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p47phox and NOXO1, the organizer subunits of the NADPH oxidase 2 (NOX2) and NADPH oxidase 1 (NOX1)

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ABSTRACT

The enzyme responsible for superoxide anion production in phagocytes is called the phagocyte NADPH oxidase. It is a multicomponent enzyme system resulting from the assembly upon activation of four cytosolic proteins (p47phox, p67phox, p40phox and Rac1 or Rac2) with two transmembrane proteins (p22phox and gp91phox, which form the cytochrome *b558*). The gp91phox is the catalytic subunit of the phagocyte NADPH oxidase and was the first NADPH oxidase to be discovered, renamed today as NOX2. Since then, a family of NOX enzymes, comprising NOX1 to NOX5 and the two DUOX, DUOX1 and DUOX2 has been characterized. NOX1 was the first homologue of gp91phox to be identified, and now refers to a multicomponent enzyme complex composed of three cytosolic proteins (NOXO1, a p47phox homologue, NOXA1, a p67phox homologue and Rac1) with two transmembrane proteins (NOX1 and p22phox). NOX1- and NOX2-derived ROS are essential for innate immunity and other physiological functions; however, excessive ROS production can induce tissue injury, contributing to inflammatory diseases. Thus, NOX1 and NOX2 activation must be tightly regulated in time and space in order to limit ROS production. p47phox and NOXO1 play a major role in the regulation and organization of the NOX2 and NOX1 complexes, respectively, through the interactions of specific protein domains and via phosphorylation. This chapter aims to provide new insights on the role of p47phox and NOXO1 in NOX2 and NOX1 regulation and activation.

Keywords: NADPH oxidase, NOX2, NOX1, p47phox, NOXO1, protein phosphorylation, neutrophil, epithelial cells.

1. Overview

Phagocytic cells such as polymorphonuclear neutrophils, eosinophils, monocytes and macrophages are the first line of defense against microbes such as bacteria, parasites and fungi [1-3]. Upon phagocytosis of microbes, phagocytes produce high amounts of superoxide anion ($O_2^{\cdot-}$), which is the source of other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [4-6]. From a historical point of view, initial experiments in 1932 by Baldrige and Gerard using a Warburg manometer found that phagocytosis was accompanied by a “burst of oxygen consumption” [7]. In 1961, Quastel *et al.* used [^{14}C]-formate, which is oxidized to $^{14}CO_2$ in the presence of catalase, and reported the production of hydrogen peroxide by neutrophils [8]. The source of hydrogen peroxide remained unknown until the findings by Babior *et al.* in 1973 [9]. Using the cytochrome c reduction assay in combination with superoxide dismutase, they discovered that neutrophils produced superoxide anion that was then converted into hydrogen peroxide. The rapid increase in oxygen uptake and the abrupt ROS production that occurred during neutrophil activation was then called the “respiratory burst”. The discovery by Babior *et al.* [9] opened up a new research field in phagocyte biology. The enzyme producing superoxide anion was called the phagocyte NADPH oxidase because it uses cytosolic NADPH as the electron donor to reduce molecular oxygen into superoxide anion [10]. The enzyme was characterized in neutrophils, monocytes and macrophages and its membrane and cytosolic components were identified in the eighties and nineties by several groups (reviewed in [11, 12]).

Today, the structure of the phagocyte NADPH oxidase is well known. It is a multicomponent enzyme system, which is composed of several membrane and cytosolic proteins that are assembled upon activation (reviewed in [11, 12]). The phagocyte NADPH oxidase has an efficient electron transporter, which was previously referred to as the cytochrome *b558* for its signature spectral absorbance, and is a heterodimer

comprised of two transmembrane proteins, e.g., gp91phox (phox: phagocyte oxidase) and p22phox. The activity of the cytochrome b558 can be switched “ON” and “OFF” and is regulated by the four cytosolic proteins, p47phox, p67phox, p40phox and Rac2 (in neutrophils) or Rac1 (in monocytes and macrophages) (Figure 1).

The gp91phox is the core enzyme as it can bind NADPH and FAD, has two hemes, and is able to transfer electrons from NADPH to oxygen to produce superoxide anion [13, 14]. p22phox is a stabilizing subunit for gp91phox and binds to p47phox during activation [15, 16]. p47phox is the regulatory subunit that organizes the assembly of the complex as it interacts with the cytosolic “p67phox-p40phox complex” to allow translocation of all three proteins from the cytosol to the membranes [17, 18]. p67phox is considered as the activator subunit as it binds and stimulates gp91phox enzymatic activity *via* an activation domain (AD) [19, 20]. The GTPases Rac1 or Rac2 are also required for gp91phox enzymatic activity in monocytes/macrophages and neutrophils, respectively [21, 22]. Finally, p40phox is an enhancer of the phagocyte NADPH oxidase activation [23, 24].

The vital importance of the phagocyte NADPH oxidase is demonstrated by the genetic immunodeficiency disorder called chronic granulomatous disease (CGD) in which phagocytes do not produce ROS and affected patients have infections often more fatal than in healthy individuals [25, 26]. CGD is a rare disease found in 1 over 200,000 to 250,000 individuals. It is caused by a gene mutation in one of the NADPH oxidase components, the most frequent CGD form being caused by mutations in the *gp91phox/CYBB* gene (65%), followed by mutation in the *p47phox/NCF1* gene (20%), the *p22phox/CYBA* gene (less than 5%), the *p67phox/NCF2* gene (less than 5%), and the *p40phox/NCF4* and *Rac2/NCF3* genes (both forms less than 5%) [25, 26].

ROS production by various non-phagocytic cells such as epithelial and endothelial cells was described for years, although the sources of ROS were not identified [27-29]. Based on a sequence homology search with the gp91phox/CYBB gene, Lambeth's group described the first human homologue of gp91phox, which was called NOX1 (for NADPH oxidase homologue 1) [30]. Later, several other homologues of gp91phox were described in several tissues such as kidney, lung, colon, inner ear and thyroid [31-37]. These homologues were then cloned and grouped under the acronym "NOX", for NADPH oxidase (NOX1 to NOX5) or "DUOX" for dual oxidase (DUOX1 and DUOX2) [38, 39]. The major difference between the phagocytic gp91phox (now called NOX2) and the other NOXs is that NOX2 requires stimulation to form an active complex between membrane and cytosolic components, and ROS production by NOX2 occurs on the external face of the plasma membrane, releasing high amounts of ROS into the phagosome or the extracellular space, whereas the other NOXs produce lower amounts of ROS ~~that are detected within the intracellular space~~ [30-39]. These data suggested that ROS play a role in several cellular functions such as local tissue-specific bactericidal activity and intracellular signaling, stimulating major scientific interest in understanding the mechanisms controlling their production.

NOX1 shares 56% amino-acid identity with NOX2 [30], and like NOX2, it has six transmembrane domains, contains two hemes, and binds FAD and NADPH. NOX1 mRNA is highly expressed in epithelial cells from the colon, but is also detected in the stomach, uterus, prostate, and smooth muscle cells [38, 39]. As with NOX2, NOX1 is expressed with p22phox in membranes and is regulated by Rac1. However, NOX1 has specific cytosolic partners, i.e., NOX organizer 1 (NOXO1, the homologue of p47phox), and NOX activator 1 (NOXA1, the homologue of p67phox) [40-42] (Figure 1).

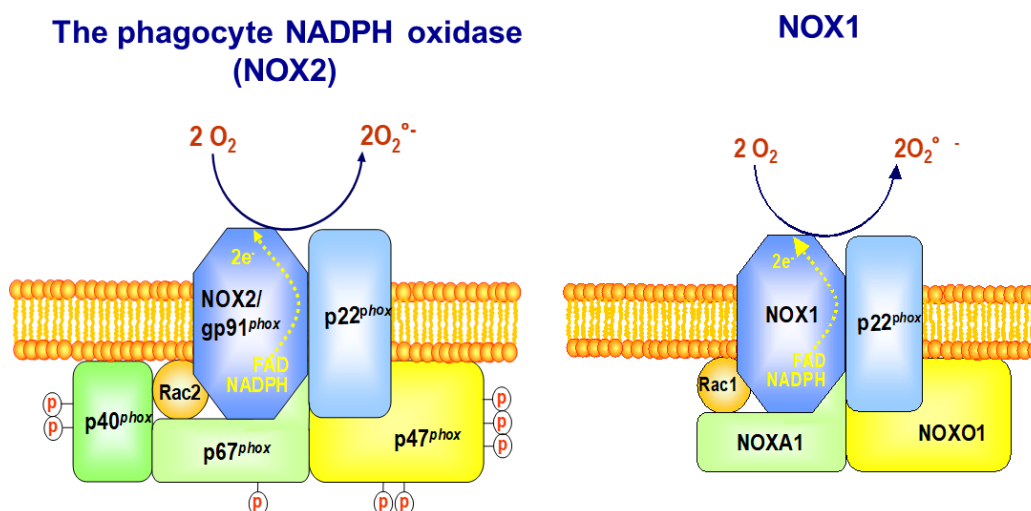


Figure 1: . Structure of the phagocyte NADPH oxidase (NOX2) and of NOX1. The active phagocyte NADPH oxidase complex is composed of two membrane proteins (gp91^{phox}/NOX2 and p22^{phox}) and four proteins (p67^{phox}, p47^{phox}, p40^{phox} and Rac1 or Rac2) that are present in the cytosol prior to activation. The active phagocyte NOX1 complex is composed of two membrane proteins (NOX1 and p22^{phox}) and three cytosolic proteins (NOXO1, NOXA1 and Rac1). The activated NADPH oxidase uses cytosolic NADPH to reduce oxygen into superoxide anion. (P) denotes phosphorylation.

p47^{phox} and NOXO are the organizer and regulatory subunits of NOX2 and NOX1, respectively, and their properties are discussed in this chapter.

2. p47^{phox}, the phagocyte NADPH oxidase organizer and regulator

2.1 History of the discovery of p47^{phox}

The discovery that the NADPH oxidase could be activated in a cell-free system [43-46], suggested that the enzyme required membrane and cytosolic factors to be catalically active [47-49]. p47^{phox} was first identified in 1985 as a 44-kDa phosphorylated protein that was missing in stimulated neutrophils from patients with autosomal recessive CGD [50]. Several groups then attempted to purify it in order to produce specific antibodies to sequence the protein and clone the gene [47-49]. Cloning of the p47^{phox}/ Neutrophil Cytosolic Factor 1 (NCF1) gene was achieved in 1989 by two different groups [51, 52].

2.2 Structure of p47phox

The human p47phox/NCF1 gene sequence encodes for a protein composed of 390 amino acids, with an estimated molecular weight of 44.7 kDa, which migrates at almost 47 kDa by SDS-polyacrylamide gel electrophoresis [51, 52]. It is abundant in the cytosol of human neutrophils as it has been estimated to be present at 100-150 ng/10⁶ cells [53, 54]. Native non-phosphorylated p47phox is a very basic protein with an isoelectric point of 9.8 [55, 56]. Its N-terminal amino acid sequence has a phox homology domain (PX; amino acids 4-121), and the center of the protein contains two Src homology 3 (SH3) domains (amino acids 159-214 (SH3A) and amino acids 229-284 (SH3B)) [11, 51, 52]. The C-terminal sequence of p47phox is very basic, rich in serines as potential phosphorylation sites and contains an auto-inhibitory region (AIR) (amino acids 292-340) and a proline rich region (PRR) (amino acids 363-368) [11, 12, 57] (Figure 2).

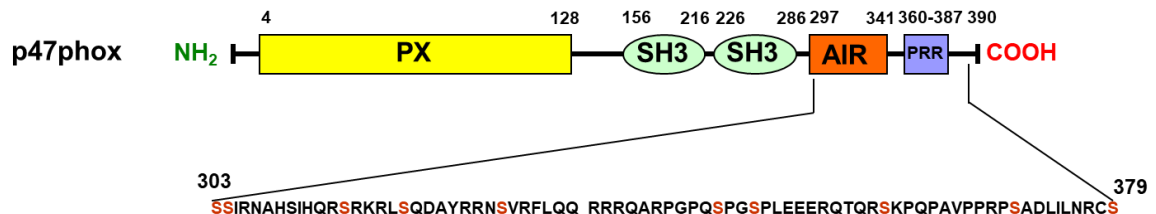


Figure 2 : Structure of human p47phox. The human p47phox protein has 390 amino acids, organized into multiple domains. A phox domain (PX) (amino acids 4-128), two Src homology 3 (SH3) domains [SH3A (amino acids 156-216) and SH3B (amino acids 226-286)], a proline-rich region (PRR) (amino acids 363-367), and an autoinhibitory region (AIR) (amino acids 397-341). The COOH-terminal sequence, including the AIR domain, contains several serines that can potentially be phosphorylated.

2.3 Phosphorylation of p47phox

Using ³²P labeling of neutrophils, it was shown that several agonists that stimulate ROS production, such as phorbol 12-myristate 13-acetate (PMA), formyl-Met-Leu-Phe (fMLF) and opsonized zymosan induced

the phosphorylation of p47phox in human neutrophils [58, 59]. Two-dimensional gel electrophoresis analyses suggested that p47phox was phosphorylated on several sites as several forms of p47phox were detected in stimulated neutrophils [55, 56]. Phosphorylation studies showed that p47phox is a good substrate for protein kinase C (PKC), protein kinase A (PKA) and other protein kinases [60-63]. The phosphorylated sites were then localized in the AIR domain at the carboxy-terminal region of the protein between Ser303 and Ser379 [64, 65]. Most of the phosphorylation sites are localized in an arginine-rich region with the consensus phosphorylation sequence "RRXSXR", which is targeted by PKC and a proline-rich region with the consensus phosphorylation sequence "PXSP", which is a substrate for the mitogen activated protein kinases (MAPK) [64, 65].

2.3.1 Phosphorylation of p47phox is required for NOX2 activation

PMA, the direct activator of PKC, induces a strong activation of NOX2 in neutrophils, a process correlating with the phosphorylation of p47phox [60, 66]. In addition, the use of PKC selective inhibitors suggested that the PKC-dependent phosphorylation of p47phox was involved in NOX2 activation [67]. The proof that the phosphorylation of p47phox was required for NOX2 activation in intact cells was provided by site-directed mutagenesis studies. These studies clearly showed that p47phox phosphorylation is required for PMA-, fMLF- and IgG-mediated activation of the NADPH oxidase in EBV-transformed lymphocytes, B lymphocytes and COS-7 cells stably expressing gp91phox, p22phox, p47phox and p67phox (COSphox cells) [68-70]. Individual mutation of each identified serine showed that only Ser379 is essential for oxidase activation [68-70], while double mutation analysis showed that two pairs of phosphorylated serines, Ser(303+304) and Ser(359+370), are necessary for optimal NADPH oxidase activation [71, 72].

The development of a cell-free system based on the phosphorylation of p47phox by PKC, provided further evidence of a direct role for p47phox phosphorylation in the activation of the NADPH oxidase. The involvement of a cytosolic PKC in the activation of the NADPH oxidase was first shown in a cell-free system by Tauber and colleagues [73]. McPhail *et al.* showed that phosphatidic acid and diacylglycerol were able to activate the NADPH oxidase in a cell-free system in a phosphorylation-dependent manner [74]. Babior and colleagues developed a PKC-dependent cell-free system using recombinant p47phox and showed that mutation of p47phox Ser379 to Ala inhibited the activation [75, 76]. Interestingly, Babior's group subsequently showed that p47phox phosphorylated by PKC could activate the NADPH oxidase not only in a cell-free system containing neutrophil membrane and cytosol, but also in a system in which the cytosol was replaced by p67phox, Rac2 and phosphorylated p47phox recombinant proteins, suggesting that the neutrophil membrane plus those three cytosolic proteins are both necessary and sufficient for NADPH oxidase activation [77]. In addition to PKC, the protein kinase Akt (protein kinase B), which depends on phosphatidylinositol 3-kinase for activation, was shown to phosphorylate p47phox on serines Ser304 and Ser328 and activate the NADPH oxidase in a cell free system [78].

2.3.2 Phosphorylation of p47phox regulates NOX2 priming

Depending on the environment where neutrophils reside, the phagocyte NADPH oxidase can either be in a "resting/dormant" state, in a "primed/pre-activated" state, or in a fully "activated" state, [6, 79]. Resting NADPH oxidase is mainly present in circulating neutrophils, while primed NADPH oxidase is found in adherent neutrophils and in neutrophils in contact with pro-inflammatory agents such as the pro-inflammatory cytokines, tumor necrosis factor alpha (TNF α), granulocyte/macrophage-colony

stimulating factor (GM-CSF), interleukin-8 (IL-8) and the toll-like receptors (TLR) agonists, lipopolysaccharide (LPS) and CL097, the highly water-soluble derivative of the imidazoquinoline compound Resiquimod [6, 79]. These pro-inflammatory priming agents do not activate the NADPH oxidase, but they prime it for full activation in response to a second stimulus such as the bacterial peptide fMLF. Priming agents have been shown to induce partial phosphorylation of p47phox on Ser345 [80-82] and translocation of the cytochrome *b558* to the plasma membrane [83, 84], which are critical for the priming of ROS production in neutrophils. Ser345 is located within a MAPK consensus phosphorylation site, and its phosphorylation involves p38MAPK under TNF α stimulation and the extracellular-regulated kinases 1 and 2 (ERK1/2) under GM-CSF stimulation [79]. Importantly, phosphoSer-345 is a binding site for the proline isomerase Pin1 [85], an enzyme that recognizes and catalyzes the cis-trans isomerization of phospho-Ser/Thr-Pro peptide bonds [86].

Pin1 is likely to play a major role in the activation of the NADPH oxidase. Pin1 induces p47phox conformational changes and NADPH oxidase hyper-activation through its activation by the priming agents, TNF α , CL097, LPS and fMLF in intact neutrophils. Furthermore, inhibition of Pin1 activation prevents the priming process [81, 82, 85]. Activated Pin1 binds to p47phox when it is phosphorylated on Ser-345, then catalyses the conformational change of p47phox necessary for the subsequent phosphorylation of p47phox on other sites by PKC, thereby allowing the hyper-activation of the NADPH oxidase [85].

2.3.3 Phosphorylation of p47phox is regulated by p67phox and p40phox

In a recent study, our team used phospho-specific antibodies we developed against five major p47phox-

phosphorylated sites (phospho-Ser304, -Ser315, -Ser320, -Ser328 and -Ser345), and found that phosphorylation of p47phox on these serine residues was dramatically reduced in neutrophils isolated from p67phox-deficient CGD patients (p67phox^{-/-}) [87]. This finding was confirmed in Epstein-Barr virus (EBV)-transformed B- lymphocytes from p67phox^{-/-} CGD patients and in COSphox cells transfected with all the NADPH oxidase components, except for p67phox. In vitro studies showed that recombinant p47phox was phosphorylated on Ser304, Ser315, Ser320 and Ser328 by different PKC isoforms and the addition of recombinant p67phox alone or in combination with p40phox potentiated this process [87]. These new data demonstrated that p67phox and p40phox are required for optimal p47phox phosphorylation on Ser304, Ser315, Ser320 and Ser328 in intact cells. Therefore, p67phox and p40phox are novel regulators of p47phox-phosphorylation.

2.3.4 Phosphorylation of p47phox induces p47phox conformational changes and its interaction with p22phox

Cloning of the p47phox gene revealed the presence of two SH3 domains (amino acids 159-214 (SH3A) and amino acids 229-284 (SH3B)) [51, 52]. The SH3 domain is a protein sequence of about 60 amino acids, first identified in the Src protein tyrosine kinases and later in other proteins involved in signal transduction [88], which mediates protein-protein interaction by binding to PRR sequences [89]. In resting cells, 100% of the p47phox are located in the cytosol and are not phosphorylated. In this form, the p47phox is in a closed, auto-inhibited state [90, 91]. X-ray structure of the auto-inhibited form of p47phox reveals that the tandem SH3 domains share an interface that forms a shallow groove, which constitutes the peptide binding surface of the p47phox AIR C-terminal domain [92-95]. Furthermore, in the cytosol, p47phox is

associated with the p67phox-p40phox complex, where the p47phox-PRR interacts with the p67phox C-terminal SH3 domain [90, 96].

During NADPH oxidase activation in intact cells, approximately 10-20% of p47phox migrate to the cell membranes after being phosphorylated, while 80-90% of p47phox remain in the cytosol [97, 98]. Binding of p47phox to the cytochrome *b558* (gp91phox/NOX2 and p22phox) is required for activation of the NADPH oxidase complex as translocation of p47phox to the plasma membrane is impaired in neutrophils from gp91phox- or p22phox-deficient CGD patients [16, 18]. The p47phox-p22phox interaction is the best-known interaction in the NOX field. Sumimoto *et al.* (90) and Leto *et al.* (91) used recombinant p47phox-SH3 domains to show that *in vitro*, they interact with the cytosolic tail of p22phox *via* the PRR sequence (amino acids 151-160: PPSNPPPRPP). The requirement of the p47phox-SH3 domains for p47phox translocation and NADPH oxidase activation was later confirmed by deletion analysis of p47phox in whole cells (99). Native, non-phosphorylated p47phox does not interact with p22phox; however, phosphorylation of p47phox in its C-terminal region introduces negative charges that inhibit the intra-protein interaction and open the closed conformation allowing the two SH3 domains of p47phox to then interact with the p22phox-PRR (90-92). In addition to the p47phox-p22phox interaction, it was shown that p47phox interacts with some specific sequences of the gp91phox/NOX2 (amino acids (86-93), (450-457), (494-498) and (554-564)) (100, 101). However the direct interaction between the native p47phox and gp91phox has not been shown yet.

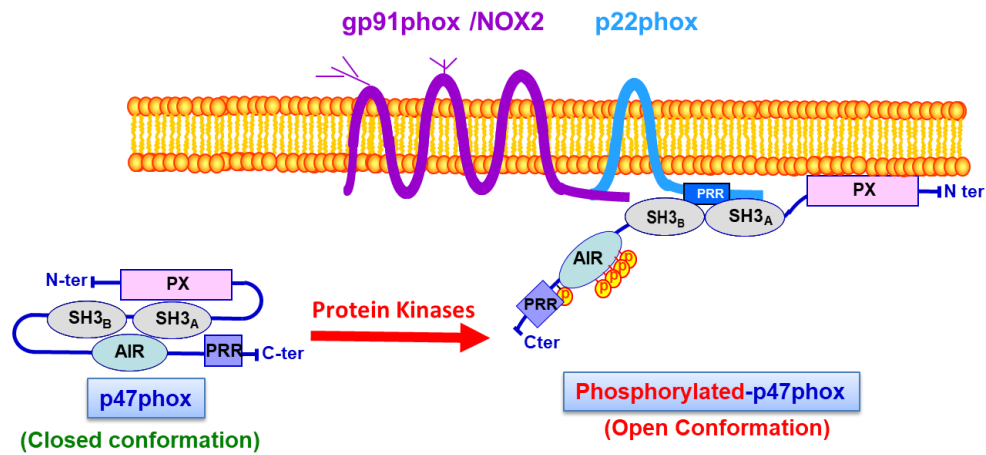


Figure 3: Closed and open conformations of p47phox. In the native non-phosphorylated state, the p47phox-SH3 tandem domains interact with the AIR domain, keeping p47phox in a closed, inactive conformation. Phosphorylation of p47phox on multiple residues in its AIR domain inhibits the two-SH3/AIR intra-molecular interaction, resulting in the p47phox-SH3 domains to be able to bind to the p22phox PRR sequence upon translocation. The p47phox-PX domain interacts with membrane phospholipids.

The p47phox PX domain (a sequence of about 125 amino acids) is known to bind to phosphatidylinositol 3,4-bisphosphate (PtdIn(3,4)-P2) and phosphatidic acid [102, 103]. Some studies had proposed that the phosphorylation of the C-terminal region of p47phox could release the PX domain, allowing its binding to (PtdIn(3,4)-P2) and phosphatidic acid [104-106]. This PX-phospholipid interaction might play a role in the translocation of the cytosolic complex to the membranes, as the proximity of p67phox and p40phox to gp91phox/NOX2 allows for the p67phox AD to promote gp91phox enzymatic activity.

Most of the studies on the p47phox conformational changes were obtained using *in vitro* systems with phosphorylated recombinant p47phox protein or in the presence of SDS or arachidonic acid [107-109]. Swain *et al.* used tryptophan fluorescence and circular dichroism spectroscopy to show that SDS and phosphorylation induced p47phox conformational changes [107]. Park and colleagues used a cysteine

labeling approach to show that PKC-mediated phosphorylation and arachidonic acid induced p47phox conformational changes [108, 109]. These data were confirmed by Marcoux *et al.* using mass spectrometry coupled to hydrogen/deuterium exchange and limited proteolysis [110]. Interestingly, Shiose and Sumimoto showed that arachidonic acid at low concentrations synergises with phosphorylation of p47phox to induce the conformational change necessary for the interaction with p22phox and the activation of the NADPH oxidase [111]. They also showed that mutation of known phosphorylated serines to alanines resulted in inhibition of PKC-dependent NADPH oxidase activation *in vitro* [111, 112].

3. NOXO1, the NOX1 organizer and regulator

3.1 History of NOXO1 discovery.

NOX1, the homologue of gp91phox/NOX2, was first cloned from the human adenocarcinoma epithelial cell line Caco-2 in 1999 [30]. In normal human tissues, its messenger was found to be mostly expressed in the gastrointestinal tract, specifically in the colon [30]. As ectopic expression of NOX1 alone resulted in minimal superoxide anion generation, it was hypothesized that NOX1, like gp91phox, might require regulatory subunits to be fully active. Banfi *et al.* demonstrated that NOX1 was able to produce superoxide anion when cells expressing NOX1 were co-transfected with p47phox and p67phox [40], indicating a functional similarity between NOX1 and NOX2. The expression of p47phox and p67phox being largely restricted to myeloid cells, it was hypothesized that homologues of p47phox and p67phox could be found in cells and tissues expressing NOX1. The cloning of human and mouse cDNAs encoding for proteins homologous to p47phox and p67phox was quickly performed by three independent teams [40-42]. These novel proteins were named NOX Organizer 1 (NOXO1) and NOX Activator 1 (NOXA1) and are the

homologues of p47phox and p67phox, respectively. The mRNAs of NOXO1 and NOXA1 were predominantly found in the colon and more precisely in the epithelium [40, 41]. It was then found that co-transfection of NOXO1, NOXA1 and NOX1 in human embryonic kidney 293 (HEK293), COS-7, Chinese hamster ovary (CHO) or HeLa cells resulted in low constitutive activity of the NOX1 complex and that the absence of one of the proteins was sufficient to prevent its activity [40, 113]. Nevertheless, studies with human proteins showed that the constitutive production of superoxide by the NOX1 complex could be increased by pharmacological agents such as PMA [41, 42]. NOXO1 can also operate in a complex with NOX3 in transfected cells. Notably, Cheng *et al.* showed that NOX3 could be strongly activated by NOXO1 in the absence of NOXA1 [114]. However, the activation of NOX3 induced by NOXO1 is less efficient than that of NOX1, and NOXO1 remains the primary partner of NOX1 [115, 116].

3.2 Structure of NOXO1

Human NOXO1, which is encoded by a gene located on chromosome 16 (16p13.3) and composed of eight exons, is a protein of 370 amino acids that migrates at almost 41 kDa by SDS-polyacrylamide gel electrophoresis [116]. Although the overall amino acids sequence of human NOXO1 has only 27% identity with p47phox, the domain arrangements are well conserved. As for p47phox, NOXO1 N-terminal amino acid sequence has a PX domain (amino acids 2-131) which is involved in the binding of membrane phosphoinositides [117]. However, while the PX domain of p47phox binds to PtdIns (3,4)-P₂, which is only produced upon cell activation, studies using phosphatidylinositol arrays showed that the PX domain of NOXO1 binds mainly monophosphorylated phosphatidylinositols such as PtdIns 4-P and PtdIns 5-P, which are present in plasma membranes of non-activated cells [113]. However, further studies using surface plasmon resonance identified PtdIns (4,5)-P₂ and PtdIns (3,4,5)-P₃ as targets of NOXO1-PX [118].

Arginine 40 (R40) is important for binding to membrane phosphoinositides as its mutation to glutamine is sufficient to interfere with lipid binding [113]. However, R40 is largely buried in NOXO1 as shown by nuclear magnetic resonance spectroscopy, and it has been suggested that the R40Q mutation is more likely to disrupt protein/lipid binding by destabilizing the PX domain rather than by abrogating the specific arginine/phosphatidylinositol interaction [118].

The center of NOXO1 contains two SH3 domains (amino acids 157-218 (SH3A) and 228-291 (SH3B), which are involved in the binding to p22phox [42]. Contrary to p47phox, the C-terminal sequence of NOXO1 does not contain an AIR domain, but it has a PRR sequence (amino acids 320-332: PPPTVPTRPSP) [41, 42] (Figure 3), which could be involved in the interaction with NOXA1 [42]. Four structural variants of human NOXO1 resulting from alternative splicing of both ends of exon 3 encoding the PX domain have been described, e.g., α , β , δ and γ [41, 116, 119, 120]. Lysine 50 is deleted in the α and δ forms, and five additional amino acids are found in the N-terminal PX domain of the δ form. The β and γ forms have no deletion of lysine 50, and γ has also a 5 amino acid insertion in the N-terminal PX domain [116]. The mRNAs encoding NOXO1 α and NOXO1 δ are found in low abundance in tissues and cells, and when expressed in *E. Coli*, the PX domains of the respective proteins show low expression, suggesting poor translation or unstability [116]. It is therefore unlikely that these isoforms have a significant biological role. In contrast, the mRNAs encoding NOXO1 β and NOXO1 γ are found in high abundance in the carcinoma T84 colon epithelial cell line and in testis, respectively, and their PX domains show stable expression in *E. Coli*. When expressed in HEK293 cells, these variants show differential subcellular localization as NOXO1 α and NOXO1 δ are found in intracellular vesicles or cytoplasmic aggregates, while NOXO1 β is prominent in the plasma membrane, and NOXO1 γ is detected in both the plasma membrane and the nucleus [120]. The NOXO1 α and NOXO1 δ isoforms support low NOX1

activity, whereas NOXO1 β and NOXO1 γ support high NOX1 activation, especially upon stimulation with PMA. Without PMA, NOXO1 γ ability to support NOX1 activity is weaker than NOXO1 β . Interestingly, purified PX domains of NOXO1 β and NOXO1 γ bind with the same affinity to membrane phosphoinositides [116, 119, 120].

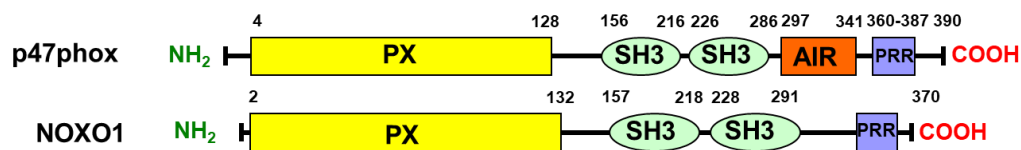


Figure 4: Comparison of the structures of human p47phox and human NOXO1. The human NOXO1 protein has 370 amino acids, organized into multiple domains. Like p47phox, it has a phox domain (PX) (amino acids 2-132), two Src homology 3 (SH3) domains [SH3A (amino acids 157-218) and SH3B (amino acids 228-291)] and a proline rich region (PRR) (amino acids 320-329), but no AIR domain.

3.3 Phosphorylation of NOXO1

Because NOXO1 is lacking the AIR domain containing the phosphorylation sites for p47phox, it was suggested that NOXO1, unlike p47phox, did not exist in an autoinhibited conformation in resting cells, thereby allowing its constitutive association with p22phox and membrane phosphoinositides [42, 113]. This structural feature could explain the constitutive localization of NOXO1 to the membrane as well as the constitutive activity of the NOX1 complex in transfected cells [41, 42, 113]. Therefore, it was commonly accepted that NOX1 activation was not regulated by the phosphorylation of NOXO1. However, in later studies, it was demonstrated by pull-down and isothermal titration calorimetry approaches with different constructs of NOXO1 that an intramolecular interaction involving the SH3 tandem and the PRR domain existed within NOXO1, despite the absence of the AIR domain, and that disruption of this interaction facilitated NOXO1 binding to p22phox [121,122]. Interestingly the binding

between NOXO1 and NOXA1 was found to be very weak as compared to that of their phagocytic counterparts, p47phox and p67phox (100-fold lower affinities), probably due to the fact that the PRR region of NOXO1 is engaged in the intramolecular interaction and is not available to NOXA1 [122]. The existence of an intramolecular interaction within NOXO1 that could prevent p22phox binding and the observation that PMA could increase NOX1 activity, strongly suggested that post-translational modifications could regulate NOX1 activation. Debbabi *et al.* demonstrated that PMA increased NOXO1 phosphorylation in ³²P labeled transfected HEK-293 epithelial cells *via* PKC and identified Ser-154 by phosphopeptide mapping as the major phosphorylated site [123]. Furthermore, PMA-induced phosphorylation on Ser-154 was shown to enhance NOXO1 binding to NOXA1 and to p22phox, allowing optimal ROS production by NOX1 in intact cells [123]. Ser-154 of NOXO1 has also been shown to be targeted by PKA *in vitro*, although this PKA-induced phosphorylation has not been demonstrated in intact cells [124]. Moreover, it has been shown that PKC could also phosphorylate NOXO1 on Thr-341 *in vitro* and in CHO-transfected cells [124]. Substitution of this threonine with alanine decreased PMA-dependent phosphorylation of NOXO1 in these cells, as assessed using the phosphoprotein dye Phos-Tag, and reduced PMA-induced activity [124]. Phosphorylation of NOXO1 on Thr341 seems to be required for its interaction with NOXA1 as shown by *in vitro* experiments using truncated NOXO1-(154-371) and purified NOXA1-SH3 domain [124].

3.4 NOXO1 expression

Cellular and tissular expression of NOXO1 has been mostly documented at the mRNA level. Except for its predominant expression in the colon, NOXO1 mRNA has also been detected in the kidney, liver, pancreas, uterus, testis, inner ear, and the vasculature [40, 42, 115, 125, 126]. Increased expression of

NOXO1 mRNA was described in experimental gastric tumor development and in human colon cancers as compared with adjacent normal bowel mucosa [127, 128]. NOXO1 protein expression most often requires a triggering signal such as inflammatory mediators or growth factors. Therefore, pro-inflammatory cytokines including interferon gamma (INF γ), interleukin (IL)-1 β , TNF α and IL-17 [129-131], and pathogens such as *Escherichia coli* LF82 can all increase NOXO1 protein expression along with ROS production in intestinal epithelial cells [132]. In addition, the prototype anti-inflammatory cytokine, IL-10, prevented increased expression of the NOXO1 protein induced by TNF α and INF γ in the colonic epithelial cell line [133]. Interestingly, increased protein expression of NOXO1 has been observed in colon biopsies of patients with Crohn disease in the inflamed and non-inflamed areas, as compared with healthy controls [131] and in TNF α -induced colitis in mice [134]. Proteasomal degradation of NOXO1 could also regulate the levels of the NOXO1 protein. The ubiquitination, i.e., the ligation of ubiquitin molecules, to proteins plays an essential role in their specific degradation by the proteasome. This process is catalyzed by three main groups of enzymes, namely the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase). Ubiquitin is activated by E1 then transferred to the carrier E2 enzyme, which in turn conjugates ubiquitin to substrate proteins with the help of a specific E3 ligase [135]. In this regard, it has been shown in HCT116 colon cancer cells that NOXO1 interacted with Grb2, which in turn recruited the Casitas B-lineage lymphoma (Cbl)-E3 ligase, leading to ubiquitination and degradation of NOXO1. Epidermal growth factor (EGF)-mediated phosphorylation of NOXO1 on Ser-154, induced its dissociation from Grb2/Cbl, thereby inhibiting the rapid degradation of NOXO1 [136]. Consequently, NOXO1 association with NOXA1 facilitated the stimulation of ROS generation. Interestingly, the expression and stability of NOXO1 were shown to significantly increase in human colon cancer tissues compared with normal colon [136]. More recently, Cyndromatosis tumor

suppressor protein (CYLD), a deubiquitinase, best known as an essential negative regulator of the NF κ B pathway, was also identified as a binding partner of NOXO1 and was suggested to act as a potential tumor suppressor by decreasing the stability of the NOXO1 protein and suppressing excessive ROS generation [137].

4. Conclusions

Superoxide production by the phagocyte NADPH oxidase (NOX2) and the epithelial NOX1 is essential for many physiological functions, including host defense. However, excessive superoxide production by these enzymes can induce tissue injury resulting in inflammatory diseases. It is clear today that the phosphorylation of p47phox and the phosphorylation of NOXO1 are crucial for NOX2 and NOX1 regulation; however, the upstream pathways involved in their phosphorylation are still not fully identified. Furthermore, little is known about the dephosphorylation of phospho-p47phox and phospho-NOXO1 by phosphatases, which could be involved in limiting NOX1 and NOX2 activation in cells and in the resolution of inflammation. Future studies are required to determine these pathways in neutrophils, monocytes/macrophages and epithelial cells. Identification of the pathways involved in p47phox and NOXO1 phosphorylation will help to develop new strategies to limit ROS production by phagocytes in inflammatory diseases.

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Conflict of interest statement

Authors declare that they don't have any conflict of interest.

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