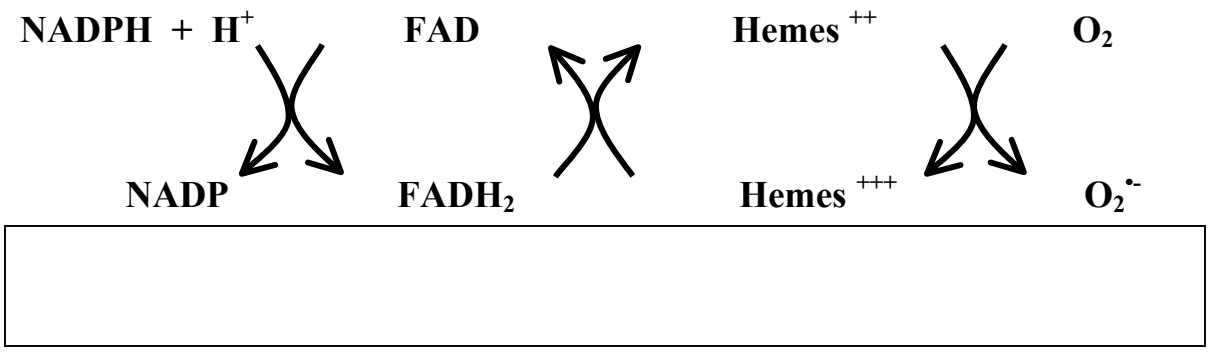



Figure 1. The heme groups of gp91phox are liganded by two pairs of histidine residues. The heme attached to His101 and His209 accepts electrons from FAD and passes them the heme attached to His115 and His222, which passes the electrons to $O_2$ to produce $O_2^{\bullet-}$. From Biberstine-Kinkade KJ *et al*. [2].




The translocation of cytosolic proteins p47phox, p67phox and GTP-binding protein Rac to the membrane bound flavocytochrome $b_{558}$ is essential for NADPH oxidase activity [6]. It is hypothesized that p47phox is the subunit chiefly responsible for transporting the cytosol components to the membrane because in neutrophils deficient in p47phox, p67phox is not transported to the membrane after stimulation. However, in p67phox-deficient neutrophils p47phox is translocated normally [6]. p47phox is a basic protein with an arginine-rich C-terminal region containing numerous serine residues that are phosphoralyted prior to translocation to the membrane [7]. In addition, p47phox and p67phox contain SH3 domains, which are well known as mediators of protein-protein interactions, functioning through binding to short proline-rich motifs [7]. Interestingly, proline-rich motifs have been identified in p22phox, p47phox and p67phox, suggesting a variety of binding interactions between these oxidase components [8]. It is believed that p47phox interacts with itself in the resting state via the SH3 domains binding to the proline-rich domains; however, upon activation the SH3 domain of p67phox occupies the C-terminal proline-rich domain of p47phox and the complex can be translocated to the membrane [8]. In addition to the interaction with p67phox, the phosphorylation of p47phox unfolds the protein and opens sites for the binding of p47phox with cytochrome $b_{558}$. The last essential cytosolic component for an active NADPH oxidase is a Ras-related protein called Rac. Rac is activated by the exchange of GDP for GTP and it is the Rac-

GTP bound isoform that is believed to be a key player in NADPH oxidase activation [9]. The essential components of the phagocyte NADPH oxidase are summarized in Table 1.

Table 1: Components of the NADPH oxidase

| Component | Structure | Interaction and role |
|---|---|---|
| gp91phox | Cytochrome subunit, 65 kDa includes carbohydrate, 2 hemes, and FAD | Binds p47phox, NADPH, FAD, membrane bound, transfers electrons from NADPH to $O_2^{\bullet-}$ |
| p22phox | Cytochrome subunit, proline-rich motifs | Provides p47phox docking site, membrane bound |
| p47phox | Cationic, serine phosphorylation sites, 2 SH3 domains, proline-rich motifs | Binds SH3 domains in p67phox, translocates from cytosol to membrane after phosphorylation |
| p67phox | 2 SH3 domains, proline-rich motifs | Binds p47phox, bind Rac, translocates to membrane |
| Rac | Ras like protein, GTP-binding protein | Binds p67phox, translocates to membrane |

As discussed above, a large collection of evidence demonstrates that gp91phox is the catalytic subunit of the NADPH oxidase. If this is indeed the case, then what roles do the essential cytosolic components play? One report suggests that p67phox participates in NADPH binding [10]. Interestingly, a separate study provides evidence for a role of p67phox in facilitating the initial transfer of two electrons from NADPH to FAD [11]. In addition, this study suggests that p47phox is required for the transfer of single electrons from the reduced flavin to the two heme groups.

## Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is an inherited disorder characterized by the inability of phagocytes to produce $O_2^{\bullet-}$, thus resulting in an increase in the susceptibility of affected patients to bacterial and fungal infections [9]. The primary cause of CGD is a mutation

that results in the loss or inactivation of one of the NADPH oxidase essential subunits, p47phox, p67phox, p22phox, or gp91phox. In patients with classical X-linked CGD, neutrophil membrane fractions lacked the characteristic heme spectrum of cytochrome $b_{558}$ [12]. However, in other CGD patients, in whom the disease was transmitted in an autosomal recessive fashion, cytochrome $b_{558}$ was normal, but the cytosolic components failed to form an active oxidase complex. It was shown that in patients with autosomal recessive CGD neutrophil cytosol lacked p47phox, and in rare cases lacked p67phox [12]. In studying the different mutations (Table 2), researchers have learned more about the mechanism of the oxidase. In cells from CGD patients lacking cytochrome $b_{558}$, neither p47phox nor p67phox translocate to the membrane upon activation [12]. However, in CGD patients with a missense mutation resulting in membrane expression of an inactive cytochrome $b_{558}$, the cytosolic subunits exhibited normal translocation. These studies confirm previous hypotheses that cytochrome $b_{558}$ is the docking site of the cytosolic components. CGD patients with the gp91phox mutation (R54S) are unable to produce $O_2^{\bullet-}$ because of the midpoint potentials of the hemes are altered preventing the electron transfer from FAD to cytochrome $b_{558}$ [3]. In the p67phox mutation (ΔK58), the subunit loses its ability to interact with Rac resulting in the inability of the cytosolic complex to translocate to the membrane [12]. These studies suggest that Rac is involved in the translocation of the cytosolic complex during oxidase activation. In the future, the transfer of a correct gene encoding the wild-type oxidase subunit into hemeopoietic stem cells, precursors of the phagocytic cell lines, may provide treatment for CGD patients.

| Oxidase Subunit | Mutation |
|---|---|
| gp91phox | P415H |
| gp91phox | D500G |
| gp91phox | A57E |
| gp91phox | R54S |
| p22phox | P156Q |
| p67phox | ΔK58 |

Table 2. Various mutations in the NADPH oxidase subunits identified in CGD patients have led to a better understanding of the oxidase. This list is not an exhaustive list of mutations. This table was modified from Babior BM [12].

**Non-Phagocyte NADPH Oxidase**

Recently, a functionally relevant non-phagocytic NADPH oxidase has been identified in various cell types including endothelial cells and vascular smooth muscle cells (VSMC). Suh *et al.* described a human expressed sequence tag that showed homology to human gp91phox that contained FAD and NADPH binding sites [13]. This gp91phox homologue, initially called Mox-1 (for **M**itogenic **ox**idase) was found to be highly expressed in human colon epithelial cell, in human colon carcinoma cells Caco-2, and in cultures of rat aortic VSMC [13]. Subsequently, a collection of gp91phox homologues have been identified and have since been given the common name Nox (for **N**ADPH **ox**idase). Important for our laboratory's research interests, there is substantial evidence that a phagocyte-like NADPH oxidase is localized throughout the vascular wall that is important in the development of hypertension. Of particular interest are the studies by Griendling *et al*. demonstrating that angiotensin II (AngII), the effector peptide of the renin-angiotensin system, activates NADPH oxidase and that chronic AngII infusion leads to an increase in blood pressure dependent on the production of NADPH oxidase-derived $O_2^{\bullet-}$ [14].

We have recently reported a role for $O_2^{\bullet-}$ in AngII signaling in the brain by demonstrating that the well-known central effect of AngII on cardiovascular and volume homeostasis was attenuated in mice over-expressing ROS scavenging enzymes, CuZn-superoxide dismutase and Mn-superoxide dismutase, in the brain [15]. In addition, we have provide evidence suggesting a role for a phagocyte-like NADPH in central AngII-induced $O_2^{\bullet-}$. We administered adenoviruses encoding either the dominant-negative (AdN17Rac1) or wildtype (AdwtRac1) form of NAD(P)H-activating Rac1 protein (described earlier) into the brains of normal C57BL/6 mice Three days later, the effects of central AngII on blood pressure and heart rate were examined in

conscious freely moving mice. Central AngII caused the characteristic pressor and bradycardic response in saline and control vector (AdLacZ) treated mice, whereas these responses were

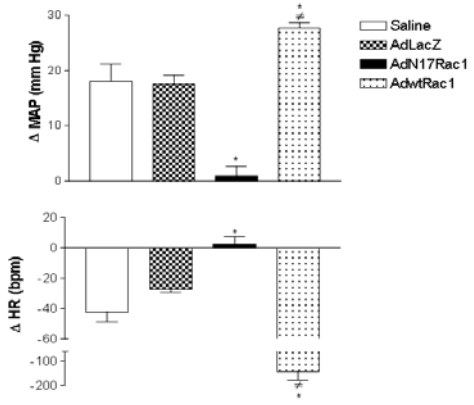



Figure 3. Rac1 mediates central AngII-induced cardiovascular responses. Central administration of AngII induced an increase in mean arterial pressure (ΔMAP) and a decrease in heart rate (ΔHR) in mice given saline or AdLacZ centrally. This response was attenuated in mice expressing dominant negative Rac1 (AdN17Rac1) and enchanced in mice over-expressing wildtype Rac1 (AdwtRac1) in the brain. This graph was obtained from Dr. Robin Davisson's laboratory (unpublished observations).

markedly attenuated in AdN17Rac1 and enhanced in AdwtRac1 treated mice (Figure 3, unpublished observations). In addition, AdN17Rac1 attenuated AngII-stimulated NAD(P)H-dependent $O_2^{\bullet-}$ production in primary neuronal cultures, while AdwtRac1 caused enhanced AngII-induced $O_2^{\bullet-}$ generation as measured by lucigenin-enhanced chemiluminescence. This is the first evidence that a phagocyte-like Rac1-NAD(P)H oxidase is a critical mediator of *central* AngII-induced cardiovascular responses, and may represent a key mechanism in neurogenic hypertension.

**Summary**

The phagocyte NAPDH oxidase, a multicomponent enzyme consisting of membrane bound subunit p22phox, the catalytic subunit gp91phox, and cytsolic subunits p47phox, p67phox and Rac, catalyzes the production of $O_2^{\bullet-}$ by the one-electron reduction of oxygen, using NADPH as the electron donor. NADPH oxidase activity is critical in innate host defense, as patients with CGD, characterized by having a dysfunctional oxidase, lack the ability to fight off bacterial and fungal infections. While it is commonly thought that the primary role of NADPH oxidase is to produce ROS to fight infection, more recently it has become appreciated that a phagocyte-like NADPH oxidase exists is numerous cell types. This non-phagocyte NADPH oxidase is implicated to play a role in systemic AngII-dependent hypertension, and as we have demonstrated central AngII-induced hypertension.

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