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Nox proteins in signal transduction

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Abstract

The NADPH oxidase (Nox) family of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) producing proteins has emerged as an important source of reactive oxygen species (ROS) in signal transduction. ROS produced by Nox proteins Nox1-5 and Duox1/2 are now recognized to play essential roles in the physiology of the brain, the immune system, the vasculature, the digestive tract, and hormone synthesis. Nox-derived ROS have been implicated in regulation of cytoskeletal remodeling, gene expression, proliferation, differentiation, migration and cell death. These processes are tightly controlled and reversible. In this review, we will discuss recent literature on Nox protein tissue distribution, subcellular localization, activation and the resulting signal transduction mechanisms.

Keywords

NADPH oxidase; Nox; Nox1; Nox2; Nox3; Nox4; Nox5; Duox1; Duox2; ROS; superoxide; hydrogen peroxide; signal transduction; signaling; Redox

I. Introduction

NADPH oxidase (Nox) proteins are membrane-associated, multiunit enzymes that catalyze the reduction of oxygen using NADPH as an electron donor. Nox proteins produce superoxide ($O_2^{\bullet -}$) via a single electron reduction. The electron travels from NADPH down an electrochemical gradient first to flavin adenine dinucleotide (FAD), then through the Nox heme groups and finally across the membrane to oxygen, forming $O_2^{\bullet -}$. Historically, the NADPH oxidase was known as the source of the phagocyte respiratory burst; however, in the past fifteen years Nox family members and the reactive oxygen species (ROS) they produce have been identified as important contributors to many signaling pathways. This review summarizes current research on the Nox enzymes in signal transduction, focusing on mammalian Nox proteins. Non-mammalian Noxes have been reviewed elsewhere [1,2].

1. NADPH oxidases: a brief history

Early Nox research was carried out in neutrophils, studying the respiratory burst NADPH oxidase complex [3]. The catalytic subunit of this protein is now known as Nox2, or gp91phox. Nox2 has been extensively studied and reviewed [4-6], so we will summarize here the role of Nox2 in signaling pathways only briefly. Although functional studies indicated the probable

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existence of Nox2 homologues, new family members have only been cloned and studied in the past decade.

The first homologue of Nox2 to be cloned was Nox1, originally described in 1999 as Mox1 [7], and almost simultaneously as NOH-1 [8]. Although the dual oxidase (Duox) enzymes (longer homologues of Nox2) were not cloned until shortly after Nox1, earlier research had characterized a putative thyroid NADPH oxidase [9], so the Duoxes were believed to exist before they were finally cloned [10,11]. Almost immediately thereafter, in 2000, Nox3 was described as a gp91phox homologue expressed in fetal kidney and a cancer cell line [12]. Nox3 was later determined to be primarily expressed in the inner ear in adults [13]. Nox4, originally Renox, was discovered in the kidney [14,15], and soon afterwards was described in osteoclasts [16]. Nox5 was discovered in 2001 by two different groups [17,18].

Structurally, all members of the Nox family contain at least six transmembrane domains and cytosolic FAD and NADPH binding domains. Nox1-4 lack extra functional domains that Nox5 and Duox1/2 contain. Nox5 contains EF-hand Ca^{2+} binding domains [17,19], while Duox1/2 have an extracellular peroxidase domain in addition to the EF-hand and gp91phox homology domains (Figure 1).

2. Nox in signal transduction: Overview

i. Activation—A number of regulatory subunits have been identified for the Noxes, and various stimuli such as angiotensin II (Ang II), thrombin, platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) have been shown to alter the activity or expression of the Nox proteins and subunits, and ultimately the amount of ROS produced. Activation mechanisms for Nox1-3 are similar, and involve complex formation with regulatory cytosolic subunits. Regulation of Nox4 is poorly understood, but may be primarily at the expression level [20], although a Nox4 regulatory protein was recently indentified [21]. In contrast, Nox5 and the Duoxes appear to be activated by Ca^{2+} [19,22]. Detailed mechanisms of activation for individual Nox enzymes are included below.

ii. Physiological targets of Nox-derived ROS—The ROS produced by NADPH oxidases seem to have two general downstream physiological roles. Superoxide produced by Nox2 is required for the respiratory burst that occurs in phagocytes. A role in host defense has been proposed for other Nox enzymes as well, including Nox1 in the colon and Duox1 and 2 in the lung [23]. This topic has been extensively reviewed elsewhere [6,24-26]. The second role of Nox is in signaling: $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) that are derived from Nox enzymes can specifically and reversibly react with proteins, altering their activity, localization, and half-life. Many signaling processes are known to be affected by Nox-derived ROS. They will be described in greater detail below with respect to the Nox family member that initiates the ROS signal.

iii. Compartmentalization—It should be noted that the roles of different Nox family members, though they all produce $O_2^{\bullet -}$, are distinct. This is due in part to compartmentalization within the cell. Antioxidants and ROS metabolizing enzymes are in place to reduce non-specific reactivity. Nox1 has been identified in caveolae on the plasma membrane [27,28]. Nox2 is found in phagosomes and on the leading edge of lamellipodia [29]. Both Nox1 and Nox2 have also been localized to “redoxisomes”, endosomes responsible for early receptor-mediated signaling in non-phagocytic cells [5,29,30]. The subcellular localization of Nox3 has only been studied in overexpression systems, where it was shown to target to the plasma membrane [31]. Nox4 has been identified in focal adhesions [27], the nucleus [32], and the endoplasmic reticulum [33], where it interacts with kinases and phosphatases distinct from those found in caveolae and endosomes. Nox5 has been found to localize to internal membranes in the absence

of stimulus; however, in response to added phosphatidylinositol 4,5-biphosphate (PIP_2), Nox5 localizes to the plasma membrane via an interaction between PIP_2 and the Nox5 N-terminal polybasic domain [34]. Duox1/2 are found on the plasma membrane [35].

ROS produced by Nox proteins can act both intra- and extracellularly. Nox2 can produce ROS extracellularly via exocytosis that occurs after agonist activation of the enzyme. Nox1 on the other hand, has been demonstrated to induce endocytosis upon activation, which produces intracellular-acting ROS in endosomes. Other Nox members primarily produce ROS intracellularly, and are believed to reside within intracellular membrane structures or vesicles, from which ROS enter the cytosol.. The mechanism by which ROS escape from these signaling endosomes is under active investigation, but has been studied most extensively for Nox1 (see section II.A.iii below).

3. Physiology and Pathology

i. Physiological roles—The Nox family of proteins has been demonstrated to be essential in normal physiology. Expression of NADPH oxidases is ubiquitous in mammals, though the individual Nox isoforms have different distributions between tissues and species. Nox proteins have been shown to regulate many fundamental physiological processes, including cell growth, differentiation, apoptosis, and cytoskeletal remodeling. In addition, they have more specialized functions, such as host defense (Nox2) [6], otoconium formation in the inner ear (Nox3) [20], iodination of thyroid hormone (Duox2) [36], and control of vascular tone (Nox2) [37]. As research in this area expands, we are bound to gain a better understanding of the myriad functions of this enzyme family.

One controversial potential role of the Nox proteins is oxygen sensing. It is clear that ROS species play a role in the hypoxia response; however, the source or sources of ROS are a matter of dispute. Early in vitro studies showed that Nox enzymes were less active in hypoxia than normoxia [38]. However, in vivo it was found that ROS production increases in low oxygen to activate the transcription factor hypoxia inducible factor-1 (HIF-1) [39] and redox sensitive K+ channels [40]. There is evidence to support both mitochondrial [41] and NADPH oxidase-derived [42] ROS in oxygen sensing, but overall, the mechanisms are not well understood.

ii. Nox proteins in disease—The misregulation or absence of certain Nox isoforms has been linked to a variety of diseases in essentially every organ system. The earliest discovery was an immune disorder, chronic granulomatous disease (CGD), caused by the absence of active Nox2 or its subunits [43-45]. Patients with CGD exhibit chronic infections and impaired wound healing [46]. Nox derived ROS have been implicated in the pathogenesis of a number of neurological diseases, including Alzheimer's disease. Nox has even been proposed as a potential pharmacological target for slowing Alzheimer's disease progression [47]. Overactivation of Nox1 and Nox2 has been shown to be involved in the development of H. pylori-induced gastrointestinal inflammation [48], hypertension [49,50], and restenosis after angioplasty [51,52], while excess ROS produced by Nox5 are related to atherosclerosis [53] and cancer [54,55]. Moreover, Duox1/2 dysregulation has been associated with thyroid dysfunction [56] and cystic fibrosis [57]. Nox proteins have also been linked to rheumatoid arthritis and diabetes. A complete description of pathologies associated with the Nox proteins is beyond the scope of this review, but can be found in several recent reviews [25,37,48,58].

4. Nox-derived ROS

i. Superoxide—Superoxide, the primary product of Nox enzymes, is produced physiologically via a one-electron reduction of molecular oxygen. Superoxide is highly reactive and short lived, which makes determining a biological half-life difficult. Superoxide can dismutate to a second signaling intermediate, H_2O_2, spontaneously (rate constant = 8 ×

10^4 $M^{-1}s^{-1}$) or enzymatically via superoxide dismutase (SOD) (rate constant = 2×10^9 $M^{-1}s^{-1}$) [59]. As a consequence, $O_2^{\bullet-}$ must be produced in very close proximity to its target to be effective as a signaling molecule. Superoxide is also capable of reacting with nitric oxide (NO), forming highly reactive and potentially damaging peroxynitrite ($OONO^-$) (rate constant = 4-16 $\times 10^9$ $M^{-1}s^{-1}$) [60]. This also inactivates NO, which can have pathological consequences, particularly in vascular endothelial cells.

Superoxide is known to react with $(FeS)_4$ clusters, which may release ferric ions [61]. In the case of aconitase, $O_2^{\bullet-}$ inactivates the enzyme, leading to reduced mitochondrial function [62]. There is *in vitro* evidence of $O_2^{\bullet-}$ reacting with heme groups such as cytochrome C; however, the physiological significance of this reaction remains to be determined. Finally, the formation of peroxynitrite from $O_2^{\bullet-}$ can then lead to reversible glutathionylation of proteins on reactive cysteines, as has been described for the Na+-K+ ATPase [63].

Superoxide is also known to react with protein thiols such as cysteine residues, but it has been pointed out that the reaction rate of SOD converting $O_2^{\bullet-}$ to H_2O_2 is much faster than that of $O_2^{\bullet-}$ with biothiols [64]. H_2O_2 also reacts with protein thiols, and although the reaction rate of $O_2^{\bullet-}$ with protein thiols is chemically faster than that of H_2O_2, the greater stability and diffusibility of H_2O_2 increases its probability of reacting with the protein thiols involved in ROS signaling. This suggests that physiological protein thiol oxidation is most likely H_2O_2-dependent.

Although production of $O_2^{\bullet-}$ is the main biological function of Nox proteins and is important in the bactericidal activity of Nox2, much of the signaling that occurs is directly mediated by its dismutation product H_2O_2. Superoxide is not able to diffuse across biological membranes due to its negative charge. There is, however, evidence for channels that are capable of transporting $O_2^{\bullet-}$, which will be discussed in section II.A.iii.

ii. Hydrogen peroxide—Hydrogen peroxide is more stable than $O_2^{\bullet-}$ and is also capable of crossing biological membranes. Because of the presence of SOD in the cell, H_2O_2 is formed rapidly from Nox-generated $O_2^{\bullet-}$, or in the case of Nox4, perhaps prior to the release of $O_2^{\bullet-}$ from the enzyme [65]. H_2O_2 is also tightly regulated biologically by catalase, glutathione peroxidase, and peroxiredoxins, which convert H_2O_2 to water and other metabolites. H_2O_2 can reversibly react with low pKa cysteine residues [66] on proteins to initially form a disulfide bond (−SSR) and sulfenic acid (−SOH). Sulfinic acid ($-SO_2H$) and sulfonic acid ($-SO_3H$) can be formed by additional oxidation; however, these latter reactions are essentially irreversible, and not useful for signaling [67,68]. Oxidation of thiols by H_2O_2 has been demonstrated to have diverse physiological consequences, as indicated by the myriad signaling pathways described in section II.

iii. Relationship of Nox to other sources of ROS—NADPH oxidases are not the only ROS-producing molecules expressed physiologically. ROS generation has been identified as a byproduct in a variety of physiological processes including cytochrome P-450 oxidase uncoupling [69], endothelial nitric oxide synthase (eNOS) uncoupling [70], xanthine oxidase activation [71], mitochondrial respiration [72,73], and activation of various peroxisome oxidases [74]. Importantly, there appears to be a reciprocal relationship between many of these sources of ROS. For example, exposure of endothelial cells to oscillatory shear stress leads to a Nox-dependent activation of xanthine oxidase [75], while Ang II stimulation results in mitochondrial ROS production that is downstream of Nox activation [65]. eNOS uncoupling has also been shown to be a direct result of Nox activation [76]. Thus, it appears that Nox enzymes play important roles as initiators and integrators of redox signaling via cross-talk with other ROS producing systems.

iv. Redox balance—A theory dubbed the "Redox Hypothesis" proposes that redox elements (like redox sensitive cysteine residues) are organized in redox circuits controlled by GSH, thioredoxin and cysteine residues [77]. Oxidative stress is defined as a disruption of these circuits, rather than an overall imbalance of oxidizing elements to reducing elements in the cell as previously described. The signaling described in this review can exist within the context of these circuits. Changes in activity and expression of NADPH oxidase proteins can influence the proposed redox potential to cause the observed physiological effects.

II. Nox proteins in signal transduction

A. Nox1

i. Tissue distribution and physiological function—Nox1 was the first of the novel NADPH oxidase catalytic subunits to be cloned. Shortly after the discovery of Nox1, an alternatively spliced form of the gene was discovered (Nox1β), which lacks exon 11 [8] and is incapable of producing $O_2^{\bullet -}$ [78]. A second splice variant (Nox1 γ) was also identified, but was later discovered to be an artifact of the technique used [78,79], likely caused by stable loop formation of Nox1 mRNA [58].

Nox1 mRNA is most highly expressed in colon epithelia [48], but is also expressed at lower levels in VSMCs, endothelial cells, uterus, placenta [80], prostate, osteoclasts [81], retinal pericytes, neurons, astrocytes and microglia [26]. There is evidence of species-specific distribution of Nox1 as well. Rodent stomach expresses Nox1, which has been shown to be upregulated by *Helicobacter pylori* lipopolysaccharide (LPS) [82,83]. However, the expression of Nox1 in human stomach has been questioned [48], though another Nox isoform could play a similar role in humans.

The physiological role of Nox1 in colon remains somewhat controversial. Two proposed roles are immune defense and cell proliferation (or pathologically, inflammatory bowel disease and carcinogensis) [48,84]. Nox1 ROS production has been shown to be increased in response to LPS and flagellin [85]. Several studies have correlated increased Nox1 activity to increased proliferative signaling processes such as mitogen-activated protein (MAP) kinase [86] and c-Src [87]. Another recent study suggests that increased Nox1 activity promotes colon adenocarcinoma migration [88].

Though Nox1 has a low basal expression in VSMCs, it has been extensively studied because it is upregulated at the mRNA level and activated by vascular pathological stimuli such as Ang II and PDGF [89-91]. Nox1 mRNA has been shown to be increased in rat arteries during restenosis after balloon injury [51], in the aortas of hypertensive rats [92], and in diabetic arteries [93]. It has been shown to regulate smooth muscle cell growth, both hypertrophy and hyperplasia, and migration [37,94]. In addition, Nox1 may be important in regulating blood pressure [95].

Nox1 is also active in the central nervous system (CNS). A study of Nox1 knockout mice found that these mice exhibit a reduction in the augmented sensitivity to pain that accompanies inflammation (hyperalgesia), which is apparently mediated by a reduction in transient receptor potential vanilloid receptor 1 (TRPV1) channel activation via impaired calcium mobilization and impaired translocation of PKCε to the membrane [96]. In microglia, like in the colon, LPS has been shown to activate Nox1, which suggests a role in host defense [97]. Nox1 in neurons has also been implicated in neurite growth [98].

ii. Mechanisms of activation—At the protein level, Nox1 associates with the membrane subunit p22phox, which is necessary for enzymatic activity [33,99,100]. Nox1 is activated by forming a complex with cytosolic activators in a similar manner to Nox2, and can interact with

p47phox [101], p67phox [101] and the small GTPase Rac [102], but is most highly activated by the p47phox and p67phox homologues, NoxO1 and NoxA1 [103]. In contrast to the cytosolic localization of p47phox in resting cells, NoxO1 is constitutively associated with Nox1, and lacks the autoinhibitory region found on p47phox, which may be responsible for some constitutive activity [104]. Analogous to Nox2 activation, NoxA1 and Rac membrane translocation are required for activation and initiation of $O_2^{\bullet -}$ production [105]. The best-studied activation of Nox1 occurs via Ang II in vascular smooth muscle cells (VSMCs). Ang II stimulates the AT-1 receptor, which rapidly activates phospholipase C (PLC) though the heterotrimeric G-protein subunit Gα/11 [106]. PLC cleaves PIP_2 into inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG and Ca^{2+} released by IP_3 activate protein kinase C (PKC), which phosphorylates p47phox [91,107]. Continued activation of Nox1 by Ang II requires ROS-sensitive, Src-mediated transactivation of the epidermal growth factor (EGF) receptor, leading to phosphatidylinositol 3-kinase (PI-3K)-dependent activation of Rac [91, 108].

iii. Subcellular localization—Various subcellular localizations of Nox1 have been reported. In keratinocytes, Nox1 was found to have a nuclear localization with some cytoplasmic distribution [109]. Recent studies suggest a plasma membrane distribution, specifically in caveolae on the cell surface [27,28]. ROS production by Nox1 in vascular cells occurs in early endosomes and requires the expression of chloride channel 3 (CLC-3) [110]. Although the mechanism of why the channel is required is not well defined, the authors suggest that CLC-3 may act to neutralize the electron flow into the endosome that occurs during Nox1-mediated $O_2^{\bullet -}$ generation. Another suggestion, based on the observation that CLC-3 can transport $O_2^{\bullet -}$ across endothelial cell membranes [111], is that CLC-3 may transport $O_2^{\bullet -}$ out of the endosome and into the cytosol [112]. This model is attractive because Nox-derived ROS are detectable in the cytosol, although the orientation of Nox1 is similar to that of Nox2, which releases $O_2^{\bullet -}$ from phagosomes extracellularly. Since $O_2^{\bullet -}$ is a charged species, it cannot freely diffuse across membranes and would require dismutation to an uncharged species such as H_2O_2, or transport.

iv. Signal transduction—The primary ROS produced by Nox1 is $O_2^{\bullet -}$, although H_2O_2 is thought to be the most important signaling molecule in Nox1 signal transduction. Due to the short-lived nature of ROS, the localization of Nox in the cell is believed to determine the downstream signaling effects [29]. In the case of Nox1-derived ROS, there is evidence for a role in inactivating phosphatases, modifying kinase pathways, regulating cell cycle proteins and altering the activity of transcription factors (Figure 2).

Before Nox1 had been formally cloned and identified, it was observed that the hypertrophic agent Ang II stimulates a NADPH oxidase in VSMCs [113] leading to activation of a variety of signaling cascades including the p38 MAP kinase/mitogen-activated protein kinase activated protein kinase-2 (MAPKAPK2)/Akt [106,114,115], Ras (via glutathionylation) [116], and EGF receptor transactivation [117] pathways. Ang II-induced hypertrophy can be inhibited by DPI and catalase [118]. Though multiple Nox members are present in the vasculature, Ang II selectively activates Nox1, so the ROS-dependent hypertrophic effects are likely mediated by Nox1 [89,90]. Additionally, protein tyrosine phosphatase (PTP) SHP-2 and Akt activation by Ang II have been demonstrated to be regulated in a Nox1-dependent manner in a study of spontaneously hypertensive rats [92].

Along with hypertrophy, Nox1 has also been implicated in cell migration. Migration in response to PDGF [119] or fibroblast growth factor (FGF) [120] is impaired in VSMCs from Nox1 knockout mice, while Nox1 siRNA attenuates arachidonate-induced migration of HT29-D4 adenocarcinoma cells [88]. The downstream targets of Nox1 with respect to migration have been studied extensively for PDGF. PDGF induced H_2O_2 formation mediates smooth muscle

cell migration via activation of c-Src, which subsequently activates phosphoinositide-dependent kinase-1 (PDK1) and p21-activated protein kinase (PAK1) [121]. A parallel pathway in which PDGF stimulates Nox1-dependent ROS-mediated regulation of actin turnover has also been described. Nox1 activates Slingshot (SSH)1L phosphatase, through disruption of an inhibitory partnership with 14-3-3 proteins [122,123]. Once active, SSH1L dephosphorylates and activates cofilin [124]. The PDGF-induced activation of SSH1L is required for cofilin activation and migration in VSMC [123]. This Nox1-dependent pathway was demonstrated to be functionally important in a femoral artery wire-injury model using Nox1 knockout mice, in which neointima formation was decreased compared to wild type mice [119]. There is also a significant amount of information about how FGF-mediated Nox1 activation affects migration. As is the case for PDGF, Nox1 appears to target cytoskeletal remodeling. FGF-induced activation of c-Jun N-terminal kinase (JNK) and subsequent phosphorylation of the cytoskeletal adapter protein paxillin were shown to be mediated by Nox1 [120]. Consistent with this theme, Nox1 mediates integrin turnover in carcinoma cells [88].

There is also substantial literature suggesting that Nox1 has role in cell proliferation. $H2O_2$ generated via Nox1 were demonstrated to mediate cell growth and transformation when overexpressed in NIH 3T3 fibroblasts [125]. More recently, cyclin D1 has been identified as a target of Nox1 regulation of the cell cycle [126]. Lung epithelial cells overexpressing Nox1 exhibit increased proliferation, higher protein expression of cyclin D1, and increased extracellular signal regulated kinase (ERK) 1/2 activity. H_2O_2 is believed to mediate these effects, as they are blocked by catalase [127]. Of interest, Nox1 is also probably important for thrombin-induced proliferation of VSMCs, given that its growth effects in the aorta, whose VSMCs express only Nox1 and Nox4, are blocked in p47phox knockout mice [128]. Finally, the activated oncogene, Ras, has been proposed to constitutively activate and upregulate Nox1, which is necessary for its oncogenic properties [129]. This activation results in upregulated vascular endothelial growth factor (VEGF) via activation of the transcription factor SP1 [130]. The disruption of stress fibers and focal adhesions associated with Ras-activation are mediated by the oxidative inactivation of low molecular weight (LMW)-PTP, which reduces Rho activity [131]. A study of human colon cancers demonstrates a correlation between activating Ras mutations and overexpressed Nox1 [132], supporting the in vitro data.

Nox1 has also been implicated in cell death and necrosis. Tumor necrosis factor-α (TNFα) promotes complex formation between Nox1, TRADD, RIP1, and Rac1, which in turn promotes necrotic cell death by prolonged JNK activation [133]. Another group showed that TNFα also regulates the Nox1 complex transcriptionally [134].

In colon epithelial cells, Nox1 and NoxO1 expression are controlled transcriptionally to allow for sustained signaling upon activation. TNFα rapidly phosphorylates p38 MAP kinase and JNK1/2, leading to phosphorylation of the transcription factors c-Jun and c-Fos and activation of AP-1. This is presumed to be the cause of increased NoxO1 expression in those cells [134]. Interestingly, in these cells Nox1 appears to function in host defense.

2. Nox2

i. Tissue distribution and physiological function—Nox2 is known to be essential in innate host defense, both by producing ROS to attack invaders after phagocytosis and by acting as a signaling molecule to initiate a number of inflammatory and immunoprotective responses [6]. Though Nox2 is most highly expressed in phagocytes, expression has also been detected in CNS, endothelium, VSMCs, fibroblasts, cardiomyocytes, skeletal muscle, hepatocytes, and hematopoietic stem cells [58]. In vascular cells, Nox2 is activated by Ang II, endothelin-1, VEGF, TNFα, and mechanical forces [135,136]. Superoxide produced by Nox2 can react with NO in the cells to regulate bioavailability with the consequence of creating the reactive

molecule $OONO^-$, which has been implicated in oxidative stress. NO is an important vasodilator and signaling molecule in endothelial cells. Misregulation of Nox2 activity can lead to endothelial dysfunction and contribute to hypertension [137].

ii. Mechansims of activation—Nox2, or the neutrophil NADPH oxidase, is the first discovered and most extensively studied of the Nox members. The Nox2 complex is composed of the membrane subunits gp91phox (Nox2) and p22phox, and is stimulated by agonists such as F-Met-Leu-Phe [138-140]. Upon exposure of neutrophils to this peptide, p47phox is phosphorylated on 8-9 serines by either proline-directed kinases or PKC [141]. S359 and S370 are phosphorylated first, and then S379 acquires a phosphate, exposing an SH3 binding site that interacts with the proline-rich region of p22phox and facilitates translocation to the membrane. Finally, S303 and S304 are phosphorylated, leading to full catalytic activity [142]. p67phox then binds to the translocated p47phox, providing a binding site for activated Rac and forming the functional enzyme. Nox2 has also been found to complex with p40phox, but the functional consequences of this interaction are controversial [58].

iii. Subcellular localization—Nox2 is localized in submembranous phagosomes in neutrophils, and in caveolae on the leading edge of lamellipodia in endothelial cells [29]. Nox2 has also been identified in endosomes [143], including those responsible for early receptor-mediated signaling called redoxisomes in non-phagocytic cells [5,29,30]. In transfected HEK293 cells, Nox2 is distributed to the plasma membrane [144].

iv. Signal transduction—Nox2 signaling in neutrophils has been extensively studied. In host defense Nox2 localized in phagosomal membranes is activated by the presence of microbes and cytokines to generate $O_2^{\bullet -}$ (Figure 3). Superoxide in the phagosome dismutates to H_2O_2, which, along with chloride ions, can be converted to hypochlorous acid (HOCl) by extracellular myeloperoxidase (MPO). HOCl is an effective antimicrobial oxidant. This pathway has clear physiological relevance in immune defense. Individuals lacking Nox2 or with mutations in other necessary components of the neutrophil NADPH oxidase are afflicted with CGD, and are highly susceptible to infection [44].

It has become clear that the production of ROS in phagosomes is not the only role of Nox2, even in the context of host defense. Numerous cytokines activate ROS production in neutrophils, which then inactivate PTPs [145] leading to cytoskeletal rearrangement [146] or other signaling consequences [147]. Nox2-derived ROS in macrophages have also been implicated in apoptosis by activating the ASK1-p38 MAP kinase pathway [148].

In other cell types, Nox2 signals to kinase/phosphatase cascades in a similar manner to Nox1 (Figure 3). For example, a recent study in fibroblasts found Nox2 in endosomes to be involved in TNFα induction of the transcription factor NF-κB [149]. The authors propose that once the TNFα receptor is activated and endocytosed, TRADD is recruited to the receptor. Nox2-derived ROS promotes TRAF2 binding to the TNFR1/TRADD complex, which then activates IkB Kinase (IKK) and promotes NF-κB activation. This pathway may contribute to cell death, as TRAF2-deficient MEFs are resistant to ROS-induced cell death [150,151].

Nox2 signaling in endothelial cells has emerged as an important angiogenesis-regulating pathway. Nox2 is activated in endothelial cells by VEGF [152], angiopoietin-1 [153], hypoxia [5] and thrombin [154]. ROS produced in this process have been implicated in endothelial cell proliferation and migration. It is clear that Nox2-derived ROS take part in VEGF-induced VEGF receptor 2 phosphorylation, activation of cSrc and Akt, and phosphorylation of VE-cadherin to promote angiogensis; however, the molecular mechanisms remain to be fully elucidated [155].

3. Nox3

i. Tissue distribution and physiological function—Nox3 was first discovered in 2000, along with Nox4 and Nox5, based on sequence homology to gp91phox [18]. The finding that Nox3 is expressed in the inner ear led to an examination of balance in a Nox3 mutant mouse model ($Nox3^{het}$) [156]. Indeed, these mice exhibit a head tilt, similar to that seen in *nmf333* mice, a mouse strain with a point mutation in p22phox [157], and in *hslt* mice, a mouse strain with a spontaneously arisen mutation in the region of the NoxO1 gene [158]. Both of these mice are unable to remain on the surface of the water during a swim test, and fail to respond to linear acceleration of the head with vestibular-evoked potentials, indicating a severe balance disorder, which has been attributed to a lack of functional Nox3 [157].

Nox3 has also been shown to have functional significance in lung endothelial cells. A study of toll-like receptor (TLR) 4 knockout mice found increased expression and activity of Nox3, which resulted in increased elastolytic activity, an indicator of emphysema development. DPI and siRNA against Nox3 reversed the phenotype [159]. This suggests that Nox3 may serve physiological roles distinct from the inner ear. Indeed, it has been detected in fetal spleen, kidney, lung and skull by PCR [13,18], which may indicate that Nox3 plays an important role in tissue development, but is turned off in adult tissue, a concept that requires further investigation.

ii. Mechanisms of activation—Nox3 three-dimensional structure is predicted to be similar to that of Nox1 and Nox2 [58]. Nox3 is highly expressed in the inner ear, along with the Nox subunits p47phox, NoxO1 and NoxA1 [13,160]. Studies on the activation of Nox3 have shown contradictory results. Ueno et. al. [161] demonstrated that p22phox is necessary for Nox3 $O_2^{\bullet -}$-producing activity. Recent studies suggest a weak constitutive activity when Nox3 is coexpressed with p22phox, but full activation requires Rac and various other combinations of cytosolic Nox subunits [162-165]. Contradictory results were obtained when the Nox3 system was reconstituted in HEK293 cells with combinations of NoxO1, NoxA1, p47phox and p67phox, but most studies agree that NoxO1 and p67phox each universally activate Nox3. The fact that the head tilt phenotype is shared in Nox3 and NoxO1 deficient mice strongly suggests a functional interaction between the two [158]. However, it is likely that the precise molecular composition of the Nox3 complex is tissue dependent.

iii. Subcellular localization —Very little information is available about the targeting of endogenous Nox3, but tagged Nox3 coexpressed with p22phox in HEK293 cells is localized to the plasma membrane [31]. In the absence of p22phox, Nox3 is detected in the cytoplasm.

iv. Signal transduction—Based on the head tilt phenotype of Nox3 mutant mice [156], a recent observation may offer a clue to downstream effects of Nox3-derived ROS. The drug cisplatin is known to induce hearing loss and increase $O_2^{\bullet -}$ production via Nox3 [13]. Mukherjea et. al. reported that the TRPV1 channel in the cochlea is upregulated in response to cisplatin [166]. The upregulation is prevented by diphenylene iodonium (DPI), a non-specific inhibitor of Nox catalytic subunits and other flavin containing proteins, and the antioxidant lipoic acid. Finally, siRNA against TRPV1 reduces cisplatin-induced ototoxicity, which suggests that TRPV1 may be downstream of Nox3 and may mediate cisplatin toxicity effects. It is not known whether this pathway also mediates the head tilt phenotype seen in the $Nox3^{het}$ mice.

4. Nox4

i. Tissue distribution and physiological function—Nox4 is highly expressed in the kidney [14], but has been found to be expressed and functionally important in many cell types including mesangial cells [167], smooth muscle cells [27], endothelial cells [168], fibroblasts

[169], keratinocytes [170], osteoclasts [16], neurons [171], and hepatocytes [172]. Nox4 tissue distribution is fairly ubiquitous [20], and in general Nox4 is highly expressed compared to other Nox homologues.

Nox4-derived ROS have been implicated in a variety of physiological processes, including cellular senescence [14,15,173], apoptosis [174], survival [175], insulin signaling [176], migration [177,178], the unfolded protein response [179], and differentiation [169,180-182]. In addition, Nox4 has been proposed to play a role in oxygen sensing by enhancing the O2 sensitivity of TWIK-related acid sensitive K channel 1 [183]. The best established functions of Nox4 revolve around cell growth, death and differentiation. Because these responses are often antagonistic, it is likely that Nox4 regulates a fundamental physiological process common to all of them, such as cytoskeletal reorganization or gene expression.

ii. Mechanisms of activation—Nox4, originally Renox [14], is unique among the catalytic Nox subunits in that it only requires the membrane subunit p22phox for ROS producing activity, and appears to be constitutively active [184]. This observation has led to the proposal that Nox4 is an inducible Nox, and its activity is proportional to Nox4 protein expression alone. In cardiac fibroblasts, lung [185] and pulmonary artery [186] smooth muscle cells, TGF-β induces increased expression of Nox4 [169]. Insulin stimulates Nox4 expression in adipocytes [187] and IGF-1 has been found to induce expression in VSMCs [177]. Importantly, Peshavariya et al. [188] recently showed that the regulation of Nox4 also occurs at the translational level by a mechanism dependent on p38 MAP kinase.

Of interest, a recent paper from our group described the identification of a p22phox-interacting protein, polymerase delta-interacting protein (Poldip2), that increases the activity of Nox4 and participates in its regulation of the cytoskeleton in VSMCs [21]. The importance of Poldip2 in Nox4 regulation of other systems remains to be determined.

iii. Subcellular localization—There have been conflicting reports on the localization of Nox4. In VSMCs, Nox4 has been identified in focal adhesions [27], the nucleus [32], and the endoplasmic reticulum [33]. Nuclear and endoplasmic reticular localization have been confirmed in other cell types, including HEK293 cells and endothelial cells [28,32,189,190]. One study identified Nox4 splice variants with potentially distinct subcellular localizations [191]. However, it is not known whether or not these variants are translated physiologically.

iv. Signal transduction—Nox4 differs from other Nox enzymes because the $O_2^{\bullet -}$ produced by Nox4 is rapidly converted to H_2O_2, so $O_2^{\bullet -}$ release from this enzyme is almost undetectable [192]. In rat VSMCs tested in basal conditions, siRNA against Nox4 does not reduce $O_2^{\bullet -}$ production as measured by DHE-HPLC, but does reduce production of H_2O_2 measured by Amplex Red assay [65]. How this occurs remains controversial; however, it is believed that H_2O_2 is responsible for the majority of Nox4 downstream effects.

With such a variety of physiological processes proposed to be regulated by Nox4-derived ROS, it is not surprising that specificity of downstream signaling dictates the final response (Figure 4). In adiposities, insulin-induced ROS production inactivates PTP1B, which enhances the phosphorylation of the insulin receptor [176]. In VSMCs, IGF-I-induced migration is dependent upon Nox4-mediated activation of matrix metalloproteinase-2 (MMP2) [177], while PDGF-induced migration requires Nox4-mediated focal adhesion turnover [21]. In contrast, Nox4 overexpression inhibits angiotensin II-induced migration of adventitial myofibroblasts by an unknown mechanism [193].

Growth and survival effects of Nox4 activation have been reported to be mediated by Akt in mesangial cells stimulated with Ang II [167]. In pancreatic cancer, LMW-PTP inactivation by

Nox4 promotes prolonged phosphorylation of JAK2, a tyrosine kinase that phosphorylates signal transducers and activators of transcription (STAT) proteins and enhances the growth response [194]. Nox4-associated ROS have also been implicated in progression through the G2/M checkpoint of the cell cycle via regulation of cdc25 phosphorylation [195]. Nox4-mediated growth and survival have also been observed in VSMCs treated with urokinase plasminogen activator [196] or TGF-β [185], and in hypoxia-mediated activation of pulmonary adventitial fibroblasts [197]. In the latter case, hypoxia induces TGF-β, which increases IGFBP-3 expression via a phosphatidylinositol 3-kinase/Akt-dependent pathway. IGFBP-3, in turn, induces Nox4, leading to proliferation [197]. Nox4 has also been shown to mediate TGF-β-induced phosphorylation of retinoblastoma protein (pRb) and the eukaryotic translation initiation factor 4E binding protein-1 (eIF4E), which regulate cell cycle progression and hypertrophy, respectively, in airway smooth muscle cells [185].

Studies performed with VSMCs [182], fibroblasts [169], adipocytes [187], and embryonic stem cells [181,198] show that ROS production by Nox4 promotes differentiation. In adipocytes, Nox4 was shown to upregulate MAP kinase phosphatase-1 (MKP-1), which reduces activation of ERK1/2 [187]. However, the detailed molecular mechanisms by which Nox4 regulates MKP-1 expression, and by which MKP-1 regulates differentiation, are not known. In mouse embryonic stem cells, Nox4-derived ROS activate p38 MAP kinase, resulting in the phosphorylation and translocation to the nucleus of MEF2C, a transcription factor important in cardiomyocyte differentiation [181]. Nox4-mediated differentiation of VSMCs appears to be related to regulation expression of the smooth muscle-specific transcription factor serum response factor (SRF) [182,198].

5. Nox5

i. Tissue distribution and physiological function—Nox5 is expressed in lymphatic tissue [199], testis [17], VSMCs [200], endothelial cells [199], spleen, uterus [18], and prostate cancer cells [54]. Several authors have proposed that Nox5 plays a role in cell proliferation [20,54,55,199-201]. Not surprisingly, Nox5 has been found to be highly expressed in several cancer cell lines [54,55,201,202].

ii. Mechanisms of activation—Nox5 differs from other Nox enzymes in its activation by calcium and possibly calmodulin based mechanisms, instead of by complexion with cytosolic subunits. Nox5 was originally identified by cloning homologues of gp91phox, and was quickly determined to be Ca^{2+} activated [17,18]. Several isoforms of Nox5 have been described, Nox5-L (α, β, δ, and γ) and a short form: Nox5-S, which lacks EF-hand motifs at the N-terminus [199]. Nox5-L contains EF-hand Ca^{2+} binding domains [19,203], and Nox5 activity increases with increasing calcium concentrations. In COS-7 cells transfected with Nox5, PMA treatment increases ROS production by stimulating phosphorylation of Nox5 residues Thr^{494} and Ser^{498} [204]. The phosphorylation increases the sensitivity of Nox5 to calcium, resulting in activation at lower calcium concentrations.

As with other Nox enzymes, the $O_2^{\bullet -}$ produced by Nox5 can be rapidly converted to H_2O_2. A recent paper points out that PIP_2 causes Nox5 to localize to the plasma membrane [34], which also results in increased activity. Nox5 activation by c-Abl has also been observed, as well as an association between them [205]. Nox5 is not found in rodents, a model that has been commonly used to study the other Nox proteins, presenting a severe limitation for physiological and pathophysiological studies.

iii. Subcellular localization—Like Nox3, little information is available about the subcellular localization of endogenous Nox5. However, in two studies in which tagged Nox5 was overexpressed in HEK293 cells, it was detected at the plasma membrane [34,206].

Membrane targeting of Nox5 is a function of the interaction of its N-terminal polybasic region with PIP_2 [34]. Mutations in this region caused Nox5 to localize internal organelles, away from the plasma membrane.

iv. Signal transduction—There are relatively few studies of the signaling pathways mediated by Nox5 (Figure 5). Although little is known about the mechanisms responsible for Nox5 upregulation in cancer, in Barrett's esophageal adenocarcinoma cells a short form of Nox5 is induced by platelet activating factor (PAF) via a STAT5-dependent mechanism [202]. The NF-κB transcription factor signaling pathway is an important mediator of inflammatory gene expression. There is evidence that Nox5 (or an isoform of Nox5) induces NF-κB in adenocarcinoma cells. It was shown that overexpressing Nox5 variant Nox5-S reduces IκBα, an inhibitor of NF-κB signaling [201]. It is not known whether the Nox5-S variant is active in terms of ROS production.

Like Nox1 and Nox4, Nox5 has been shown to inhibit the phosphatase PTP1B in epithelial cells. Stimulation of the interleukin-4 (IL-4) receptor activates Nox1 and Nox5 via IP_3-mediated calcium release. The resulting ROS inactivates PTP1B, which enhances the phosphorylation and activation of the IL-4 receptor [207]. In human VSMCs, proliferation induced by PDGF activation of JAK2 and STAT3 phosphorylation was determined to be Nox5 dependent [200].

6. Duox1/Duox2

i. Tissue distribution and physiological function—The Duox proteins are described as having dual nature due to an extracellular peroxidase domain in addition to the EF-hand Ca^{2+} binding and gp91phox homology domains [10]. Originally isolated from the thyroid, they produce the H_2O_2 that is used to oxidize iodide during thyroid hormone synthesis [10,11]. More recently, it has been shown that Duox2 is necessary functions as a heme peroxidase in respiratory epithelium [208]. The additional potential peroxidase activity of the Duox proteins has not been well studied, and has even been suggested to be inactive [35]. Others have proposed that the peroxidase domain may contribute to the enzyme's ability to form H_2O_2 by two-electron reduction [22].

Duox2 is more highly expressed than Duox1 in the thyroid, and is believed to be mainly responsible for thyroid hormone synthesis. Clinical data support this, as several mutations in Duox2 have been implicated in hypothyroidism, whereas no such mutations have been found in Duox1 [209]. The Duox proteins are also widely expressed in epithelial cells. Both Duox proteins are present in airway epithelial cells and the respiratory tract. Duox1 is more highly expressed than Duox2 in lung and airway epithelia, but Duox2 expression is inducible in colon and the salivary gland [209]. In airway epithelium, it was discovered that IL-4 and IL-13 increase Duox1 mRNA, while interferon-γ highly induces Duox2. This led the authors to hypothesize that Duox1 is constitutively expressed to maintain normal epithelial function, whereas Duox2 is induced in response to infection [210].

ii. Mechanism of activation—The major mechanism of Duox1 and 2 activation is Ca^{2+} binding to Duox EF-hand binding pockets [22]. There are some distinctions between the Duoxes in activation, however. Duox1 is activated by forskolin in thyroid, which leads to phosphorylation by protein kinase A. This mechanism of activation is absent in Duox2; instead, phorbol esters induce PKC-mediated phosphorylation of this protein [211]. Of importance, Duoxes produce H_2O_2 directly via a two electron reduction of oxygen, rather than by producing $O_2^{\bullet -}$ first as an intermediate [212].

Although no other proteins are known to be required for Duox activity, $p22^{phox}$ and EFP1 were found to interact with the Duox proteins, but without a known functional consequence [213].

NoxA1 is expressed in airway cells and inhibits Duox1 activity, possibly by preventing Ca^{2+} binding [214], and therefore functions much differently from its role in the regulation of Nox1 and Nox3.

iii. Subcellular localization—In cell types expressing endogenous Duox proteins, they are localized to the plasma membrane [10]. When Duox is transfected into cells lacking the Duox maturation factors DuoxA1 or DuoxA2, Duox remains in the endoplasmic reticulum. The maturation factors are required for Duox1/2 to make the journey from the endoplasmic reticulum to the plasma membrane [215,216].

iv. Signal transduction—As noted above, Duox2 has been demonstrated to be functionally important in thyroid hormone (T_4) production. The generation of H_2O_2 is essential in the iodination step of thyroid hormone generation. Clinical studies have found the loss of functional Duox2 to be linked to cases of congenital hypothyroidism [56,217]. Patients with the most severe disease are homozygous for a mutant Duox2 that lacks all H_2O_2 producing ability. Duox1 is functional in these patients, so Duox1 is not able to compensate for the loss of Duox2.

In the airways there is evidence that the Duox enzymes aid in defense and inflammation processes. It has been proposed that H_2O_2 produced by Duox can be converted to a bactericidal ROS by lactoperoxidase (LPO) [213]. LPO converts H_2O_2 and SCN- to the bactericidal HOSCN. One theory about why cystic fibrosis patients have chronic lung infections is that defects in the cystic fibrosis chloride channel impair SCN- transport. Without SCN- at the cell surface, the Duox/LPO system is not able to carry out its protective function, as H_2O_2 is not sufficiently bactericidal [213].

The role of the Duox enzymes in signal transduction is still relatively unknown. Recent findings in lung and airway cells demonstrate the importance of Duox outside the thyroid [213,214], and ongoing studies promise to broaden our understanding of Duox signaling.

III. Cell and tissue specificity of Nox proteins

One of the puzzling observations in the Nox field is that cells and tissue often express multiple Nox proteins in the same cell, but that these enzymes regulate different functions in different cell types. It is clear from the previous discussion that Noxes are involved in a plethora of signaling pathways and cellular responses. Yet, they all produce the same ROS. This suggests that the complement of Nox proteins within a cell, and more importantly, their subcellular localization and coupling to external stimuli, are critical determinants of the integrated response to Nox activation.

In many, if not most, cell types, different Nox homologues are coupled to different agonists and therefore different physiological responses. For example, in VSMCs, Ang II and PDGF activate Nox1 [90], while TGF-β and serum withdrawal activate Nox4 [169,218]. In this cell type, Nox1 is growth-promoting, while Nox4 is pro-differentiating. In contrast, Nox4 is activated by Ang II and mediates hypertrophy of mesangial cells [167]. While Nox1 is acutely activated by agonists in VSMCs, it is regulated by transcriptional control of NoxA1 expression in the gut [48]. In endothelial cells, Nox2 and Nox4 appear to play antagonistic roles. Nox2 is activated by Ang II and TGF-β, and Nox4 is upregulated by serum withdrawal and insulin [144]. Finally, in cardiac fibroblasts, Nox4 and Nox5 are oppositely regulated by TGF-β and appear to mediate the transition to myofibroblasts and inflammatory pathways, respectively [169]. In all of these cell types, activation of individual Noxes leads to activation of specific signaling pathways that are dictated by their subcellular localization. Given these non-redundant functions and tissue-specific responses, it is imperative to study Nox enzymes in specific cellular contexts.

IV. Conclusions

The explosion of knowledge over the decade since the discovery of novel Nox homologues has been astounding, but much remains to be learned about these important proteins. Given their association with numerous diseases, it is essential that we learn more about the specific molecular pathways whose function is altered by activation of specific Nox proteins. This will be greatly aided by the creation of additional genetically modified mouse models, as well as homologue-specific chemical inhibitors. Once these pathways are identified, detailed investigation into how ROS alter molecular function is needed. Because both $O_2^{\bullet -}$ and H_2O_2 have potential signaling functions, it is important to understand which ROS is predominantly produced by a given homologue, and how that particular ROS interacts with kinases, phosphatases and transcription factors to coordinate the final response of the cell. Moreover, it is clear that subcellular localization of the Nox proteins plays a critical role in their functional impact. More information is needed on how these proteins are targeted to different subcellular compartments and how this transport process is regulated. Only when we understand the full spectrum of events that regulate Nox activation and their full impact on discrete signaling pathways and cellular functions can we move into the clinical realm and thoughtfully design specific new therapeutic approaches targeting Nox proteins.

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List of Abbreviations

Ang II	angiotensin II
CGD	chronic granulomatous disease
CLC-3	chloride channel 3
CNS	central nervous system
DPI	diphenylene iodonium
Duox	Dual oxidase
EGF	epidermal growth factor
elF4E	eukaryotic translation initiation factor 4E binding protein-1
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal regulated kinase
FAD	flavin adenine dinucleotide
FGF	fibroblast growth factor
HIF-1	hypoxia inducible factor-1
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
IKK	IκB kinase
IL-4	interleukin-4
IP_3	inositol trisphosphate
JNK	c-Jun N-terminal kinase

LPO	lactoperoxidase
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MAPKAPK2	MAP kinase activated kinase-2
MMP2	matrix metalloproteinase-2
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
Nox	NADPH oxidase
NoxO1	Nox organizer 1
NoxA1	Nox activator 1
$O_2^{\bullet -}$	superoxide
$OONO^-$	peroxynitrite
PAF	platelet activating factor
PAK1	p21-activated protein kinase
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDK1	phosphoinositide-dependent kinase-1
PIP_2	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
PTP	protein tyrosine phosphatase
pRb	retinoblastoma protein
RIP1	receptor-interacting protein 1
ROS	reactive oxygen species
SOD	superoxide dismutase
SSH1L	slingshot 1L phosphatase
STAT	signal transducers and activators of transcription
TGF-β	transforming growth factor-β
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR1	TNF receptor-1
TRADD	TNFR1-associated death domain
TRPV1	transient receptor potential vanilloid receptor 1
VEGF	vascular endothelial growth factor

VSMCs vascular smooth muscle cells

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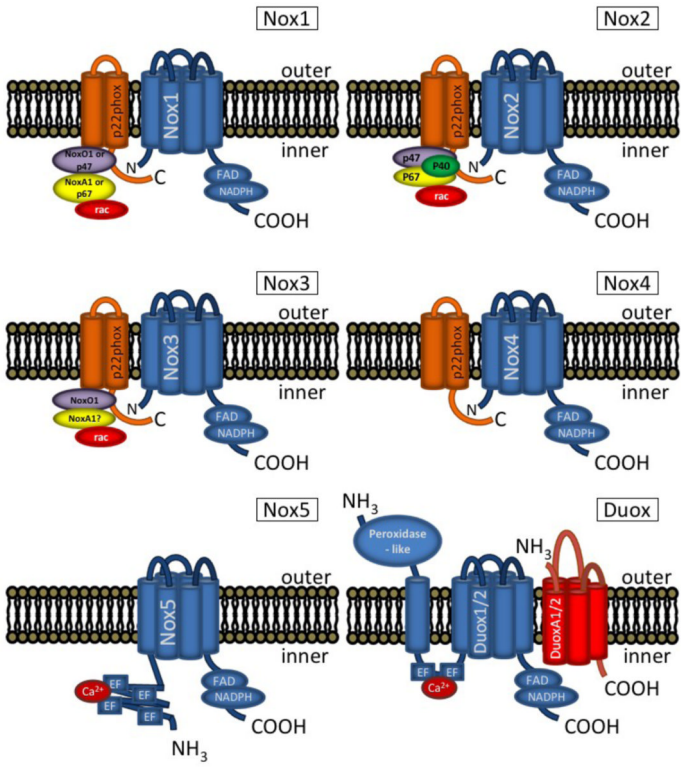

Fig 1. Nox family members and their regulatory subunits

Although no 3-dimensional crystallization of Nox proteins has been performed, they are believed to contain six transmembrane domains based on hydrophobicity analysis (seven for Duox1/2). Oxidase activity occurs when NADPH binds to Nox on the cytosolic side, where it transfers electrons to FAD and the heme centers (not shown) and finally to oxygen on the outer membrane surface, resulting in $O_2^{\bullet -}$ formation. In Nox1-4, the transmembrane subunit p22phox associates with active and inactive Nox. It is believed to have between two and four transmembrane segments. Nox1 is believed to primarily interact with the cytosolic subunits NoxO1, NoxA1 and GTP-Rac upon activation; however p47phox and p67phox can replace NoxO1 and NoxA1, respectively. Nox2 activation involves association with GTP-Rac, p47phox, p67phox and p40phox. Nox3 activation is less well defined, but is believed to primarily involve GTP-Rac, p47phox and NoxA1 in the inner ear. Nox4 is constitutively active when associating with the cytosolic p22phox subunit. Nox5 and Duox1/2 activation involves $Ca2^+$ binding to EF-hand domains in the cytosol. Duox1/2 require the association of DuoxA1/2, respectively, for localization to the plasma membrane.

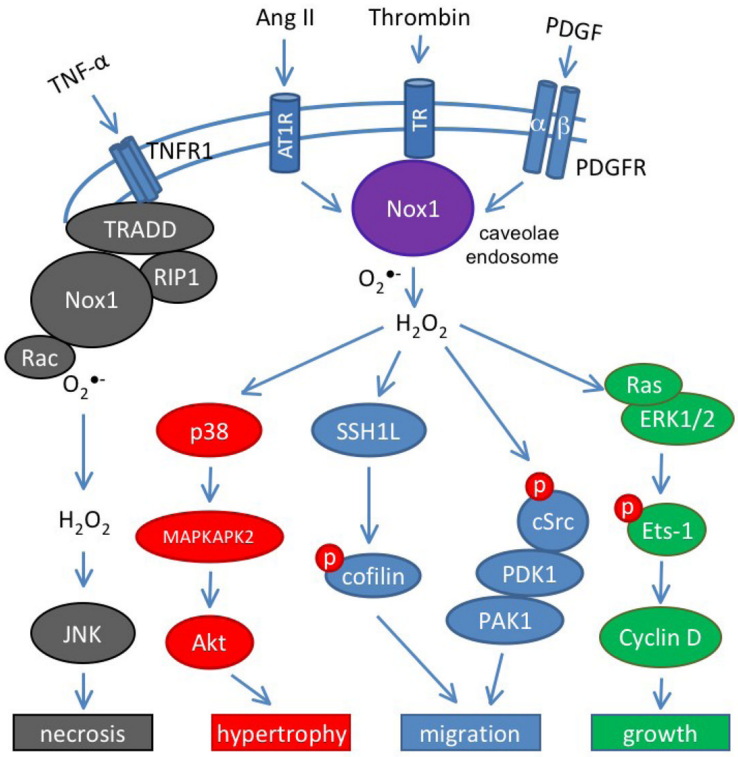

Fig 2. Nox1 signal transduction pathways

Nox1 is localized to cavaolae in the plasma membrane and endosomes. TNF-α stimulates TNF receptor-1 (TNFR1), resulting in the recruitment of TRADD, RIP1, Rac and Nox1 to the receptor. The complex produces ROS that activate JNK to initiate necrosis. Other activators of Nox1 include Ang II, thrombin, and PDGF. H_2O_2 produced by Nox1 activation initiates hypertrophy by activating p38 MAPK, which associates with MAPKAPK2 and Akt. Nox1 also activates SSH1L, which activates cofilin by dephosphorylation to promote cell migration. In a parallel pathway, Nox1-derived ROS increase cSrc phosphorylation, which activates PDK1, followed by PAK1. Nox1 also stimulates growth by activating Ras and ERK1/2, which activate the transcription factor Ets-1 by phosphorylation. Ets-1 upregulates Cyclin D, promoting passage through the cell cycle.

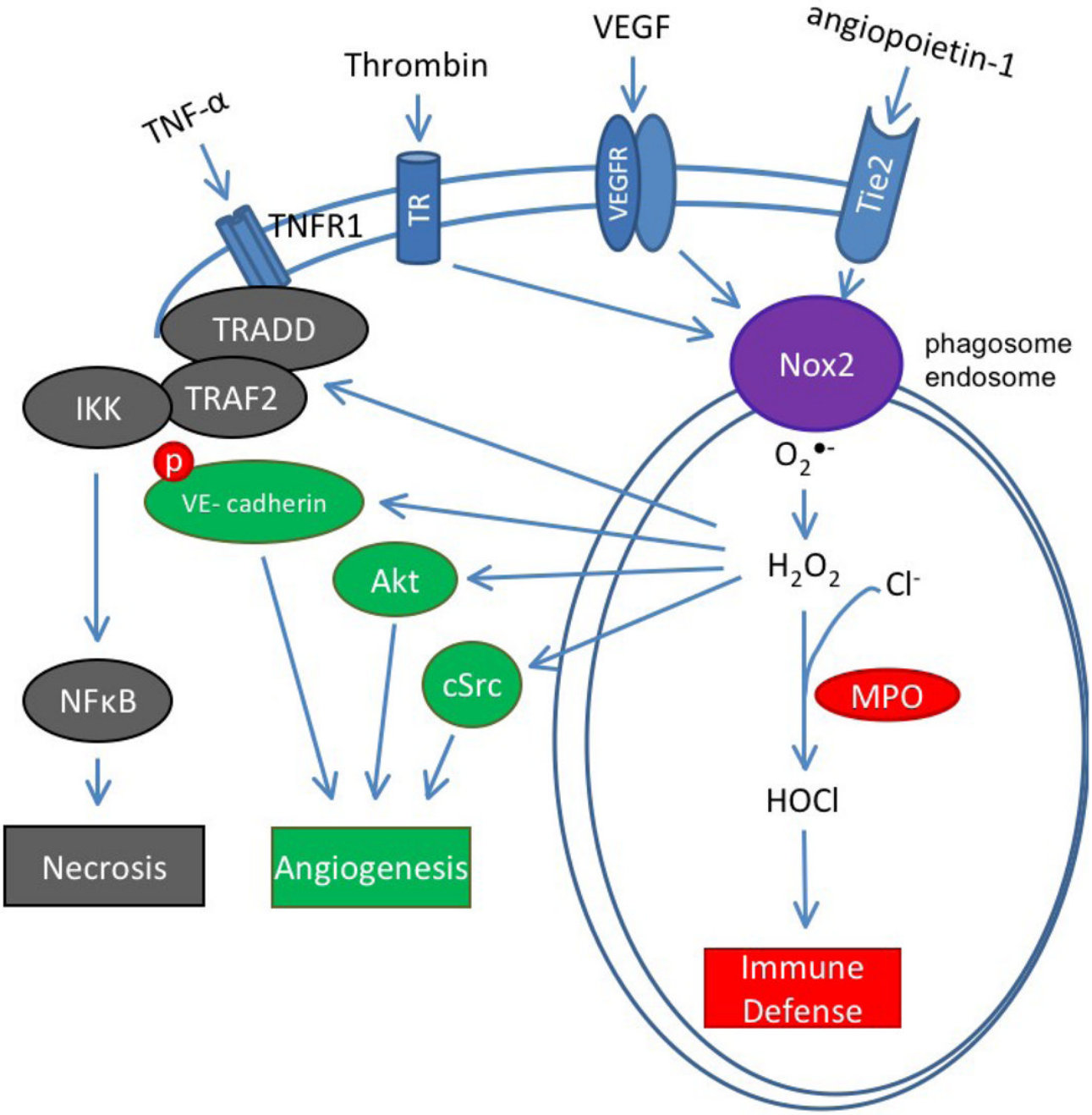

Fig 3. Nox2 signal transduction pathways

Nox2 is localized to endosome and phagosome membranes. In necrosis, TNF-α activates TNFR1, which recruits TRADD to the receptor. TRAF2 then binds to TRADD in a Nox2-derived ROS dependent manner and activates IKK, leading to NFκB activation and necrosis. Nox2 in endosomes is also activated by thrombin, VEGF and angiopoietin-1. Nox2-derived ROS promote angiogenesis by activating VE-cadherin, Akt and cSrc. Nox2 acts in host defense in phagosomes by producing $O_2^{\bullet-}$, which is dismutated to H_2O_2. The reaction of H_2O_2 with Cl^- is catalyzed by MPO to form HOCl, which is bacteriocidal.

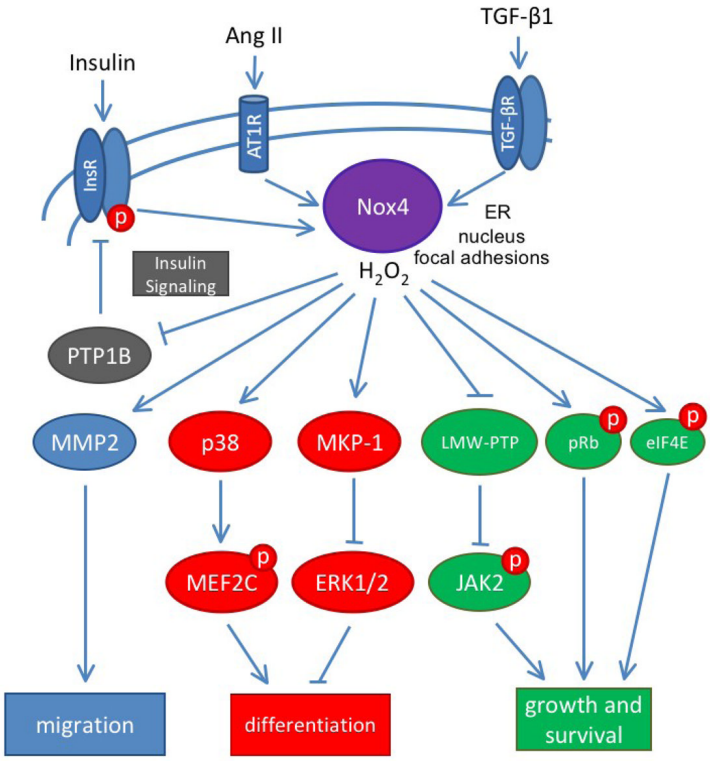

Fig 4. Nox4 signal transduction pathways

Nox4 is constitutively active, but activity and/or expression can be increased by insulin binding to the InsR, Ang II activating the AT1R and TGF-β1 binding to TGF-βR. Nox4 is involved in the inhibition of insulin signaling by inhibiting the phosphatase PTP1B, which prolongs the phosphorylation of the insulin receptor. Nox4 promotes migration by activating MMP2. Nox4 promotes cell differentiation by multiple mechanisms. Nox4 derived-ROS activate p38 MAP kinase, which phosphorylates and activates MEF2C to promote differentiation. In addition, H_2O_2 produced by Nox4 activates MKP-1, which inhibits the activation of ERK1/2. Since ERK1/2 normally promotes growth, its inhibition may allow for differentiation to occur. Nox4 also promotes growth and survival by several pathways. Nox4 derived ROS inhibit LMW-PTP, which prolongs the phosphorylation of JAK2 to promote growth. Nox4 also promotes phosphorylation of pRb and elF4E to promote growth and hypertrophy.

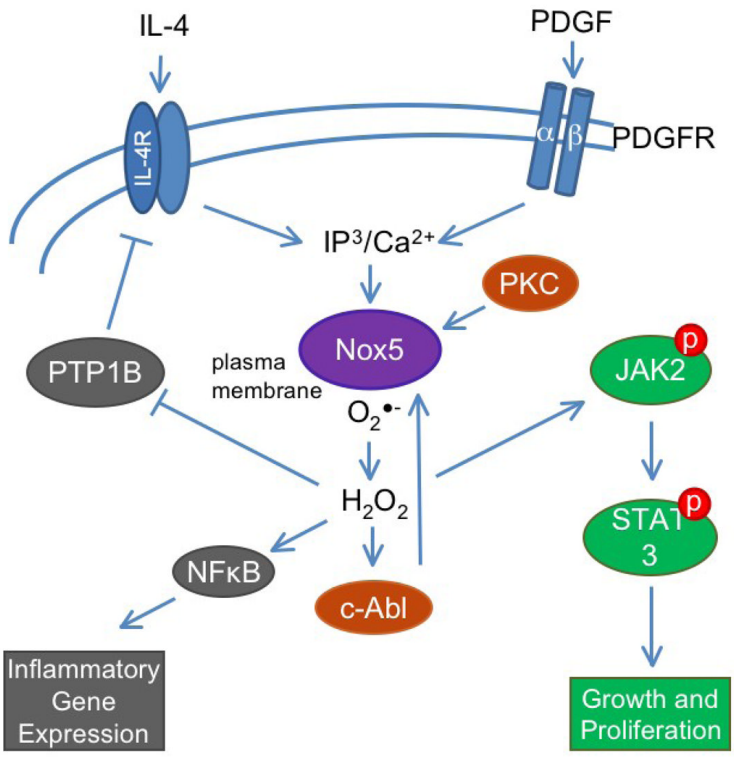

Fig 5. Nox5 signal transduction pathways

Nox5 is activated by PKC, IP_3 and Ca^{2+} produced by PDGFR activation and cytokine receptor activation (IL-4/IL-4R). Nox5-derived ROS can increase inflammatory gene expression by activating NFκB and through positive feedback of the IL-4R by inhibiting PTP1B. H_2O_2 activates ROS production by Nox5 via an association between Nox5 and c-Abl. Nox5-derived ROS activate growth and proliferation by phosphorylating JAK2, which phosphorylates STAT3 to promote proliferation.