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Regulation of Nox and Duox Enzymatic Activity and Expression

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Summary

In recent years, it has become clear that reactive oxygen species (ROS, which include superoxide, hydrogen peroxide and other metabolites) are produced in biological systems. Rather than being simply a byproduct of aerobic metabolism, it is now recognized that specific enzymes --- the Nox (NADPH-oxidase) and Duox (Dual oxidase) enzymes ---- seem to have the sole function of generating ROS in a carefully regulated manner, and key roles in signal transduction, immune function, hormone biosynthesis and other normal biological functions are being uncovered. The prototypical Nox is the respiratory burst oxidase or phagocyte oxidase, which generates large amounts of superoxide and other reactive species in the phagosomes of neutrophils and macrophages, playing a central role in innate immunity by killing microbes. This enzyme system has been extensively studied over the past two decades, and provides a basis for comparison with the more recently described Nox and Duox enzymes, which generate ROS in a variety of cells and tissues. This review first considers the structure and regulation of the respiratory burst oxidase, and then reviews recent studies relating to the regulation of the activity of the novel Nox/Duox enzymes. The regulation of Nox and Duox expression in tissues and by specific stimuli is also considered here. An accompanying review considers biological and pathological roles of the Nox family of enzymes.

The Respiratory Burst Oxidase of Phagocytes

a. The respiratory burst

The “respiratory burst” refers to the early observation that when professional phagocytes such as neutrophils and macrophages are exposed to microbes, they consume large amounts of oxygen. Unexpectedly, this oxygen consumption was not inhibited by cyanide, an inhibitor of mitochondrial electron transport. This observation led to a more than 25 year search for the enzymatic origin of the respiratory burst and to the eventual discovery and molecular characterization of the phagocytic NADPH-oxidase or “respiratory burst oxidase”. The phagocyte oxidase generates superoxide via the one electron-reduction of oxygen by NADPH, with secondary production of hydrogen peroxide, HOCl and other activated forms of oxygen. Together, these reactive oxygen species (ROS) participate in host defense by killing invading microbes.

b. gp91^{phox}, the catalytic moiety of the respiratory burst oxidase

The phagocytic NADPH-oxidase consists of a membrane-localized glycosylated, catalytic subunit, gp91^{phox} (which has also come to be known as Nox2, a terminology that will be used in this review), along with a second membrane-associated subunit, p22^{phox}, both depicted in Fig. 1. Nox2 and p22^{phox} stabilize one another in a tightly associated heterodimer which is referred to as flavocytochrome b_{558}. The C-terminal half of Nox2 forms a domain that is

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homologous to known flavoprotein dehydrogenases, and contains a bound FAD and an NADPH binding site. The N-terminal half of Nox2 consists of six predicted transmembrane α-helices containing four histidine residues that are absolutely conserved among all known Nox enzymes (T. Kawahara and D. Lambeth, unpublished). These histidine residues, located in a-helices 3 and 5, provide the axial and distal ligands for binding to the irons of two nonidentical [1] hemes (red in Fig. 1), as demonstrated by mutational analysis [2]. Molecular models [*e.g.*, [3]] of the locations of the conserved histidine residues in the transmembrane helices indicate that the two hemes are located approximately in the inner and outer leaflets of the membrane bilayer, with the hemes oriented perpendicular to the surface of the membrane, providing a conduit for electrons to pass from the cytosolic NADPH, through FAD and across the membrane via the hemes to reduce molecular oxygen to form superoxide. The reaction with oxygen is thought to occur from the edge of the second heme in an outer sphere reaction rather than directly from the heme iron [4], accounting for lack of inhibition by cyanide and carbon monoxide.

The interaction of the membrane components with cytosolic regulatory subunits is important for the activation of electron flow. $p22^{phox}$ plays a central role in this process, via interaction of its proline-rich domain (PRD in Fig. 1) with $p47^{phox}$, as described in detail below. An additional region of importance is the B-loop on Nox2 (Fig. 1), a polybasic sequence that also participates in the interaction with $p47^{phox}$ [5].

c. Regulatory subunits for the Nox2 system

Early attempts to reconstitute the NADPH- oxidase activity demonstrated that proteins in the cytosolic as well as the membrane were required for activity [6–8]. Subsequently, $p47^{phox}$, $p67^{phox}$, and the small GTPase Rac1 [9] or Rac2 [10] were identified as essential regulatory subunits. $p47^{phox}$ and $p67^{phox}$ were demonstrated to be important for NADPH oxidase activity, based on impairment of ROS generation in forms of chronic granulomatous disease in which these components were missing or mutated [11–14]. Using knockout mice, it was found that Rac2 was needed for optimal activation of the respiratory burst in neutrophils [15,16], but Rac1 also functions *in vitro* [17] and in other cell types such as macrophages [9]. $p40^{phox}$ was later discovered as an additional member of the complex that translocates from cytosol to membrane to activate Nox2 [18]. In resting cells, these subunits reside in at least two cytosolic complexes: one containing $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ [18,19] and a second containing Rac and its inhibitory protein RhoGDI (GDP Dissociation Inhibitor for Rho) [9,20,21]. For the phagocyte system, all except RhoGDI assemble in a membrane complex with the flavocytochrome following cell activation, resulting in activation of electron transfer reactions within the complex. Neither $p47^{phox}$ nor $p40^{phox}$ are absolutely required for *in vitro* activity [22,23]. However, $p47^{phox}$ markedly enhances the binding of the other regulatory subunits [22,24] and it is an essential protein in intact cells [13]. In activated neutrophils, $p47^{phox}$ is phosphorylated at 7–8 sites in its C terminus [25–28] releasing autoinhibitory internal interactions and allowing its binding to $p22^{phox}$ [29–31]. By simultaneously binding to $p67^{phox}$, $p47^{phox}$ helps to organize the regulatory subunits in the active complex, leading to the concept that $p47^{phox}$ functions as an “adapter” or “organizer protein” [22]. In addition, $p40^{phox}$ also functions to increase the interaction of other regulatory subunits [32,33], although its *in vivo* role does not appear to be as critical as that of $p47^{phox}$ since no mutations in $p40^{phox}$ have been detected in chronic granulomatous disease. Cell activation causes Rac-GDP to separate from RhoGDI, as a result of the phosphorylation of GDI and through the action of lipid mediators [34,35], and this is accompanied by exposure to guanine nucleotide exchange factors which catalyze the formation of Rac-GTP. Rac-GTP translocates to the plasma membrane independently of the ^{phox} components [20,36–38], where it interacts with $p67^{phox}$ and wth flavocytochrome b_{558}. Thus, activation involves an assembly of regulatory subunits with the catalytic subunit, resulting in the activation electron flow through the prosthetic groups of the catalytic subunit Nox2.

Because both hemes as well as FAD remain mostly in the oxidized form during catalytic turnover of the activated flavocytochrome [39–41], the rate-determining step in the active enzyme is the reduction of FAD by NADPH [41]. An "activation domain" was identified in $p67^{phox}$ (Fig. 2) that regulates the hydride transfer step from NADPH → FAD to form $NADP^+$ plus $FADH_2$ without affecting NADPH binding [41]; this sequence is highly conserved among $p67^{phox}$ homologs in multiple species, pointing to its general importance (T. Kawahara and D. Lambeth, unpublished studies). In addition, Rac, which is modified at its C-terminus with the hydrophobic geranyl-geranyl membrane anchoring moiety [42,43], binds in its active GTP-associated form simultaneously to membrane and to $p67^{phox}$ [44–46], tethering $p67^{phox}$ to the membrane and probably simultaneously to the cytochrome [47]. To date, it has not been possible to replace the function of Rac by using other methods to tether $p67^{phox}$ to the membrane, leading to the proposal that Rac has a specific role in Nox2 catalysis. Two theories that need not be mutually exclusive have been advanced for a specific role for Rac. First, Rac binding induces a conformational change in $p67^{phox}$ [48] which may permit the activation domain of $p67^{phox}$ to act on Nox2. Second, studies using a diaphorase electron acceptor to intercept electrons from the FAD support the view that Rac plays a direct role in stimulating electron transfers that is independent of $p67^{phox}$ [49].

d. Domain structures of the ^{phox} regulatory subunits

Binding and structural investigations have been used in combination with mutational approaches to understand the domain structure of the regulatory subunits. These interactions have been summarized in detail recent excellent reviews [50–52] and are considered in less detail briefly here.

i. $p47^{phox}$—Phosphorylation of $p47^{phox}$ serves as one of several triggers for the assembly of an active oxidase. As shown in Fig. 2, this protein contains a polybasic C-terminal autoinhibitory region (AIR) that contains multiple serine residues that are the targets for phosphorylation [30,53]. In the resting cell, $p47^{phox}$ is dephosphorylated and is maintained in an inactive conformation by an intramolecular interaction between the AIR and the centrally-located adjacent SH3 domains [31] (referred to as the *bis*-SH3 domain, Fig. 2), which function together to form a single binding site for its target proline-rich regions (PRR) in the AIR. Phosphorylation of $p47^{phox}$ releases this binding, freeing the *bis*-SH3 domain to bind to the PRR of $p22^{phox}$, thereby triggering the initial phase of oxidase assembly. Although less well understood, another, autoinhibitory intramolecular interaction occurs in the non-phosphorylated $p47^{phox}$ that prevents the N-terminally located PX domain [54] from binding to membrane lipids [55]. Phosphorylation of $p47^{phox}$ releases this inhibition, allowing the PX domain to interact with membrane via a site that binds to the 3-phosphorylated phosphatidylinositol PtdIns $(3,4)P_2$ [56,57] and a second site that binds to phosphatidic acid [58]. Phosphatidyl inositol 3,4,5-trisphosphate (PIP_3) is generated in activated phagocytes through the action of PI 3-kinase, serving as a second type of "trigger" for oxidase assembly. The C-terminus of $p47^{phox}$ also utilizes a PRR to bind to the C-terminal SH3 domain of $p67^{phox}$ in a "tail-to-tail" interaction (Fig. 2) [59,60], "taking it along for the ride" when it binds to $p22^{phox}$ and membrane lipids. While the other regions of $p47^{phox}$ are highly conserved, this tail-to-tail mode of binding $p47^{phox}$ to $p67^{phox}$ has not been stringently conserved during evolution, with various isologs of $p47^{phox}$ and $p67^{phox}$ utilizing a variety of different modular interactions to accomplish the same interaction (T. Kawahara and D. Lambeth, unpublished).

ii. $p67^{phox}$—In addition to the activation domain discussed above, $p67^{phox}$ contains four tetratricopeptide repeats (TPR) that together form a binding site for Rac-GTP (Fig. 2) [61, 62]. $p67^{phox}$ also contains a C-terminal SH3 domain for binding to the PRR of $p47^{phox}$ (see above), a second centrally located SH3 domain of unknown function, and a PB1 domain located between the SH3 domains. A PB1 domain is also found in $p40^{phox}$. This domain, was originally

described in p47^{phox}, Bem1p proteins and various eukaryotic cytoplasmic signaling proteins, and is thought to mediate protein-protein interactions with other proteins containing a PB1 counterpart; reviewed in [63]. The PB1 domain of p67^{phox} forms a complex with the PB1 domain of p40^{phox}, thereby mediating the binding of p40^{phox} to the ^{phox} regulatory protein complex [64].

iii. p40^{phox}—p40^{phox} was identified as a co-purifying component present in a 250 kDa cytosolic complex that also contained p67^{phox} and p47^{phox} [18]. In addition to its PB1 domain mentioned above, p40^{phox} contains a PX domain and an SH3 domain [54,65] (Fig. 2). The SH3 domain of p40^{phox} can compete with the C-terminal SH3 domain of p67^{phox} for binding to the proline rich region of p47^{phox} [66,67], which suggests some sort of rearrangement of binding partners must occur upon assembly and activation. When cells are activated, p40^{phox} becomes phosphorylated [68] and translocates along with the p47^{phox}-p67^{phox} complex to the membrane [32,33]. The PX domain of p40^{phox} binds to phosphatidylinositol-3 phosphate and other 3-phosphorylated lipids in the membrane [56,58,69], helping to anchor the other ^{phox} regulatory components to the membrane in cells in which PI 3-kinase has been activated [32].

Identification and Domain Structure of Novel NADPH oxidases

a. Reactive oxygen in non-phagocytic cells

During the 1990s, probably as a result of more sensitive methods for detection of H_2O_2 and O_2^-, several groups reported the occurrence of ROS in a wide variety of cell types other than professional phagocytes [70–76]. Although frequently attributed to mitochondrial respiration, in many cases ROS generation was inhibited by diphenylene iodonium (DPI), an inhibitor of the phagocyte NADPH oxidase and some other flavoprotein dehydrogenases [77]. However, although ^{phox} regulatory subunits were sometimes detected in these tissues, gp91^{phox} (Nox2) was usually absent. Based on the assumption that that ROS originated from a homolog of gp91^{phox}, Lambeth and colleagues [78] identified a homolog of gp91^{phox}, now called Nox1 (originally Mox1), and this group and others subsequently and independently identified the additional homologs Nox3, Nox4, Nox5, Duox1 and Duox2 in humans and other species [79–85]. A large number of isologs have now been catalogued in species ranging from plants, fungi, amoeba, nematodes, insects, and vertebrates. Based on sequence homology of the catalytic regions (*i.e.*, excluding non-catalytic domains, see below), these can be classified into 7 subfamilies (T. Kawahara and D. Lambeth, unpublished), and the regulated generation of ROS by these enzymes performs a range of biological functions in addition to innate immunity, considered in the accompanying article. Interestingly, no Nox isologs have been identified in bacteria, indicating that defense against ROS evolved earlier than enzymes that deliberately generate ROS.

b. Domain structure of the mammalian Nox enzymes

The mammalian Nox enzymes can classified into three groups, based on the presence of domains in addition to the Nox flavocytochrome domain (i.e., the transmembrane region containing 2 hemes plus the flavoprotein domain). Nox1, Nox2, Nox3, and Nox4 all contain the flavocytochrome catalytic moiety depicted in Fig. 1 and are ~65–66 kDa in size. Nox enzymes 1–4 all require p22^{phox} for stabilization and/or function [51,86–88]. In addition to Nox enzymes 1–4, a short form of Nox5 encoded by a short mRNA splice form conforms to the same domain structure [82].

A long form of Nox5 is the single member of the second group which has, in addition to the basic Nox catalytic moiety, an amino-terminal calmodulin-like domain that contains four calcium binding EF-hand structures [83]. While present in most mammals, a Nox5 isolog is absent in rodents where its function may have been taken over by another Nox or Duox.

A third group of Nox's are the "dual oxidases" or Duox, so-named because they contain not only an NADPH-oxidase domain but also a domain that is homologous to heme-containing peroxidases such as myeloperoxidase and lactoperoxidase [79,81,89]. These enzymes are comprised of the basic Nox5-like structure fused at the N-terminus with an additional transmembrane α-helix that is linked at its N-terminus to the peroxidase-like domain. Topologically, the arrangement of transmembrane sequences places the peroxidase domain on the opposite side of the membrane from the FAD domain, immediately adjacent to the site of ROS generation at the second heme group. The Duox peroxidase domain has been proposed [90] to utilize H_2O_2 generated by its Nox catalytic moiety (presumably formed via the dismutation of 2 superoxide molecules, although only H_2O_2 has thus far been detected [89, 91,92]). The Duox peroxidase domain of *C. elegans* and insects contains a histidine at the homologous position as the axial histidine in myeloperoxidase, and is therefore assumed to function in these species as a typical peroxidase. Indeed, the expressed peroxidase domain of the nematode Duox peroxidase domain shows peroxidase type activities including tyrosine cross-linking [90]. While the expressed human Duox1 peroxidase domain also catalyzes peroxidase reactions [90] and [Inoue and Lambeth unpublished], the activity and heme binding of this subunit is more controversial because of the absence of a histidine at the position homologous to the axial heme iron-binding histidine of myeloperoxidase. Supporting the view of mammalian Duox enzymes as peroxidases, it was recently reported that treatments that increased or decreased Duox2 levels in airway epithelial cells resulted in parallel increases and decreases in peroxidase activity [93]. Countering this view is the suggestion that the peroxidase-like domain could serve as a heterodimerization site for another peroxidase (M. Geiszt, personal communication). A catalytic or other role for the peroxidase domain of the human Duox enzymes remains an area for further investigation.

c. Homologs of ^{phox} regulatory subunits

Several groups [94–97] reported homologs of $p47^{phox}$ and $p67^{phox}$, whose structures are summarized in Fig. 3. Like $p47^{phox}$, NOXO1 (referring to Nox organizer protein 1) contains an N-terminal PX domain, a central *bis*-SH3 domain and a proline-rich region at its C-terminus. The PX domain of NOXO1, like that of $p47^{phox}$, binds lipids, although its specificity favors the 4- and 5- monophosphorylated forms of phosphatidylinositol, species that are present in membranes without cell activation. This domain mediates the localization of NOXO1 to the plasma membrane [97]. The *bis*-SH3 domain binds like that of $p47^{phox}$ to the PRR of $p22^{phox}$ [95], although with some subtle differences in binding specificity [87]. NOXO1, however, lacks the polybasic autoinhibitory region and regulatory phosphorylation sites that are present in $p47^{phox}$, and this is thought to explain the association with membrane in the absence of cell activation [97].

NOXA1 (referring to Nox Activator Protein 1) has a domain structure that is similar in most respects to $p67^{phox}$. The N-terminal TPR repeat in NOXA1, like that in $p67^{phox}$, functions to bind to Rac [95,98]. In addition, there is a conserved activation domain that is critical for Nox1 activation [99], a C-terminal SH3 domain that binds to NOXO1 [95], and a PB1 domain of unknown function. NOXA1 differs from $p67^{phox}$ primarily in that it lacks the first SH3 domain that is present in the *phox* protein. The significance of this structural difference is not known, but might relate to association of the *phox* complex with other cellular proteins.

To date, no homologs of $p40^{phox}$ or of $p22^{phox}$ have been reported. However, it should be noted that the presence of a PB1 domain in NOXA1 suggests the existence of an unknown dimerization partner. The PB1-like domain in NOXA1 lacks a conserved lysine residue that is present in $p67^{phox}$, and does not bind to $p40^{phox}$ [95].

Regulation of the Activity of Non-phagocytic Nox/Duox Enzymes

A summary of the known modes of regulation of Nox and Duox enzymes is shown in Table 1, and is detailed below.

a. Regulation of Nox 1

Nox1 forms a complex with $p22^{phox}$ [86], and its activity requires $p22^{phox}$, since depletion of $p22^{phox}$ using RNA interference inhibits Nox1 activity [87]. NOXO1 and NOXA1 are co-expressed in colon epithelium along with Nox1 [94], and co-transfection studies demonstrated that these subunits can indeed activate Nox1 in a manner that is dependent on the activation domain of NOXA1 [98]. Activation does not absolutely require phorbol esters, although phorbol esters typically increase activity by up to 2-fold in many cell systems [97,98]. The considerable constitutive activation of Nox1 in the absence of cell stimulation is explained in part by the absence of regulatory phosphorylation sites on NOXO1 and the ability of NOXO1 to localize to the resting cell membrane via its PX domain [97]. NOXO1 must also bind to the PRR of $p22^{phox}$, because mutation of the PRR prevents NOXO1-dependent activation of Nox1 [87]. Like NOXO1, NOXA1 is also constitutively located in the plasma membrane, in a manner dependent on membrane localization of either NOXO1, or, when NOXO1 is absent, the activated form of Rac1 [100]. As implied by the Rac-binding TPR repeat domain of NOXA1, Nox1 is regulated by Rac1-GTP [98,100,101]. Unlike the Nox2-$p47^{phox}$-$p67^{phox}$-Rac system that requires protein phosphorylation, lipid phosphorylation and guanine nucleotide exchange on Rac, the Nox1 system seems to have relatively few regulatory “triggers”. Currently only guanine nucleotide exchange on Rac1 is convincingly documented as a regulatory trigger [98], although as mentioned above, phorbol esters exert a modest stimulatory effect, which points to phosphorylation of an unknown component in modulating activity. While Nox2 is completely dependent upon active Rac, Nox1 activation by Rac1 is less stringent and is obvious only when cellular levels of NOXO1 and NOXA1 are low [98]. Immunoprecipitation studies have demonstrated an active complex containing Nox1, NOXO1, NOXA1 and Rac1, and direct associations of Nox1-$p22^{phox}$ have been shown with each of the individual regulatory subunits (i.e., these interactions are independent of the presence of the other subunits) [98].

In addition to NOXO1 and NOXA1, Nox1 shows a modest capacity to be activated when NOXO1 is expressed in cells along with $p67^{phox}$ or when $p47^{phox}$ is combined with NOXA1 [99]. In a similar manner, heterologous combinations of regulatory subunits are capable of activating Nox2 [99]. While heterologously regulated Nox1 and Nox2 show lower activity than homologously regulated Nox enzymes, these observations raise the possibility of some flexibility for Nox1 and Nox2 regulation. While the *in vivo* significance is unclear, this may explain studies in $p47^{phox\ (-/-)}$ mice that demonstrate $p47^{phox}$-dependent regulation of ROS production in some tissues where Nox2 does not appear to be expressed, e.g., see [102,103].

b. Regulation of Nox3

Nox3-dependent ROS production requires $p22^{phox}$, based on co-transfection and RNA interference studies [87]. Nox3 forms a complex with $p22^{phox}$, based in co-immunoprecipitation [87,104]. While Nox3 may produce a small amount of ROS without co-expressed regulatory subunits [104,105], subunits markedly increase activity. Subunit regulation differs depending on species: human NOXO1 dramatically stimulates Nox3 activity in absence of NOXA1 or $p67^{phox}$ [99]. However, mouse Nox3 activity requires both NOXO1 and NOXA1 [105]. The human Nox3 can also be regulated in transfected cell models by the combination of $p47^{phox}$ and $p67^{phox}$ [99]. In the major site of Nox3 expression, the inner ear, NOXO1 is the physiologically relevant regulator of Nox3, based on the impairment of Nox3-dependent otoconia formation in a mouse harboring an inactivating mutation in NOXO1 [106]. A possible role for Rac1 in regulation of Nox3 has been investigated by several groups.

Neither the dominant negative nor the constitutively active forms of Rac1 affected Nox3-dependent activity in transfected CHO and HeLa cell models [101,104]. On the other hand, a constitutively active form of Rac1 stimulated the activity of Nox3 alone or when NOXA1 was co-expressed with Nox3 [100]. Thus, it appears that Nox3 has a quantitatively less signficant requirement for Rac compared with Nox1 or Nox2.

c. Regulation of Nox4

Amino acid sequence comparisons show that Nox1 and Nox3 are both closely related to Nox2, while Nox4 is more distantly related [79]. Nox4 is unique among the human Nox and Duox enzymes in that it shows high constitutive activity when expressed in cell models in the absence of activators such as PMA [80,87,88] or calcium (T. Kawahara and D. Lambeth, unpublished). In addition, Nox4 activity was not affected by co-expression any of the known regulatory subunits including $p47^{phox}$, $p67^{phox}$, NOXO1, NOXA1 and Rac [87,88]. Nox4 does, however, require $p22^{phox}$ for activity, since depletion of $p22^{phox}$ inhibits Nox4 activity without affecting Nox4 protein level [87]. Unlike Noxes1–3, Nox4 activity is independent of the PRR of $p22^{phox}$ [87], consistent with the lack of a requirement for $p47^{phox}$ or NOXO1. Based on co-immunoprecipitation, Nox4 forms a complex with $p22^{phox}$ [86,88], and Nox4 co-localizes with $p22^{phox}$ in human kidney tissue [87]. While these observations indicate that the Nox4-$p22^{phox}$ complex can produce ROS without the aid of any known regulatory subunits, other studies indicate that Nox4 activity can be acutely regulated in some cells by unknown mechanisms. For example, insulin activates Nox4-dependent ROS generation within 5 min [107], a response that is too rapid to be accounted for by increased Nox4 protein expression. Lipopolysaccharide also activates Nox4 via a TLR receptor signaling cascade within 30 min [108]. Thus, it seems likely that unknown mechanisms acutely regulate Nox4 activity, and that these mechanisms do not funtion in transfected cell systems wherein overexpressed Nox4 is constitutively active. This remains an important unresolved area for future exploration.

d. Regulation of Nox5

In addition to the basic Nox catalytic moiety of Nox1–4, Nox5 encodes the N-terminal domain containing four calcium-binding EF-hand motifs. Calcium-dependent ROS production by Nox5 has been shown in Nox5-transfected cell lines [83]. Binding of calcium changes the conformation of the EF-hand domain and allows it to bind to the catalytic domain, which is proposed to activate electron transfer from NADPH to oxygen to form superoxide [109]. Nox5, at least the full-length form, does not form a functional complex with $p22^{phox}$, because depletion of $p22^{phox}$ does not affect the Nox5 activity [87] and because no complex between the two proteins can be detected by co-immunoprecipitation (T. Kawahara and D. Lambeth, unpublished). One puzzling aspect of the calcium activation was that higher than biologically realistic concentrations of calcium were required to activate the enzyme. However, a recent study showed that PMA triggers phosphorylation of T494 and S498 of Nox5, and that this increases the sensitivity of the enzyme for calcium, allowing activation at levels of calcium that are seen in the cytosol following agonist stimulation of cells [110]. Thus, the regulation of Nox5 seems to be more complex than originally appreciated and involves synergy between calcium-dependent and phosphorylation-dependent pathways.

e. Regulation of Duox enzymes

Human Duox enzymes, like Nox5, contain a calcium-binding domain, although only two, rather than four canonical calcium-binding EF-hands are present. The presence of the calcium-binding domain probably explains the observation that calcium ion triggers ROS production in thyroid cells and human bronchial epithelial cells, both of which express abundant Duox proteins [92]. Duox2-transfected HEK293 cells also produce ROS in response to calcium ion using a cell membrane preparation [111]. Characterization of the function and regulation of

Duox proteins has been hindered because the ectopically expressed Duox protein failed to become properly glycosylated and was retained in the endoplasmatic reticulum. Recently, proteins that permit the proper post-translational processing and trafficking of Duox1 and Duox2 (DuoxA1 and DuoxA2, respectively) were identified [112]. These maturation proteins allow the Duox enzymes to be expressed in their appropriate location at the plasma membrane. Interestingly, the chromosomal location of each of the DuoxA genes is adjacent to the respective Duox, and operon-like co-regulation of the enzyme and its maturation factor has been proposed. Human Duox2 protein reportedly co-immunoprecipitates with $p22^{phox}$ [111], but there is little or no effect of $p22^{phox}$ on Duox enzymatic activity. Since $p22^{phox}$ is needed for the proper glycosylation and localization of Nox2, DuoxA proteins may serve a function analogous to that of $p22^{phox}$ for Duox enzymes [112].

Regulation of Nox/Duox expression

Although the activity of Nox and Duox enzymes is acutely regulated by subunits or calcium, the maximum capacity of a cell to generate ROS will be determined not only by the activation state, but also by the protein expression of Nox/Duox enzymes and their regulatory subunits. Misregulation of Nox protein expression is a component of certain disease states and stress responses, as discussed in the accompanying review. Therefore, the transcriptional and perhaps translational regulation of Nox family proteins is an important part of the consideration of Nox regulation.

a. Regulation of Nox1 expression

Nox1 expression is normally highest in the colon, but it is also seen in a variety of other tissues, including vascular smooth muscle. Nox1 transcript is up-regulated by growth factors and growth-related agonists including angiotensin II, urokinase plasminogen activator, platelet-derived growth factor, bone morphogenic protein 4, prostaglandin F2, keratinocyte growth factor-α, phorbol ester, and mutationally activated K-Ras (RasG12V) (see Table 2). Nox1 is also induced by inflammatory mediators such as interferon gamma (INF-γ) [113,114] and pathogen-associated bacterial molecules including a flagella filament of *Salmonella enteritidis*, and lipopolysaccharide (LPS) of *Helicobacter pylori*. Interestingly *H. pylori* LPS and IFN-γ also induce expression of NOXO1. Vitamin D3, which induces differentiation of colon epithelium, also induces Nox1. Hypoxia leads to increased Nox1 expression in a human pulmonary epithelial cell line, and up-regulation of Nox1 also correlates with activation of hypoxia-inducible factor 1 (HIF-1α)-dependent pathways, but it is not clear whether HIF-1α induces Nox1 expression.

Several groups recently reported studies of the promoter of human Nox1. GATA transcription factors (GATA-4 to 6) are regulators of intestine-specific gene expression and participate in intestinal proliferation and differentiation [115]. Nox1 expression in the human colon cancer cell line Caco2 was mainly driven by elements in the −306-bp proximal promoter region in the 5′ flanking sequence [116]. This region contains two GATA-binding sites, and GATA-6 was implicated (Fig. 4). The IFN-γ-responsive region of the human Nox1 gene was localized in the range between −4331 and −2552 in the 5′ flanking sequence, and regulation was found to be due to a single γ-activated sequence (GAS) element (−3818 to −3810 bp) [113] (Fig. 4). This region is regulated by the transcription factor STAT1. Mouse Nox1 transcripts consist of three splice variants of 5′ untranslated regions [117]. The murine colon expresses the a-type that possesses a short UTR. Tow other types (f- and c-types) have longer 5′ UTRs, which are specifically expressed in smooth muscle. As in human, all three mouse transcripts are driven by the proximal promoter region, but the responsible cellular transcription factor(s) is not clear.

b. Regulation of Nox2 expression

Nox2 expression is transcriptionally up-regulated during the maturation of myeloid cells and in response to pro-inflammatory cytokines such as IFNγ. The principal *cis*-regulatory elements are clustered in the proximal promoter region of the gene between positions −450 and + 12 bp (Fig. 4). Several transcription factors bind these regions. A transcriptional repressor, CCAAT displacement protein (CDP), binds to fivesites in the proximal promoter and constitutively suppresses Nox2 expression in immature cells. CDP protein expression decreases during maturation of monocytes and macrophages resulting in increased expression of Nox2. In addition, CCAAT-binding factor CP1 also binds to the proximal promoter region in the absence of the suppressor CDP [119]. PU.1 is a key transcription factor for Nox2. PU.1 binds to the hematopoietic-associated factor (HAF)-1-binding element. The importance of PU.1 in Nox2 regulation was demonstrated with the discovery of a patient with chronic granulomatous disease harboring four single-base mutations within PU.1 binding sites [118]. These mutations inhibited the binding of PU.1 to the promoter, markedly decreasing Nox2 expression. Binding of the transcription factor YY1 to two YY1-binding sites in the proximal promoter was detected and shown to increase Nox2 promoter activity *in vitro*. However, the function of YY1 during myeloid cell development is not clear because YY1 protein levels do not correlate with differentiation and Nox2 expression [120]. Co-treatment with IFN-γ and LPS strongly activates Nox2 expression in phagocytes. Interferon regulatory factor (IRF) and STAT1 have been identified as important transcription factors mediating the IFN-γ response. Both IRF1 and STAT1 also activate the Nox2 promoter through two interferon-stimulated response elements (ISRE) interacting with IRF, and the single γ-activated sequence (GAS) element interacting with STAT1 in the absence of CDP binding. NF-κB was also recently identified as a regulator of Nox2 expression [121]. The *cis*-regulatory elements are situated in the distal promoter region of the gene between positions −1908 and −1600 bp. Thus, as shown in Fig. 4, Nox2 expression is regulated in a complex manner in a phagocyte and stimulus-specific manner.

c. Regulation of Nox3 expression

Nox3 mRNA is expressed almost exclusively in the inner ear including cochlea and vestibular sensory epithelium [105,122]. As of this writing, there are no reports examining the transcriptional regulation of Nox3 and its restriction to inner ear.

d. Regulation of Nox4 expression

Nox4 expression is most strongly observed in the kidney. Tissue-staining studies showed that human Nox4 protein expression is observed in renal distal tubules [85], whereas in situ hybridization demonstrates that murine Nox4 transcript in renal proximal tubules [80]. Moderate levels of Nox4 are seen in a wide variety of tissues and cells including vascular endothelial cells, vascular smooth muscle, murine osteoclasts, murine adipocytes, and pancreas [82]. Activators of Nox4-trancription in smooth muscle cells include urokinase, plasminogen activator, angiotensin II, transforming growth factor-β1, and TNF-α, whereas bone morphogenic protein 4 (BMP4) decreased Nox4 expression in human endothelial cells (Table 4). We are not aware of published reports focusing on the promoter structure or transcriptional factors regulating Nox4.

e. Regulation of Nox5 expression

Nox5 mRNA is expressed in human in relatively high levels spleen, testis, and vascular smooth muscle. As of this writing, no reports have characterized the active promoter region of the human Nox5 gene.

f. Regulation of Duox expression

Duox1 and Duox2 are expressed in thyroid [92], and an ascidian ortholog of Duox is expressed in the endostyle, which is regarded as the primitive homolog of the thyroid gland in vertebrates [123]. Human Duox1 is also highly expressed in the lung, pancreas, placenta, prostate, testis, and salivary gland [90]. Human Duox2 is highly expressed in the trachea, stomach, colon and rectum [90,91,122,124]. The two human Duox genes are arranged head-to-head, separated by 15.8-kb. Duox1 and Duox2 expression are regulated in both thyroid and non-thyroid cells by a promoter region within −150 bp of the first exon of Duox1 and within −250 bp of Duox2 [125]. More distal promoter regions within −6.1 kb of Duox1 and −14.6 kb of Duox2 were also important, showing increased activity in non-thyroid cells, but not in thyroid cells. In dog [81]and pig [126] thyrocytes, cAMP, which is downstream of the TSH receptor, increased Duox protein and mRNA. However, stimulation of the cAMP pathway did not affect human Duox promoter activity [125], in agreement with the absence of a significant cAMP effect on Duox mRNA expression in human thyrocytes [125,127]. Thyroid transcription factor-1 (TTF-1) and Pax8 control the expression of genes involved in differentiated thyroid function, such as thyroglobulin or thyroid peroxidase [128]. Interestingly, a competition assay using transfection of dominant negative form of TTF-1 or Pax8 displayed no reduction in transcriptional activity of Duox1/2 genes in the differentiated thyroid cell line PCCl3 [129], indicating that regulation of human Duox1/2 gene expressions is not responsive to these thyroid-specific transcription factors. Duox1 expression in airway epithelium was strongly induced by the Th-2 dominant cytokines including IL-4 and IL-13 [130]. In contrast, Duox2 expression was regulated by the Th-1 dominant cytokine IL-1α, IL-1β and IFN-γ.

Conclusions

This review of the mechanism and regulation of ROS generation by Nox and Duox enzymes makes evident that ROS levels in cells and tissues are carefully controlled, both by tissue- and stimulus-specific expression of Nox and Duox proteins and their regulatory subunits, and by acute regulation via calcium, protein phosphorylation, guanine nucleotide exchange on Rac and the assembly of regulatory subunits. Both the expression and the acute activation are regulated by complex mechanisms, and much remains to be explored, particularly with regard to the non-phagocytic Nox and Duox enzymes. Despite incomplete knowledge, it is clear that these regulatory mechanisms provide a variety of cell types with the capacity to carefully regulate ROS generation, a capacity that evolved with eukaryotic cells. As will be discussed in the accompanying review, this ROS generation is beneficial for a variety of physiological processes. However, biology's "choice" to use the chemical properties of ROS in signaling and other biological processes has proven to be a double edged sword.

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List of Abbreviations

Nox
NADPH oxidase

Duox
Dual oxidase

ROS
reactive oxygen species

phox
phagocyte oxidase

LPS
lipopolysaccharide

IFN-γ
interferon gamma

NOXO1
Nox organizer protein 1

NOXA1
Nox activator protein 1

FAD
flavin adenine dinucleotide

NADPH

nicotinamide adenine dinucleotide phosphate, reduced form

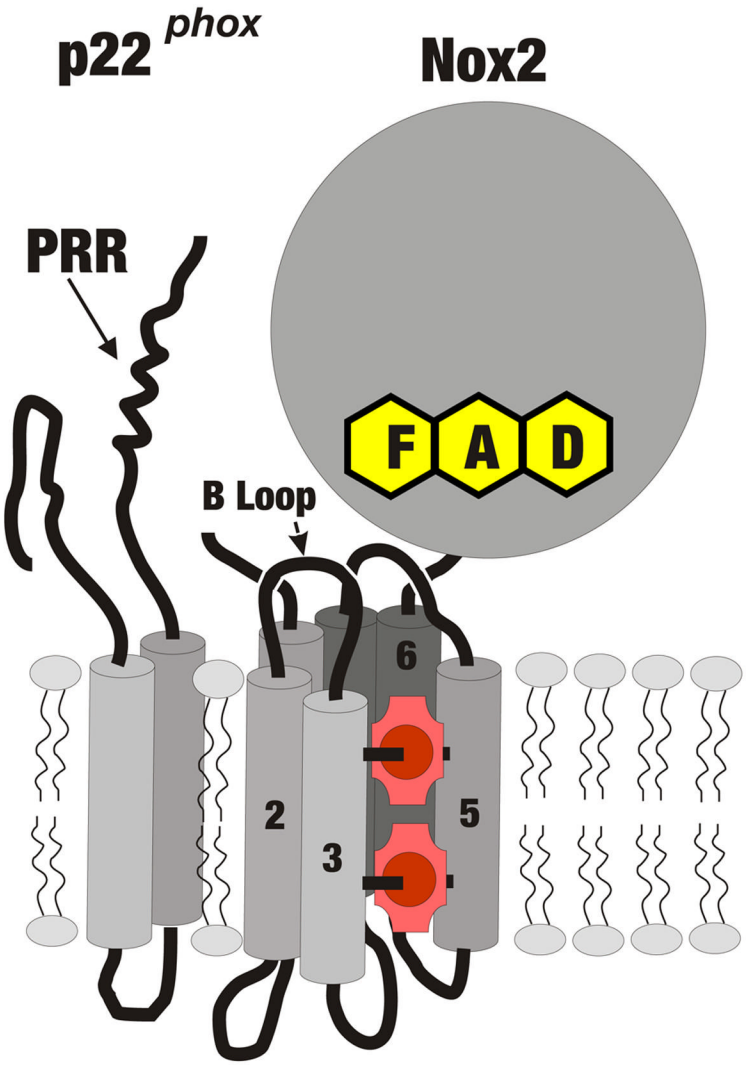

Fig 1. Schematic Structure of Nox2 and p22*phox*

Indicated are Nox2 and p22*phox*, with predicted α-helical transmembrane regions depicted as cylinders, with the cytosol-facing B loop connecting helices 2 and 3, and hemes indicated in red, with conserved histidine residues in helices 3 and 5 providing axial and distal ligands to the heme irons (red circles). Helix 6 is connected to the predicted globular flavoprotein domain containing flavin adenine nucleotide (FAD) and the NADPH binding site, located on the cytosolic side. Nox2 is associated in the membrane with p22*phox*, which has 2 predicted α-helical transmembrane segments and whose C-terminus contains a proline-rich region (PRR) which is the target for binding by the *bis*-SH3 domain of p47*phox*.

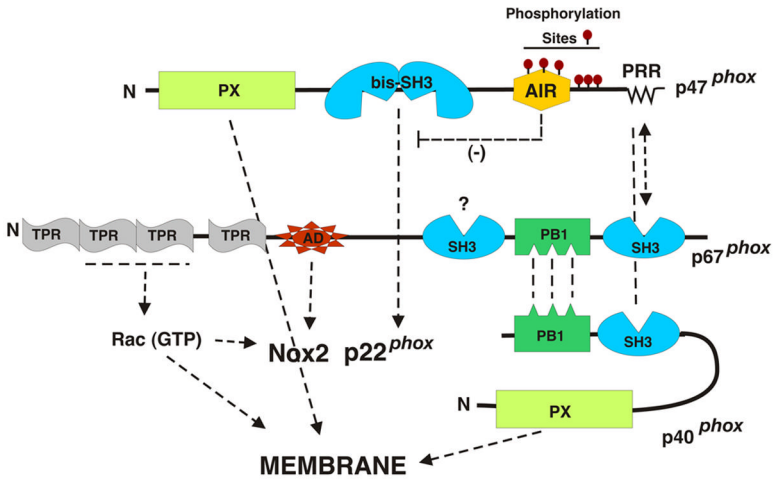

Fig 2. Regulatory Subunits for Nox2

Indicated are domains of the cytosolic factors required for Nox2 activation along with arrows indicating the domains or components with which they interact. Abbreviations are: AD, activation domain; TPR, tetratricopeptide repeats; SH3, Src Homology 3 domain; *bis*-SH3 refers to the tandem SH3 domains that function as a single domain; PX, ^{phox} domain; PB1, a domain originally described in p47^{phox} and Bem1p; AIR, autoinhibitory region.

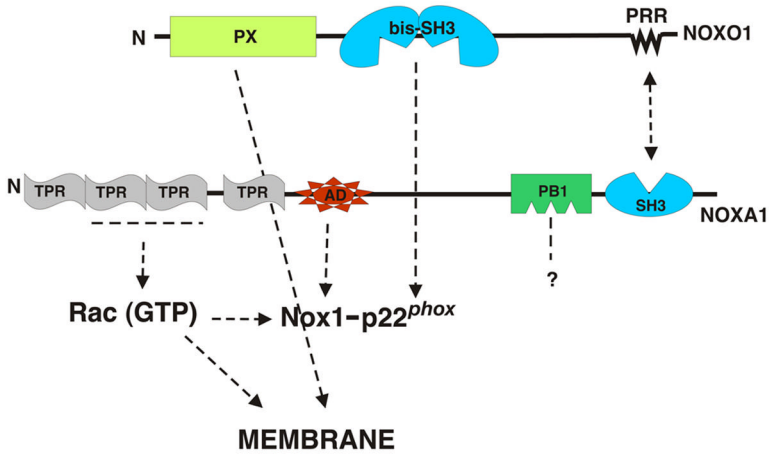

Fig 3. Regulatory Subunits for Nox1

Indicated are domains of the regulatory factors required for Nox1 activation along with arrows indicating the domains or components with which they interact. Abbreviations are as in Fig. 2.

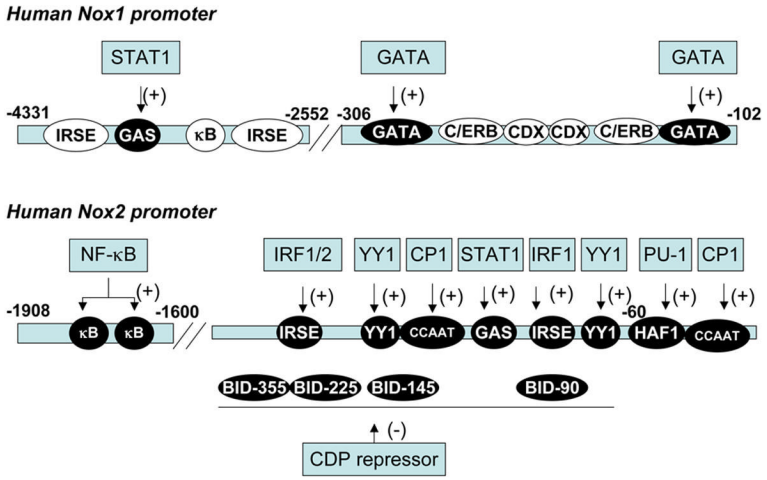

Fig 4. Promoter regions of human Nox1 and Nox2

Shown are schematic drawings of promoter regions of human Nox1 and Nox2. White letters in a filled circle indicate the region identified as regulating responsible gene expression. Black letters in an open circle indicate the region containing a consensus sequence for a given transcription factor. Boxes indicate transcription factors. The abbreviations of responsive element in promoter regions are: IRSE, IFN-γ-responsive stimulated elements; GAS, γ-activated sequence; C/ERB, CCAAT/enhancer binding protein; CDX, caudal-related homeobox; HAF-1, hematopoietic-associated factor 1; CCAAT, CCAAT box binding, BID, binding increased during differentiation.

Table 1

Summary of major mechanisms for regulating the activity of Human Nox and Duox enzymes.

Nox or Duox	$p22^{phox}$-Requiring	Mode of Regulation: Additional Protein Subunits
Nox1	Yes	Regulatory Subunits: NOXO1, NOXA1, Rac1
Nox2	Yes	Regulatory Subunits: $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac2/1
Nox3	Yes	Regulatory Subunits: NOXO1
Nox4	Yes*	Constitutively active; unknown activator(s)
Nox5	No	Calcium, phosphorylation
Duox1	No	Calcium
Duox2	No	Calcium

* PRR not required.

Table 2

Regulation of Nox and Duox Expression by Agonists and Other Stimuli.

Inducer	Nox/Duox	Tissues or cells	References
Angiotensin II	Nox1, Nox4	r-Aortic vascular smooth muscle cells	[131]
Urokinase plasminogen activator	Nox1, Nox4	r-Aortic vascular smooth muscle cells	[132]
Platelet-derived growth factor	Nox1	r-Aortic vascular smooth muscle cells	[133] (7)
Bone morphologenic protein 4	Nox1	m-Aortic endothelial cells	[134]
Prostaglandin F2α	Nox1	r-Aortic vascular smooth muscle cells	[135]
Phorbol ester	Nox1	r-Aortic vascular smooth muscle cells	[133]
K-Ras G12V	Nox1	r-, m-fibroblast cell line	[136]
Vitamin D3	Nox1	h-Colon caner cell line	[114]
Interferon-γ	Nox1	h-Colon caner cell line	[113,114]
Flagellin (*S. enteritidis*)	Nox1	h-Colon caner cell line	[137]
Lipopolysaccharide (*H. pylori*)	Nox1	gp-Gastric mucosal cells	[138]
Hypoxia	Nox1	h-Pulmonary epithelial cell line	[139]
Lipopolysaccharide (*E. coli*)	Nox2	h-Macrophage, h-Granulocyte	[140]
IFN-γ; TNF-γ; GM-CSF	Nox2	h-Macrophage, h-Granulocyte	[140]
M-CSF	Nox2	h-Macrophage	[140]
TGF-β1	Nox4	h-Cardiac fibroblast cells	[141]
TNFα	Nox4	h-Aortic vascular smooth muscle cells	[142]
Low pH	Nox5-short	h-Barret esophagus adenocarcinoma	
Th-2 cytokines (IL-4, IL-13)	Duox1	h-Tracheobronchial epithelial cells	[130]
Th-1 cytolikes (IFN-γ, IL-1)	Duox2	h-Tracheobronchial epithelial cells	[130]

The abbreviations used are: h, human; r, rat; m, mouse, gp, guinea pig.