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REVIEW ARTICLE

NADPH oxidase family proteins: signaling dynamics to disease management

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Reactive oxygen species (ROS) are pervasive signaling molecules in biological systems. In humans, a lack of ROS causes chronic and extreme bacterial infections, while uncontrolled release of these factors causes pathologies due to excessive inflammation. Professional phagocytes such as neutrophils (PMNs), eosinophils, monocytes, and macrophages use superoxide-generating NADPH oxidase (NOX) as part of their arsenal of antimicrobial mechanisms to produce high levels of ROS. NOX is a multisubunit enzyme complex composed of five essential subunits, two of which are localized in the membrane, while three are localized in the cytosol. In resting phagocytes, the oxidase complex is unassembled and inactive; however, it becomes activated after cytosolic components translocate to the membrane and are assembled into a functional oxidase. The NOX isoforms play a variety of roles in cellular differentiation, development, proliferation, apoptosis, cytoskeletal control, migration, and contraction. Recent studies have identified NOX as a major contributor to disease pathologies, resulting in a shift in focus on inhibiting the formation of potentially harmful free radicals. Therefore, a better understanding of the molecular mechanisms and the transduction pathways involved in NOX-mediated signaling is essential for the development of new therapeutic agents that minimize the hyperproduction of ROS. The current review provides a thorough overview of the various NOX enzymes and their roles in disease pathophysiology, highlights pharmacological strategies, and discusses the importance of computational modeling for future NOX-related studies.

Keywords: NADPH oxidase; Reactive oxygen species; Inflammation; Inhibitors; In silico

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INTRODUCTION

Reactive oxygen species (ROS)/free radicals are generated endogenously during mitochondrial oxidative phosphorylation as byproducts of aerobic metabolism [1]. Several external factors, such as environmental pollution, cigarette smoke, radiation, certain foods, and drugs, contribute to the generation of free radicals (Fig. 1). ROS include a variety of highly reactive molecules, such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), per hydroxyl radicals ($HO_2^{\cdot}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\cdot}$), and peroxynitrite ($ONOO^-$) [2] (Fig. 1). In general, ROS play dual roles: (a) at moderate levels they participate in multiple cellular processes such as- signal transduction, direct cellular interactions, cellular interactions with the extracellular matrix (ECM), immune regulation, apoptosis, and autophagy [1]; and (b) elevated ROS levels lead to oxidative stress, which results in protein/lipid disruption, DNA damage, and genotoxic stress [3]. ROS generation and scavenging are tightly regulated to maintain homeostasis. The antioxidant system for scavenging ROS consists of several enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. Alternately, nonenzymatic antioxidants include vitamins A, C, and E; flavonoids; and carotenoids. These factors convert ROS and their byproducts into stable nontoxic molecules [1] (Fig. 1). Additionally, various enzymes such as NADH-cytochrome *b5* reductase, dihydroorotate dehydrogenase (DHODH), complex II (succinate dehydrogenase), monoamine oxidases (MAO), xanthine oxidoreductase (XOR), and urate oxidase (UO), are responsible for ROS generation. However, in this review, we will focus on NADPH oxidase (NOX), which is the major and most widely studied source of ROS [4]. The role of NOX enzymes has been studied extensively in various disease states and pathologies, including neurological, cardiovascular, and pulmonary conditions [1, 2]. In addition, NOX inhibitors are being designed and tested as therapies for several of these conditions [5–8]. The current review provides a comprehensive account of the various NOX isoforms, specifically focusing on their role in disease pathophysiology and the associated molecular mechanisms. We also discuss the future of NOX-related research, and the role of in silico studies in paving the way in this area.

STRUCTURE AND COMPONENTS OF NADPH OXIDASES

NOX is a multisubunit protein complex that generates superoxide anions or H_2O_2 by transferring electrons from cytosolic NADPH to molecular oxygen. The classic NOX structure can be broadly divided into two membrane-associated ($gp91^{phox}$ aka NOX2 and

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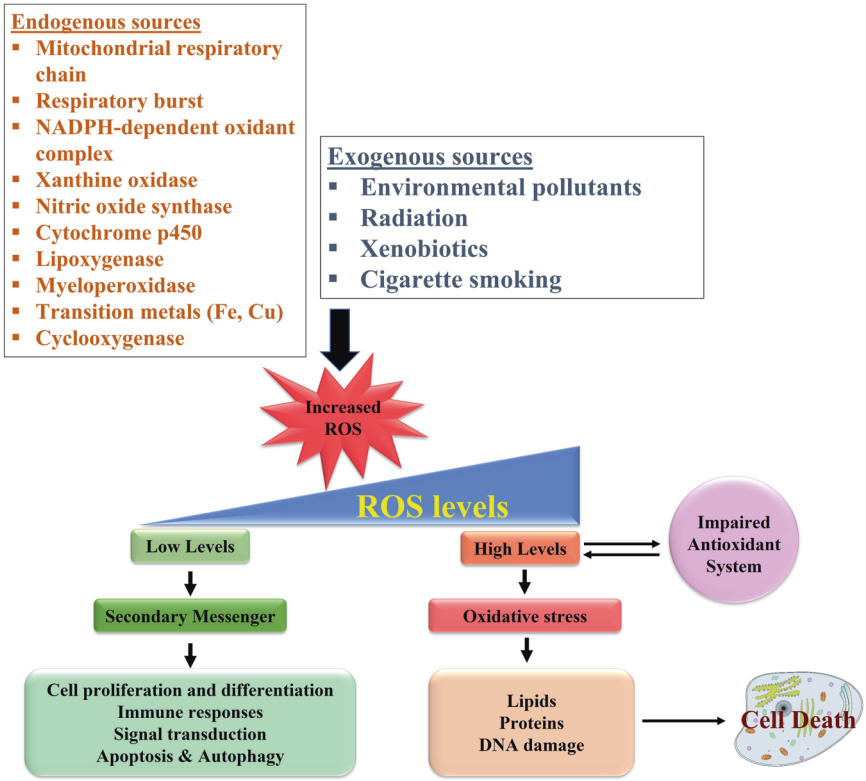

Fig. 1 Factors regulating ROS production. Several endogenous and exogenous factors can trigger cells to produce ROS. Low/moderate levels of ROS act as second messengers and play significant roles in regulating cell growth, immune responses, signal transduction, and autophagy. To maintain cell homeostasis, the antioxidant system is actively involved in eliminating excess levels of ROS in cells. However, excessive ROS production or depletion of the antioxidant system reults in oxidative stress-induced damage to lipids, proteins or DNA which is followed by cell death (apoptotic or necrotic)

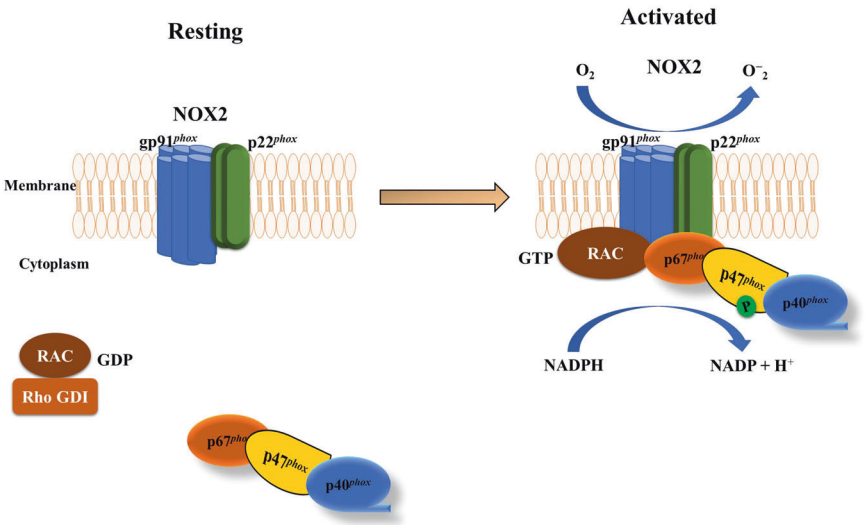

Fig. 2 Activation of NADPH oxidase. The classical NOX is constituted of three cytosolic proteins ($p47^{phox}$, $p40^{phox}$ and $p67^{phox}$), Rac and two membrane subunits ($gp91^{phox}$ aka NOX2 and $p22^{phox}$). When NOX is activated, the cytosolic subunits translocate to the plasma membrane and interact with membrane subunits. This multisubunit enzyme produces $O_2^{\cdot-}$ after electrons are transferred from NADPH through FAD and hemes to molecular oxygen

$p22^{phox}$) and three cytosolic ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) subunits and the G-protein *Rac*, as shown in Fig. 2.

NOX subunits

Flavocytochrome b_{558} consisting of $gp91^{phox}$ and $p22^{phox}$ is the redox center of the NOX enzyme. It is a membrane-bound protein containing a glycosylated 91-kDa (β-subunit/CYBB gene) and a nonglycosylated 22-kDa (α-subunit/CYBA gene) subunit [9]. $gp91^{phox}$ contains a cytoplasmic N-terminal domain with 6-transmembrane helices that bind two hemes and a C-terminal domain consisting of binding sites for NADPH and FAD [10]. In the resting state, heterodimeric flavocytochrome b_{588} is located at the membrane as

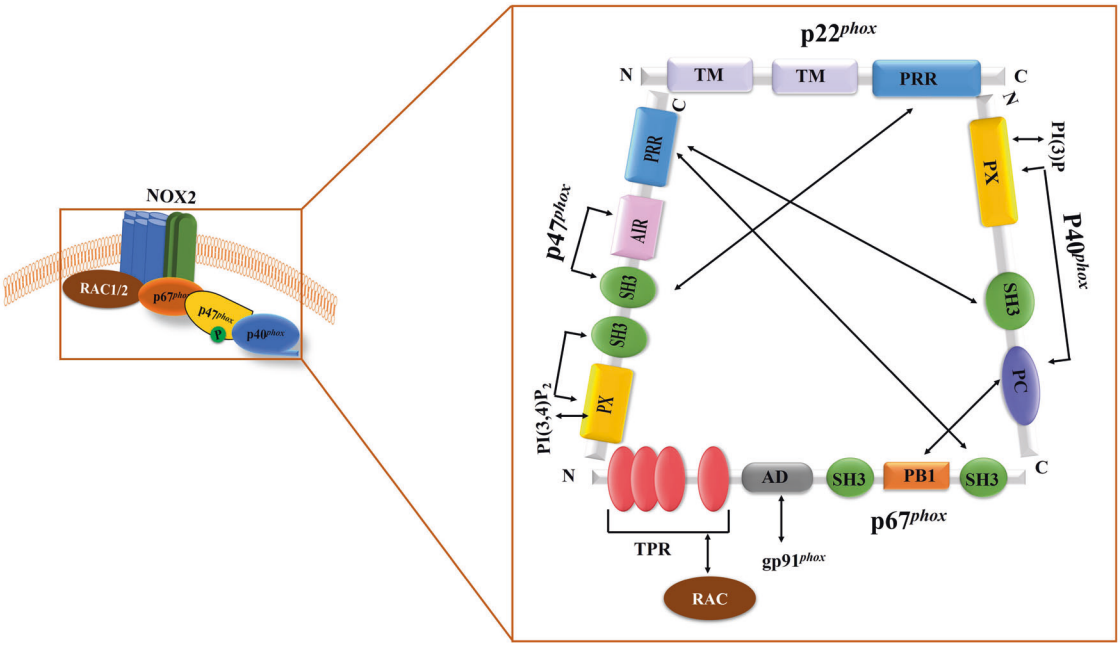

Fig. 3 Molecular interactions that are critical for NOX assembly and activation. $p67^{phox}$ is constitutively associated with $p40^{phox}$ through complementary PB1 (phox and Bem1)/PC motifs in their C-terminus. $p67^{phox}$ is also linked to $p47^{phox}$ through a tail-to-tail interaction involving an SH3 domain and a proline-rich region (PRR) in the respective C-termini of these subunits. $p67^{phox}$ contains four tetratricopeptide repeat (TPR) motifs that form a binding surface for Rac-GTP. $p67^{phox}$ activates $gp91^{phox}$ using the activation domain (AD). The NOX subunit $p47^{phox}$ includes tandem SH3 domains to bind its target PRR motif in $p22^{phox}$, as well as a regulatory PX (*phox* homology) domain that binds phosphoinositides including $PI(3,4)P_2$, and, less strongly to other phosphoinositides as well as to phosphatidylserine (PS) and phosphatidic acid (PA)

an inactive complex. However, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are attached as heterotrimers, and a small GTPase such as Rac1/Rac2 are in the cytosol (Fig. 2) [11]. NOX2 activation is mediated by a complex series of protein/protein interactions (Fig. 3). NOX2 constitutively binds to $p22^{phox}$ and is unstable in its absence. During phagocytosis, the receptor ligation leads to the phosphorylation of $p47^{phox}$, facilitating structural changes by exposing the Src-homology (SH) domain to the $gp91^{phox}$ subunit, which interacts with proline residues on $p22^{phox}$. The activation of $p47^{phox}$ mediates the binding of the $p67^{phox}$ to the $gp91^{phox}$ subunit, which regulates the transfer of NADPH-derived electrons to the flavin domain and functions as a regulator of NOX. Next, $p40^{phox}$ binds to phosphoinositides in PI3P-rich membrane regions, further strengthening the interaction of the membrane and the NADPH oxidase complex. Finally, Rac-GTP translocates to the membrane and binds to $gp91^{phox}$ and $p67^{phox}$, inducing the $gp91^{phox}$ subunit to generate superoxide (Fig. 2) [11–14]. The functional domains of the subunits of NOX enzymes are shown in Fig. 4.

However, apart from the basic structural subunits, the redox center NOX has various homologs. To date, seven NOX isoforms have been identified: NOX1-5 and DUOX1/2 (dual oxidase 1/2) [15]. Although all NOX enzymes generate ROS, they differ in the activity and type of ROS generated. While NOX1-3 and NOX5 primarily generate $O_2^{\cdot-}$, NOX4, DUOX1, and DUOX2 produce H_2O_2 [9]. A brief overview of each of these isoforms is provided in the subsequent section.

BRIEF OVERVIEW OF NOX ISOFORMS

NOX1

NOX1 was the first homolog of NOX2 to be described. It is highly expressed in the colonic epithelium, smooth muscle cells, endothelial cells, uterus, placenta, osteoclasts, retinal pericytes, neurons, astrocytes, and microglia [16]. The NOX1 complex is composed of the NOX1 catalytic subunit (a homolog of $gp91^{phox}$), NADPH oxidase organizer 1 subunit (NOXO1, a homolog of $p47^{phox}$), NADPH oxidase activator 1 subunit (NOXA1, a homolog of $p67^{phox}$), $p22^{phox}$ subunit, and a small GTPase Rac1 subunit. NOX1 is associated with the transmembrane subunit $p22^{phox}$ and mediates its stability and enzymatic activity (Fig. 5). Moreover, NOX1 is activated by forming a complex with the activators NOXA1, NOXO1, and Rac1 GTPase, resulting in the reduction of molecular oxygen to superoxide [17]. The generation of superoxide by NOX1 requires the phosphorylation of NOXO1 by cyclic AMP-dependent protein kinase A at the Ser154 residue or protein kinase C (PKC) at the Thr341 residue, which then interacts with NOXA1 to regulate the activity of NOX1. Additional studies have shown that the phosphorylation of NOXO1 by PKC-β is crucial for NOX1 complex activation and the concurrent increase in the rate of superoxide production [9,17].

NOX2

NOX2 is expressed in inflammatory cells (monocytes, macrophages, neutrophils) and in a variety of tissues, including the brain, neurons, microglia, heart, kidney, gastrointestinal tract, liver, and pancreas. NOX2 signaling has been extensively studied in neutrophils and was described in the previous section [9].

NOX3

NOX3 is structurally similar to NOX1 and NOX2. It is activated either by regulatory subunits ($p47^{phox}$ and $p67^{phox}$) or by NOXO1 and NOXA1 (Fig. 5) [10]. Recent studies have shown that $p22^{phox}$ is crucial for the activation of NOX3 and the generation of superoxide. In terms of its expression profile, NOX3 is highly expressed in the inner ear and is involved in the development of otoconia crystals in the vestibular systems of the inner ear. NOX3 is also found at low levels in the liver, lung, spleen, and fetal kidney [9, 10].

NOX4

NOX4 is distinctive from the other NOXs, as it solely depends on the $p22^{phox}$ subunit for ROS generation [11]. NOX4 is constitutively active and does not require cytosolic subunits to function (Fig. 5).

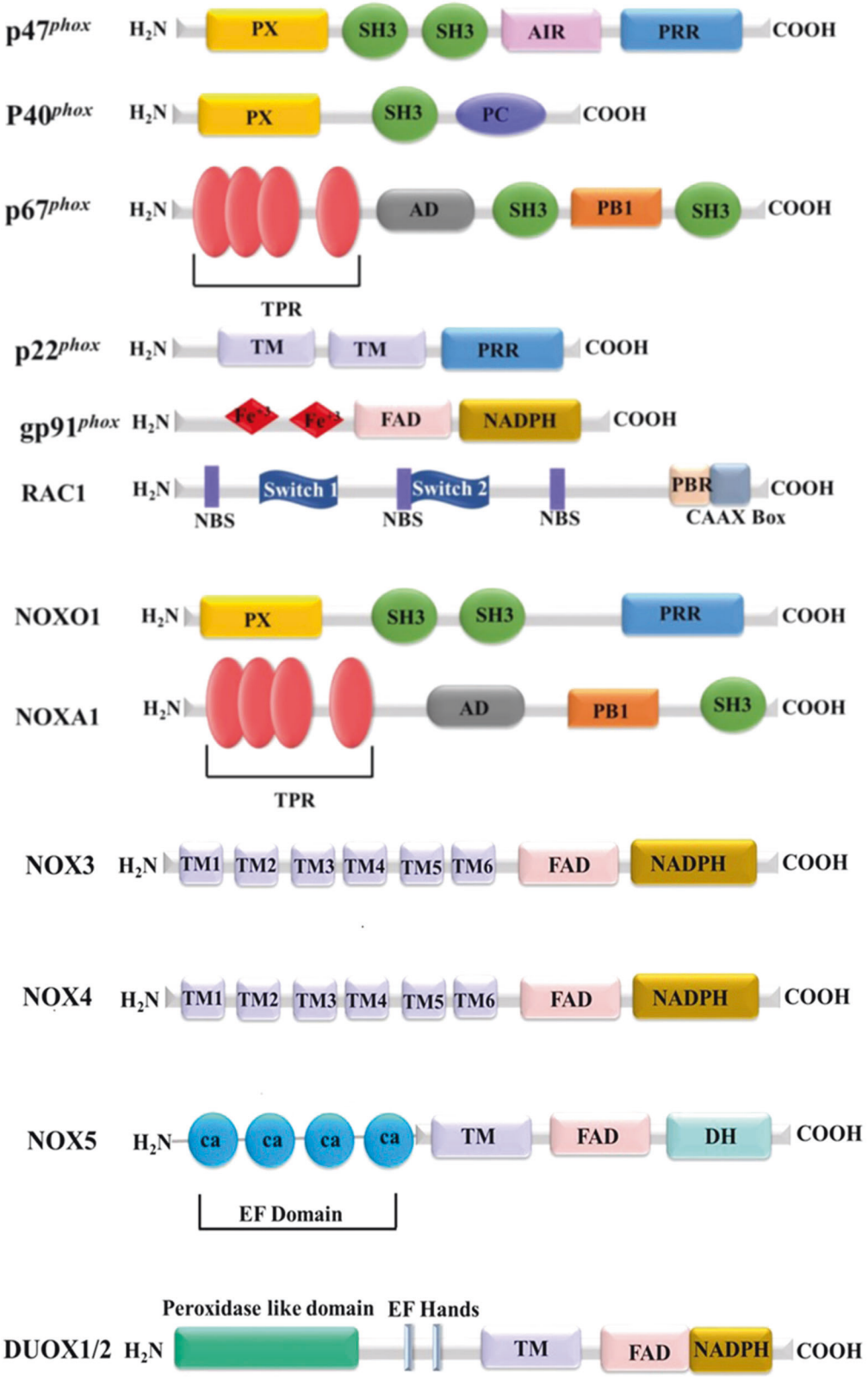

Fig. 4 Functional domains of NADPH oxidase isoforms and subunits. PX- phox homology domain of NOXO1 and NOX subunits bind to PtdIns-phosphates in the membrane; SH3 - Src homology three domain; AIR - autoinhibitory region that is inactivated upon phosphorylation; PC-phox and cdc motif; AD of NOXA1 and subunits are activation domains; PB1-phox and Bem1 domain; PPR-proline rich region; TPR-tetricopeptide repeat domains that interact with Rac; TM-transmembrane; FAD-flavin adenine dinucleotide; NBS-nuclear binding site; PBS-Poly-basic Region; Switch1-Interacts with GEFs(Guanine nucleotide exchange factors) of Switch2; RAC1 becomes activated in its GTP-bound state. NOX3 and NOX4 are equal in size with an amino-terminal hydrophobic domain that is predicted to generate six transmembrane helices. NOX5 has the same gp91^{phox}-like catalytic core, as well as an amino-terminal calcium-binding domain. The DUOX enzymes elongate the NOX5 structure by introducing an extra transmembrane helix at the amino terminus, followed by a domain homologous to peroxidases such as MPO

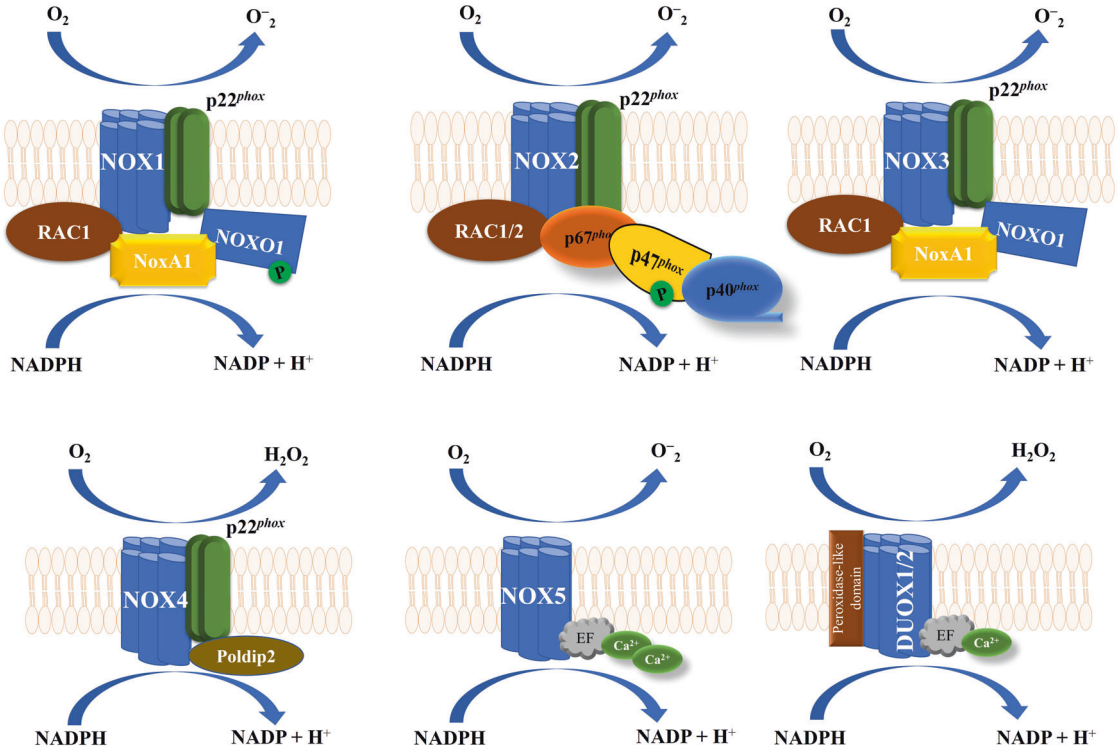

Fig. 5 Assembly of NOX isoforms. NOX2 and NOX2-like isoforms (NOX1, NOX3, and NOX4) are coupled to the membrane subunit $p22^{phox}$. Cytosolic subunits activate NOX1, NOX2, and NOX3 whereas NOX4 activation involves $p22^{phox}$ and Poldip2. NOX5, DUOX1, and DUOX2 require Ca^{2+} binding to their EF-hand domains

It is abundant in the kidneys and various cell types, such as mesangial cells, smooth muscle cells, fibroblasts, osteoclasts, endothelial cells, neurons, and hepatocytes [10, 11, 14, 15].

NOX5

NOX5 has a unique N-terminal domain containing four Ca^{2+} binding sites that help in its activation via additional elongation factor (EF)-hand motifs (helix-loop-helix motifs) [11] (Fig. 5). In fact, emerging evidence suggests that NOX5 is activated when intracellular calcium levels increase in response to calcium/calmodulin-dependent kinase signaling and through posttranslational modifications (phosphorylation, S-nitrosylation, SUMOylation, and oxidation). Once stimulated, Ca^{2+} binds to the C-terminal NADPH domain and transfers electrons to FAD and heme molecules, resulting in the production of superoxide. NOX5 is expressed in the spleen, testis, and endothelial cells [11, 17].

DUOX1/2

DUOX1/2 varies from other NOX isoforms and contains an extra N-terminal domain with peroxidase activity and intracellular EF hand-type Ca^{2+}-binding pockets. Both DUOX1 and DUOX2 have *N*-glycosylation states, a high mannose glycosylated form found in the ER and a fully glycosylated form found at the plasma membrane [18]. Similar to NOX5, DUOX 1 and 2 are calcium-dependent (Fig. 5) [9, 19] and are expressed in the thyroid, respiratory and gastrointestinal tract. In addition to its role in the oxidation of iodide by thyroid peroxidase (TPO), DUOX1/2 plays a key role in host defenses [20]. Mutations in DUOX1/2 disrupt thyroid hormone biosynthesis, leading to congenital hypothyroidism [17, 20].

ROLE OF NOX FAMILY PROTEINS IN INFLAMMATION

NOX family proteins are crucial in regulating antimicrobial host defenses and inflammation. Considering that NOX proteins are responsible for generating ROS, several proteins that trigger innate immune responses affect their expression levels [21–23]. In this context, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), have been shown to modulate NOX expression/activation in various in vivo and in vitro systems [24].

NOX and TLRs

TLRs detect endogenous DAMPs (damage-associated molecular patterns) and exogenous PAMPs (pathogen-associated molecular patterns) to allow ligand-mediated signal transduction that ultimately leads to an inflammatory response [25]. To date, 10 human (Fig. 6) and 13 mouse TLR family homologs have been identified, although TLR10 is known to be nonfunctional in mice [26, 27].

Earlier studies demonstrated a link between TLR activation and NOX homologs [21–23]. In this context, Singh et al. (2017) showed a TLR4-mediated increase in the expression of NOX4, NF-κB, AP-1, and TNF-α in human hepatoma cells and mouse hepatocytes following intraperitoneal (i.p.) administration of LPS (*Escherichia coli* 0127:B8, once per week for 6 weeks) [28]. Another study demonstrated that increased susceptibility to infection and an increase in the mortality rates of cirrhosis patients were due to decreased expression of the NOX catalytic core flavocytochrome b_{558} ($gp91^{phox}$ and $p22^{phox}$) and cytosolic partner-$p47^{phox}$ in patient neutrophils. Furthermore, superoxide production by neutrophils in these patients was shown to be restored by treatment with a TLR7/8 agonist [21], thus indicating the interdependence of these two signaling pathways.

Reports further suggest that TLR-dependent NOX-mediated ROS production could induce neutrophil extracellular trap (NET) formation in vivo [29–31]. In this regard, Al-Khafazi and coworkers (2016) demonstrated increased citrullination of histone H3 (a

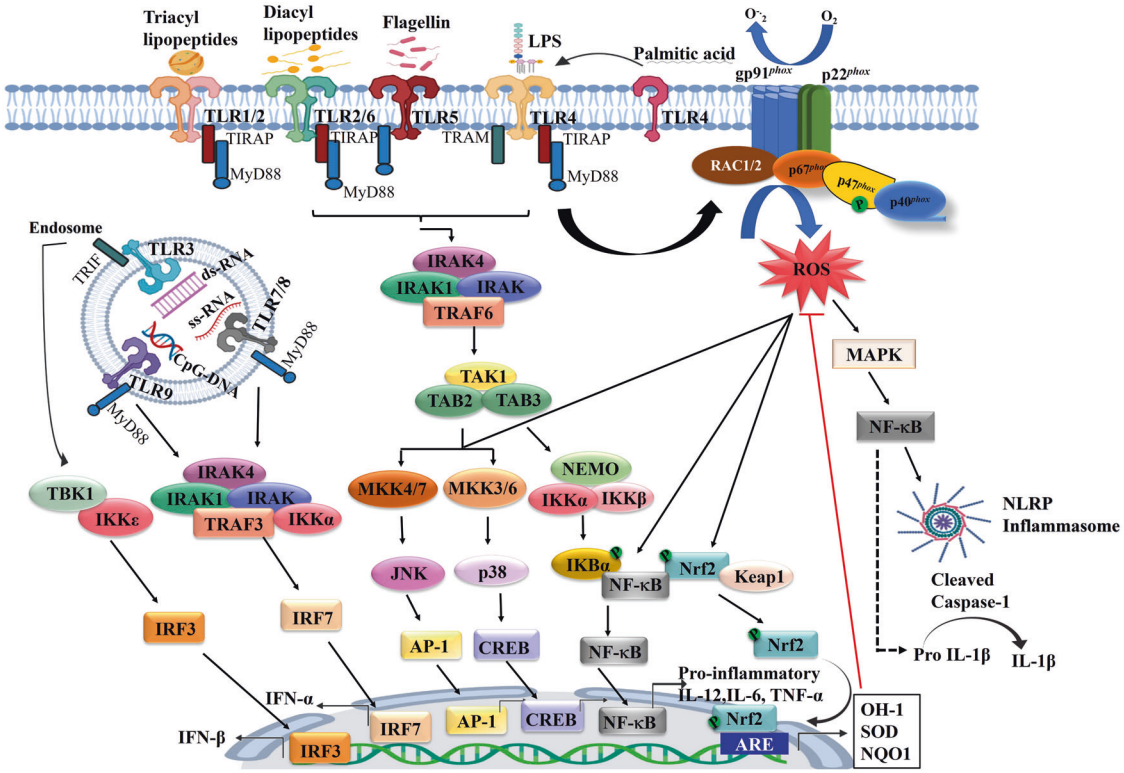

Fig. 6 Crosstalk between NOX-mediated ROS production and downstream inflammatory mediators. TLRs are stimulated by their respective ligands and activate downstream molecules, leading to the activation of nuclear factor-kappa B (NF-κB), interferon (IFN) response factors (IRFs), and mitogen-activated protein kinases (MAPKs). The activation of NF-κB, IRFs, and MAPKs causes a significant increase in cytokines [interleukin (IL)-6, IL-8, IL-12, and tumor necrosis factor-alpha (TNF-α)]. TLR-dependent NOX-generated $O_2^{\bullet-}$ activates the MAPK signaling pathway and induces NF-κB translocation from the cytosol to the nucleus, where it promotes the synthesis of pro-inflammatory cytokines. Additionally, ROS upregulate and activate the NLRP3 inflammasome via MAPK and NF-κB, inducing the release of proinflammatory IL-1β and IL-18 cytokines following caspase-1 cleavage of their pro-forms

specific marker of NET formation) and myeloperoxidase (MPO)-DNA complex formation in neutrophils isolated from xanthine oxidase- and hypoxanthine-treated C57BL/6 mice. This group further reported that inhibiting superoxide generation by allopurinol or NOX by diphenyleneiodonium prevented TLR4-dependent NET formation in vivo [32].

NOX and NLRs

The NLR family is known to mediate innate immune responses to cellular injury or stress [33]. The NLR family is composed of twenty-two members, consisting of nucleotide-binding oligomerization domain (NOD), caspase recruitment domain (CARD), pyrin domain (PYD), and leucine-rich repeat (LRR)-containing proteins that have an N-terminal pyrin domain. Among NLR family members, NLRP1, NLRP2, NLRP3, NLRP4, NLRP6, NLRP7, and NLRP12 have been shown to form multiprotein complexes known as inflammasomes. Inflammasomes are responsible for caspase-1-mediated maturation and the secretion of proinflammatory cytokines such as IL-1β and IL-18 (Fig. 6) [34–37]. The NLRP3 inflammasome is by far the most studied and has the most complex signaling [33]. In fact, dysregulated NLRP3 inflammasome activation has been implicated in the pathogenesis of several inflammatory diseases [38]. Thus, the regulation of the NLRP3 inflammasome during inflammation and/or infection plays a critical role in providing adequate immune protection without causing tissue damage in the host [35]. Evidence from the literature suggests the involvement of signaling pathways such as ionic flux, mitochondrial dysfunction, ROS generation, and lysosomal damage in the activation/assembly of the NLRP3 inflammasome [39–41].

Ulcerative colitis (UC) is associated with dysregulated NLRP3 inflammasome formation that is triggered by excessive ROS generation and the activation of the NF-κB pathway [37]. The interdependence between NOX4-mediated ROS generation and NLRP3 inflammasome activation has been demonstrated in the pathogenesis of oral submucosal fibrosis (OSF) using in vitro (human oral mucosal fibroblasts; HOMFs) and in vivo models (Sprague Dawley rats aka SD rats) in a study conducted by You et al. [42]. This group demonstrated an increase in ROS levels and induction of the NLRP3 inflammasome/IL-1β axis in arecoline-treated SD rats and HOMFs. Interestingly, arecoline-induced inflammation was reduced in HOMFs that were pretreated with either the NOX4 inhibitor VAS2870, the ROS scavenger N-acetylcysteine (NAC), or NOX4-small interfering RNA (siRNA) [42]. These observations provide evidence of arecoline-induced ROS-mediated NLRP3 inflammasome activation in vitro.

A similar association was found between NLRP1 and NOX2 in hippocampal neuronal damage. NOX2 is a major contributor to oxidative stress in the brain, and the NLRP1 inflammasome has been shown to induce the production of proinflammatory molecules in neurons [43–45]. Thus, Xu and colleagues (2019) demonstrated that NLRP1 signaling was responsible for the induction of neuronal damage during prolonged culture (6, 9, and 12 days) in primary hippocampal neurons. This study demonstrated that the upregulation of NLRP1, Apoptosis-associated speck-like protein (ASC), caspase-1 and IL-1β expression during neuronal senescence was triggered by NOX2-mediated ROS generation in hippocampal neurons (12-day culture), highlighting the role of NOX proteins in regulating NLR-mediated signaling [46]. In another study, Sun and colleagues

reported that the inhibition of NOX2-NLRP1 signaling by tempol (ROS scavenger) and apocynin (NOX inhibitor) ameliorated damage to dexamethasone-induced primary hippocampal neurons isolated from male ICR mice [47]. These findings further demonstrate the potential crosstalk between NOX proteins and NLR signaling. In fact, similar associations between NLR and NOX have been shown in various diseases, including Parkinson's disease, atherosclerosis, and inflammatory bowel disease [48–50].

Overall, there is sufficient evidence suggesting that TLR- and NLR-mediated inflammatory responses are regulated by ROS produced by NOX enzymes. Despite these studies, substantial work needs to be conducted to identify the therapeutic molecules that specifically target NOX enzymes to modulate downstream signaling and prevent tissue damage caused by uncontrolled inflammation. One of the major challenges in targeting the NOX system is their ubiquitous presence and indispensable nature. In the subsequent sections, we will summarize the roles of NOX enzymes in various disease states and review NOX inhibitors that are currently in trials to be used in the clinic.

ROLE OF NOX FAMILY PROTEINS IN NEURODEGENERATIVE DISEASES

Neurological disorders (NDs) are the leading cause of disability and the second-largest cause of death worldwide [51]. NDs are recognized as multifactorial disorders that are characterized by progressive neuronal damage in distinct brain areas [52–56]. Although the basic mechanisms of each of these disorders remain unknown, it is clear that oxidative stress is the key factor leading to NDs [57, 58].

Due to their high oxygen demand and relatively low levels of antioxidants, neurons are at high risk of oxidative stress. The crucial role of oxidative stress in the pathogenesis of NDs is associated with the dysregulation of several proteins (e.g., α-synuclein, DJ-1, amyloid β, and tau protein) and signaling pathways [including the extracellular protein kinase and phosphoinositide 3-kinase (PI3K)/protein kinase B pathways]. Since NOX proteins are the most significant sources of ROS, their activation and subsequent superoxide production play a vital role in neurotoxicity [59–61]. We discuss the currently available literature elaborating the role of NOX enzymes in the occurrence of various NDs in the following subsections and Table 1.

Alzheimer's disease (AD)

AD is a rapidly growing medical problem associated with neurological deficits such as memory failure, loss of cognitive function, and personality change. The main pathological characteristic of AD is the presence of extracellular β-amyloid (Aβ) plaques that are capable of activating microglial cells and generating ROS. In this context, a significant role of the NOX2 enzyme has been identified in the pathogenesis of AD [62]. Chay and colleagues observed an increase in the expression level of $gp91^{phox}$ in $Aβ_{25-35}$ (a fragment of Aβ with an equivalent neurotoxic effect)-treated mixed cortical cell cultures (neurons and astrocytes), proving the involvement of the NOX2 enzyme in increased ROS production in vitro. Importantly, $Aβ_{25-35}$-induced neuronal death was attenuated by treatment with NOX inhibitors [apocynin and AEBSF (4-(2-aminoethyl)-benzene sulfonyl fluoride)], which suggested the potential of targeting NOX enzymes to treat AD [63]. Furthermore, Aβ-oligomers have been shown to activate β1-integrin receptor-mediated activity in cultured astrocytes, inducing cytosolic Ca^{2+} levels, activating PI3K/PKC/Rac/NOX2 signaling, and resulting in the upregulation of NOX2 expression in an in vivo model of AD (Fig. 7) [64].

Similarly, chronic (6 weeks) i.p. administration of scopolamine in male Wistar rats (in vivo model for AD) resulted in the overexpression of NOX2, Nrf2, and NF-κB in the hippocampal regions of the rat brain. In contrast, these effects were reversed by treatment with apocynin (16 mg/kg/day) or galantamine (1 mg/kg/day) during the last 3 weeks of scopolamine administration, as indicated by low Aβ production and diminished levels of oxidative stress [65].

At the molecular level, NOX-mediated ROS production has been shown to affect capillary constriction during AD. Cerebral blood flow is reduced due to vascular constriction during AD. Nortley et al. (2019) observed that constriction in capillaries specifically by pericytes results from excessive NOX-4-mediated ROS generation in brain slides of humans and rats with AD. Excessive ROS trigger the release of endothelin-1 (ET_A), which acts on ET_A receptors to induce pericyte contraction, thereby resulting in reduced cerebral blood flow and a diminished supply of oxygen and glucose to the brain (Fig. 7) [66].

Parkinson's disease (PD)

The major hallmark of PD is the loss of dopaminergic neurons in the midbrain region (substantia nigra) resulting from α-synuclein accumulation (Lewy bodies) [67–70]. It has been demonstrated that α-synuclein binds to the microglial P2X7 receptor, which leads to PI3K/Akt activation and the upregulation of oxidative stress, ultimately resulting in neurodegeneration [71]. Interestingly, NOX2 activation produces extracellular superoxide and intracellular ROS (iROS). iROS are important second messengers that influence downstream signaling pathways such as STAT, MAPK, and NF-κB, thereby increasing the production of proinflammatory factors, resulting in microglial M1 polarization (the transition from beneficial M2 to deleterious M1 phenotype) and eventually causing neuronal damage [72–74].

In vitro and in vivo studies indicate that genetic deletion of $gp91^{phox-/-}$ or $p47^{phox-/-}$ or pharmacological inhibition of NOX2 leads to dopaminergic neuroprotection in C57BL/6 J mouse models of PD, suggesting a potential target for PD intervention [75, 76].

Amyotrophic lateral sclerosis (ALS)

ALS is a severe paralytic condition characterized by the loss of motor neurons in the cerebral cortex, brain stem, and spinal cord and ultimately leading to heart defects and respiratory failure. The commonly used animal model for studying ALS is mice bearing the human gene for the mutant Cu^{2+}/ Zn^{2+}-SOD enzyme 1 (SOD1) [77, 78]. Under normal conditions, SOD1 regulates NOX-dependent $O_2^{\cdot-}$ production by binding to Rac1. However, in ALS, the mutant SOD1 has a glycine-to-alanine substitution at position 93 (G93A) that leads to increased Rac1/NOX activation and the overproduction of ROS [79, 80]. In the spinal cords of SOD1-G93A mice, the transcription and translation of NOX2 and its subunits ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1) were significantly increased with disease progression. In fact, an increase in the expression of the NOX3 isoform has also been observed in these mutants, linking disease pathology to a dysregulated ROS-generating system in vivo [81].

These studies provide evidence for the involvement of NOX proteins and related pathways in regulating the onset and progression of various neurodegenerative conditions and highlight the need for further exploration of these molecules to design better interventions in the future.

ROLE OF NOX ISOFORMS IN PULMONARY DISEASES

The primary role of the lungs is to promote gaseous exchange across alveolar membranes. Oxygen is essential for biological life, and oxygen breakdown by aerobic respiration contributes to the generation of ROS, including $O_2^{\cdot-}$ and H_2O_2 [82]. NOX family proteins are widely expressed in different airway cells and play key roles in the maintenance of physiological processes in the airways. Dysregulated NOX activation can lead to various acute and chronic lung-related pathologies [83–89]. Therefore, an improved

Table 1. NADPH oxidase inhibitors in neurodegenerative diseases. Section 1: in vitro studies. Section 2: in vivo and clinical studies

Disease	Study Model	Type of treatment/disease model	NOX inhibitor/ Neuroprotective agent	Major Findings	References
Parkinson's Disease	murine microglial BV2	LPS (1 μg/mL); α-synuclein (5 μM); 6 h	Hydroxytyrosol (1, 10, 25, and 50 μM)	LPS-induced microglial activation, NOX subunits and MAPK expression, production of ROS. These effects were abrogated by hydroxytyrosol.	[68]
Parkinson's Disease	murine microglial BV2	α-synuclein (0, 50, 100, and 200 nM; 0, 15, 30, and 60 min)	None	Integrin CD11b mediates α-synuclein-induced NOX2 activation through a RhoA-dependent pathway	[69]
Parkinson's Disease	murine microglial BV2	2,5-hexanedione (HD) (1, 4, 8, and 16 mM; 1, 3, and 5 weeks)	Apocynin	Inhibition or genetic deletion of $\alpha_M\beta_2$ reduced NOX2-generated superoxide and $p47^{phox}$ membrane translocation.	[60]
Alzheimer's Disease	mouse mixed cortical cultures	$A\beta_{25\text{-}35}$ (10, 20, 40, and 80 μM; 24 and 48 h)	Apocynin (1 mM), AEBSF (4-(2-aminoethyl)-benzene sulfonyl fluoride) (50 μM)	$A\beta_{25\text{-}35}$ increased neuronal death, iROS, and the expression of $gp91^{phox}$. These effects were inhibited by apocynin and AEBSF.	[63]
Alzheimer's Disease	cortical neurons and astrocytes	Tau (300 nM, s oluble and insoluble fractions of early time-points (30 min, 1.5, 3 or 7 h) or late time-points of tau aggregation (1, 3, 7, 14, 26 days)	AEBSF (20 μM; 20 min)	AEBSF abrogated Tau (late insoluble aggregate) -mediated NOX-dependent ROS production.	[53]

Disease	Sample size	Study Model	Type of treatment /disease model	NOX inhibitor/ Neuroprotective agent	Major Findings	References
Alzheimer's Disease	$n = 23$	Both genders (2 months old) TgCRND8 mice (mutant human APP)	Double mutation K670N/ M671L + V717F	None	Increased expression of NOX subunits $p47^{phox}$ and $p67^{phox}$, PKC-α in the cerebellum of transgenic mice.	[54]
Alzheimer's Disease	$n = 38$ humans and $n = 24$ mice	AD patients (both genders); 3xTg-AD mice	Aβ-oligomers (5 μM; 6 and 24 h)	DPI (0.5 μM)	DPI pretreatment abolished Aβ induced expression of NOX2, NOX1, and the glial fibrillary acidic protein (GFAP).	[64]
Alzheimer's Disease	$n = 56$	Female Nulliparous SD rats	$A\beta_{1-42}$ (10 mM; intracerebroventriculy injected)	Electroacupuncture (once a day for 30 min, 28 days)	Electroacupuncture attenuated abnormal increase in the levels of ROS, MDA and 8-OH-dG; ameliorated neuronal injury and counteracted aberrant increase of NOX2 levels in the hippocampus of the AD rats	[56]
Amyotrophic lateral sclerosis	$n = 8$	Male B6. Cg-Tg mice	(SOD1-G93A)1Gur/J mice	Clemastine (10 mg/kg 5 times/week; i.p starting from 40 days of age)	Clemastine decreased the expression of proinflammatory M1 markers; NOX2 and ERK1/2 activity, and simultaneously increased M2 anti-inflammatory markers such as arginase-1 and BDNF.	[78]
Amyotrophic lateral sclerosis	$n = 9$	ALS patients (both genders); B6SJL-Tg mice	(SOD1-G93A)1Gur/J mice	Thioridazine (10 mg/kg; i.p)	In murine model, the expression of NOX2 and its subunits- $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1; NOX3 and DUOX1 subunit; and DUOXA1 were significantly increased. Increased levels of NOX2 was observed in different regions of the spinal cord of human subjects. Thioridazine decreased the generation of ROS in the spinal cord of SOD1-G93A mice, by inhibiting NOX2.	[81]
Parkinson's Disease	$n = 14$	Male SD rats	LPS (5 μg/ 5 μL; injected in substantia nigra; 21 days)	Apocynin (10 mg/kg; i.p)	Apocynin improved redox ratio, as shown by a substantial increase in the (GSH/GSSG) levels, decreased NF-κB, TNF-α, IL-1β, caspase-3, caspase-9 expression in LPS-treated SD rats.	[67]

Table 1. continued

Disease	Sample size	Study Model	Type of treatment /disease model	NOX inhibitor/ Neuroprotective agent	Major Findings	References
Parkinson's Disease	$n = 220$	Male SD rats	AAV9-α-syn A53T vector for overexpression of α-synuclein	Telmisartan; candesartan (1 mg/kg/day)	NOX, CD68, M1 microglial phenotype markers (inducible NO synthase, TNF-α) and number of OX-6 positive microglial cells were induced in the study model. In addition, major decline in the expression of M2 microglial phenotype markers such as the enzyme arginase 1 was observed. These effects were attenuated by AT1 blockers candesartan or telmisartan.	[72]
Parkinson's Disease	$n = 24–30$	C57BL/6 J or gp91$^{phox-/-}$ mice	LPS (15 × 106 EU/kg, i.p) or MPTP (15 mg/kg, sc. for 6 consecutive days)	Apocynin, DPI	Microglia activation preceded astroglia in the substantia nigra upon treatment with LPS or MPTP. Inhibition by apocynin/DPI in mice attenuated astrogliosis.	[75]
Parkinson's Disease	$n = 24–30$	Male C57BL/6 J mice	Paraquat (10 mg/kg) and maneb (30 mg/kg for consecutive 6 weeks; twice per week)	Taurine (150 mg/kg; i.p)	Taurine significantly reduced paraquat and maneb-induced microglial activation, polarization with M1, release of proinflammatory cytokines, activation of NOX in mice's brainstem.	[74]
Parkinson's Disease	$n = 40–60$	Male C57BL/6 J mice	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) (50 mg/kg), Paraquat (10 mg/kg) and maneb (30 mg/kg for consecutive 4 weeks; twice per week)		DSP-4 treatment along with paraquat and maneb decreased GPx4) and glutathione content, increased lipid peroxidation and the expression of gp91^{phox} and p47^{phox}. In addition, exaggerated microglial activation and polarization with M1	[73]
Parkinson's Disease	$n = 30–40$	Male Wistar rats	6-hydroxydopamine (OHDA) (10 μg/2 μL; intrastriatal injection)	Ang1-7 (240 pg/0.5 μL; intrastriatal administration)	Ang1-7 upregulated MASR, PI3K/Akt/CREB/BDNF/TrKB and downregulation/AT-1R/MAPK p38/NF-κB p65/NADPH oxidase pathway	[55]
Parkinson's Disease	$n = 30–36$	Male C57BL/6 J mice	MPTP (20 mg/kg; i.p. for four times at 2 h intervals)	Capsaicin (0.01, 0.1, 0.5, 1 and 2.5 mg/kg; i.p for 1 day)	Capsaicin reduced expression of TNF-α, IL-1β, NADPH oxidase, inducible NO synthase or reactive astrocyte-derived myeloid peroxidase.	[61]
Parkinson's Disease	$n = 18–27$	Male C57BL/6 J mice	Paraquat (10 mg/kg) and maneb (30 mg/kg for consecutive 6 weeks; twice per week)	Taurine (150 mg/kg, i.p, 5 μl/g body weight)	Taurine inhibited NOX2 activation by interfering with cytosolic subunit, p47^{phox}, and NF-κB translocation.	[70]
Parkinson's Disease	$n = 80$	Both genders (60–72 years)	PD patients	None	Increased serum concentrations of NOX1, ferritin, and Selenium in PD patients	[58]
Parkinson's Disease	$n = 40$	Male SD rats	2,5-hexanedione (400 mg/kg/day; i.p. for 1,3 and 5 weeks)	Apocynin	HD activated NOX2 by inducing membrane translocation of NOX2 cytosolic subunit, p47^{phox}, increased superoxide levels. These effects were attenuated by apocynin.	[60]

MAPK Mitogen activated protein kinase, *ERK* Extracellular-signal-regulated kinase, *LPS* Lipopolysaccharide, *IL-1β* Interleukin-1β, *TNF-α* Tumor necrosis factor α, *MDA* Malondialdehyde, DCF-DA- 2',7'-dichlorodihydrofluorescein diacetate, *DPI* Diphenyleneiodonium, *ETA* Endothelin A, *BDNF* Brain-derived neurotrophic factor, *CREB* cAMP response element-binding protein, *MPTP* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

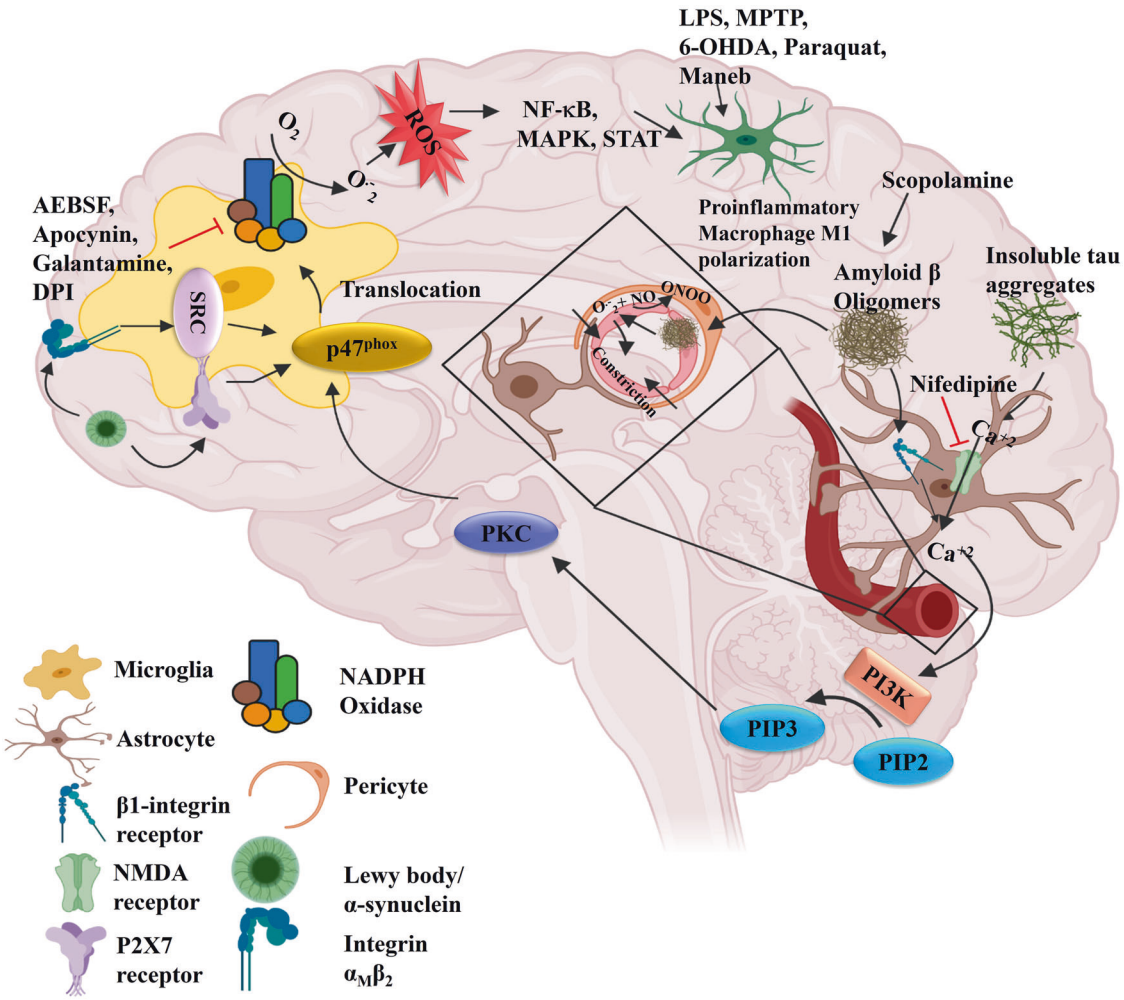

Fig. 7 Role of NOX family proteins in neurodegenerative diseases. Several cell surface receptors, including P2X7, N-methyl-D-aspartate (NMDA), and β1-integrin, can be involved in the activation of astrocytes and microglia by α-synuclein, β-amyloid oligomers, and insoluble tau aggregates. This in turn activates PKC and Src-ERK kinases, which facilitate $p47^{phox}$ phosphorylation, translocation and the subsequent activation of NOX

understanding of the expression, regulation, and inhibition of NOX isoforms is imperative [90]. The role of NOX isoforms in some of the major lung pathologies is discussed in subsequent sections.

Idiopathic pulmonary fibrosis (IPF)

IPF is a progressive and lethal disease. IPF is characterized by excessive scar tissue formation and the destruction of the lung parenchyma, resulting in abnormal gas exchange, which may lead to respiratory failure [91]. There is a plethora of information suggesting a role of intrinsic (genetics, age, sex, microbiome) and extrinsic (cigarette smoke, environmental exposures, air pollution) risk factors in the development and progression of IPF (Fig. 8) [92]. However, physiologically, most of these factors promote ROS formation and lead to biological aging, thereby resulting in gradual disease progression and development. To date, the roles of NOX1-2 and NOX4 have been implicated in the pathogenesis of IPF [82, 93]. A study assessing the role of NOX proteins in bleomycin-induced pulmonary fibrosis showed increased mRNA and protein expression of NOX1/2/4 in the lung tissues of female Kunming mice (Table 2) [94]. In this context, NOX2 has been primarily studied in phagocytic cells (macrophages and neutrophils) and is considered a critical component of the innate immune response. However, another report demonstrated increased expression of $p47^{phox}$ and $p67^{phox}$ in neutrophils isolated from the BALF of IPF patients (15 patients) compared to healthy controls (7 patients) [95].

Similarly, a study conducted by Jarman and colleagues provides evidence that NOX4-dependent ROS generation results in alveolar epithelial cell damage in IPF. This group demonstrated that genetic ablation of NOX4 protected against bleomycin-induced pulmonary fibrosis in male SD rats with a concomitant reduction in alveolar epithelial cell apoptosis [96]. Additionally, pharmacological targeting of NOX4 with a dual NOX4/NOX1 inhibitor (GKT136901) significantly reduced TGF-β1-mediated ROS generation in murine primary alveolar epithelial cells [97]. Overall, these studies suggest that NOX4 may be expressed and activated in different types of lung cells and can contribute to fibrogenic responses [98].

Acute lung injury (ALI)

ALI is a heterogeneous disease characterized by the loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration, and the release of proinflammatory and cytotoxic mediators. The etiological factor for ALI is typically parenchymal lung infection or hemorrhage [99]. Early studies revealed that NOX2-mediated ROS generation also plays a central role in the pathophysiology of ALI [100–102]. In this context, the role of glucose-6-phosphate dehydrogenase (G6PDH), a key

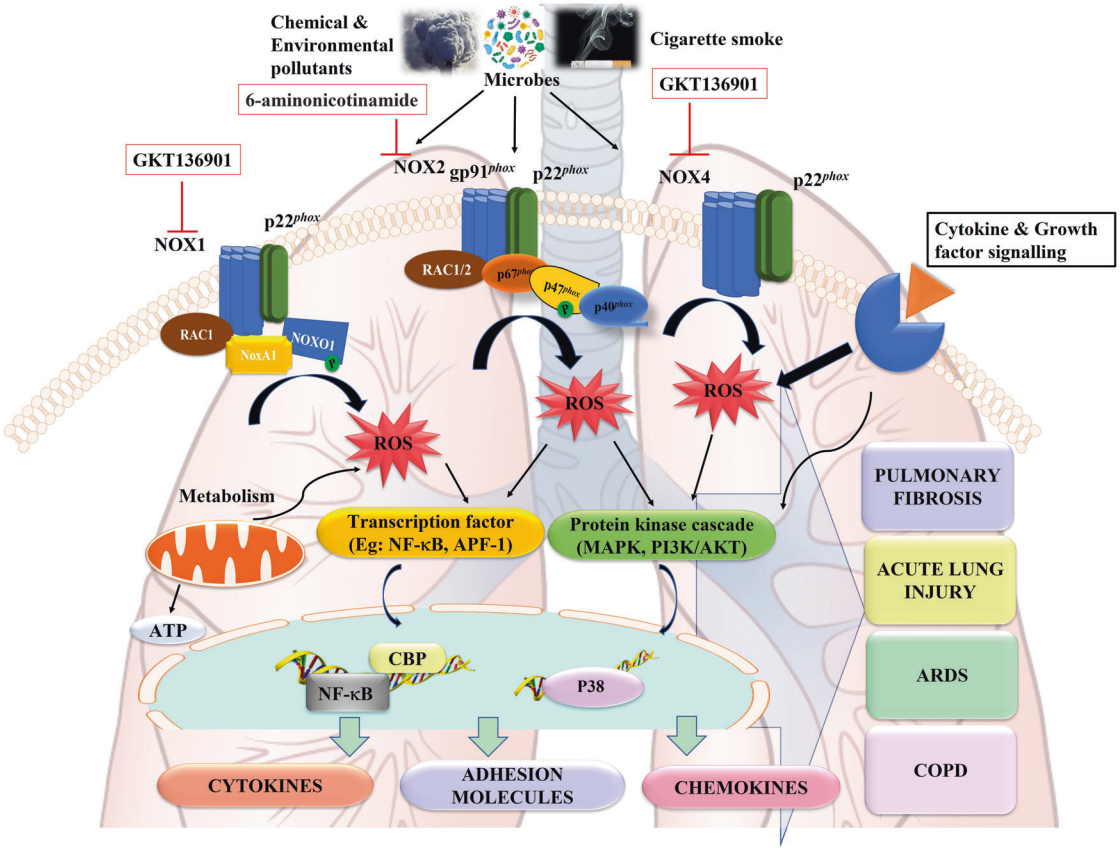

Fig. 8 Role of NOX family proteins in lung diseases. Exposure to pathogens, cigarette smoke, chemicals, and environmental pollutants induce different NOX isoforms. NOX-derived ROS in turn activate transcription factors and MAPK signaling, resulting in the release of several cytokines and chemokines. NOX inhibitors abrogate responses induced by several stimulants

enzyme involved in the regulation of redox balance through the reduction of $NADP^+$ to NADPH, has been documented. The G6PDH enzyme not only provides reducing equivalents to NOX but also scavenges ROS [103]. In 2018, Nadeem and colleagues demonstrated that G6PDH activation regulated ROS flux through NOX2 in alveolar epithelial cells (AECs) during LPS-induced ALI in male BALB/c mice. Furthermore, pharmacological inhibition of G6PDH by 6-aminonicotinamide was shown to inhibit NOX2-derived ROS and subsequent oxidative stress (Fig. 8 and Table 2) [104].

Acute respiratory distress syndrome (ARDS)

ARDS is a serious noncardiogenic syndrome characterized by impaired alveolar liquid clearance, fluid hyperpermeability, increased production of proinflammatory factors, and increased expression of the adhesion molecules needed for leukocyte recruitment and neutrophil migration across the endothelium into the lung [105]. Activated neutrophils induce tissue damage by secreting cytotoxic agents such as granular enzymes, ROS, and bioactive lipids. Oxidative stress can cause endothelial and epithelial barrier dysfunction, resulting in massive neutrophil penetration across the barriers followed by the secretion of cytotoxic agents. Increased ROS production upregulates the expression of proinflammatory cytokines and adhesion molecules and amplifies tissue damage and pulmonary edema. A healthy oxidant-antioxidant balance is therefore essential for vascular homeostasis [106]. The analysis of lung sections and BALF from patients with ARDS showed massive accumulation of PMNs (polymorphonuclear leukocytes), especially neutrophils, in the lungs. These cells produced very high levels of ROS that exacerbated the inflammatory response. Interestingly, these ROS were NOX1- and NOX2-dependent [107]. Therefore, the factors underlying the overproduction of ROS can be therapeutic targets for treating ARDS.

Chronic obstructive pulmonary disorder (COPD)

COPD is a complex disease characterized by airflow limitation, inflammation, and airway remodeling [108]. It is well recognized that oxidative stress is one of the major causes of COPD. An increase in ROS production is involved in changes in vasoreactivity, endothelial dysfunction, and vascular remodeling, including vascular cell proliferation and vasoconstriction [109]. Clinically, it has been shown that increased NOX4, α-SMA, and TGF-β1 expression is associated with an increase in the volume of the pulmonary vascular wall in COPD lungs. Furthermore, COPD patients show an increase in serum malondialdehyde and a decrease in SOD expression/activation [110]. Importantly, NOX4 is a relevant NOX homolog in human airways that can be induced by transforming growth factor-beta (TGF-β1) in human pulmonary artery smooth muscle cells (HPASMCs) (Table 2) [111]. NOXO1 has been shown to be upregulated in both clinical samples and animal models of COPD, while NOXO1-knockout mice were protected against tobacco smoke-induced pulmonary hypertension and emphysema [112]. These studies underscore the importance of NOX family proteins in COPD.

ROLE OF NOX ISOFORMS IN CARDIOVASCULAR DISEASES

NADPH oxidase and its isoforms are important players that mediate vascular diseases, and NOX-generated ROS act as second messengers that regulate vascular function and structure under physiological conditions [113–118]. Of the seven known isoforms

Table 2. Role of NOX-inhibitors in Pulmonary diseases. Section 1: in vitro studies. Section 2: in vivo and clinical studies

Disease	Study Model	Type of treatment/ disease Model	NOX inhibitor/ protective agent	Major Findings	References
Pulmonary arterial hypertension	Human pulmonary arterial EC (HPAECs)	3 weeks of hypoxia (10% O_2), followed by 1 week of normoxia (21% O_2)	NOXA1ds	Presence of NOXA1ds reverted hypoxia-induced H_2O_2 and superoxide production	[185]
Pulmonary arterial hypertension	Rat pulmonary arterial smooth muscle cell (PASMC)	platelet-derived growth factor BB (PDGF-BB) (20 ng/mL; 6, 12, 24, 48, and 72 h)	Liraglutide (1, 5, 10 nM; 6, 12, 24, 48, and 72 h)	Liraglutide reduced PASMC proliferation by inhibiting cellular Drp1/NOX pathways and Atg-5/Atg-7/Beclin-1/LC3-dependent autophagy pathways.	[85]
Pulmonary arterial hypertension	Human microvascular endothelial cells (HMEC-1) and pulmonary artery smooth muscle cells (PASMC)	Dexamethasone (0.1, 1, 10, 100, 1000 nM for 4 h)	GKT137831 (50 μM)	Treatment with the GKT137831 decreased dexamethasone-induced superoxide production, hydrogen peroxide levels. Depletion of NOX2 and NOX4 efficiently diminished superoxide production by dexamethasone in both cell types,	[86]
Pulmonary fibrosis	Human embryonic lung fibroblast (HELF)	TGF-β1 (24 h)	Costunolide (CN) (10, 20 mg/kg; 24 h)	CN suppressed the NF-κB dependent inflammation and regulated TGF-β1/Smad2/ NOX4-Nrf2 signaling pathways	[84]
Chronic lung diseases	Human Alveolar Epithelial Cell Carcinoma (A549)	Leptin (1 μg/mL for 1 h)	Apocynin/NAC (10 μM for 1 h)	Treatment with apocynin or NAC attenuated leptin increased lung cPLA2/COX-2 expression and leukocyte recruitment through the NADPH oxidase/ROS/AP-1 pathway.	[88]
COPD	Human primary artery smooth muscular cells (HPASMCs)	TGF-β1 (1, 2, 5, 10 ng/mL for 0, 6, 12, 24, 36, 48 h)	None	TGF-β1 induced expression of NOX4 and αSMA by various doses and different time periods at both transcriptional and translational level. Whereas the expression of collagen I was increased at protein and in cell culture supernatant.	[110]
COPD	Airway smooth muscle cells (primary cells)	TNF-α (50 ng/mL for 24 h)	GKT137831 (10 μM)	Pre-incubation with NOX4 inhibitor decreased TNF-α induced NOX4 expression.	[87]

Disease	Sample size	Study Model	Type of treatment/ disease Model	NOX inhibitor/protective agent	Major Findings	References
Pulmonary arterial hypertension	$n = 32$	Male SU5416 (Sugen) rat model	3 weeks of hypoxia (10% O_2), followed by 1 week of normoxia (21% O_2)	NOXA1ds	Hypoxia induced NOX1 expression, ROS production, PKA activity, CREB phosphorylation, and CREB:CRE motif binding. These responses were abrogated by selective NOX1 inhibitor NOXA1ds,	[185]
Pulmonary fibrosis	$n = 72$	Male Kunming mice	Bleomycin (5 mg/kg, intratracheal injection)	Costunolide (CN) (10 and 20 mg/kg for 19 days)	Bleomycin increased expression of NOX4, TGF-β1, smad2, NF-κB, IL-6, and IL-17. These effects were attenuated upon administration of CN at both the doses.	[84]
Acute Lung Injury	$n = 24–32$	Male BALB/c mice	LPS (single dose of 50 μg/ 50 μl/mouse intranasally (i.n)	G6PD inhibitor, 6-aminonicotinamide (200 μg/mouse i.n. 1 h before and 12 h after administration of LPS)	LPS induced airway inflammation, bronchoalveolar lavage fluid protein content, NOX2 derived ROS, and consequent oxidative stress were all reduced by G6PD inhibition.	[104]

Table 2. continued

Disease	Sample size	Study Model	Type of treatment/ disease Model	NOX inhibitor/protective agent	Major Findings	References
Acute Lung Injury	$n = 24$	Male SD rats	LPS (3 mg/kg, intratracheal)	Diphenyleneiodonium (DPI) (5 mg/kg, i.p)	DPI mediates antioxidative and anti-inflammatory effects via inactivation of NF-κB, ERK1/2, and SAPK/JNK pathway in LPS-induced ALI.	[202]
Acute Lung Injury	$n = 48$	C3H/HeJ and C57BL/ 10ScNJ mouse	Hyperoxia (100% O_2 in a Plexiglass exposure chamber)	None	NOX3 is regulated by heat shock protein 70 (Hsp70) signaling via a TLR4-Trif-signal transducer and activator of transcription 3 (STAT3) pathway. NOX3 induction leads to increased oxidant injury and death in mice	[89]
Acute Lung Injury	$n = 55$	Male C57BL/6 J mice	ischemia-reperfusion (IR)	GKT137831 (5 mg/kg, subcutaneous injection)	Elevated expression of inflammatory and autophagy genes (LC3 and Beclin-1) following IR was significantly rescued by NOX1/4 inhibitor and contributed towards the protection of lung tissue damage after IR.	[7]
Chronic lung diseases	N.S	Male ICR mice	Leptin (2 mg/kg, intratracheally injected for 0, 4, 24, 48 h)	Apocynin/NAC (2 mg/kg, i.p)	Elevated cPLA2α/COX-2 expression and leukocyte infiltration via the NOX-dependent production of ROS was reduced by apocynin	[88]
COPD	$n = 34$	Both genders (Human Subjects) with COPD	None	None	An increase in expression of NOX4, TGF-β1 and α-SMA was observed in COPD lungs at both transcriptional and translation level.	[110]
COPD	$n = 50$	Human Subjects with COPD	TNF-α (50 ng/mL for 24 h)	GKT137831 (10 μM)	Inhibition of intrinsic NOX4 demonstrating the therapeutic role of NOX-inhibitor in COPD.	[87]

COPD Chronic obstructive pulmonary disease, *ROS* Reactive oxygen species, *H_2O_2* Hydrogen peroxide, *TGF-β1* Transforming growth factor beta 1, *Nrf2* Nuclear factor erythroid 2, *NF-κB* nuclear factor kappa B, *NAC* N-acetylcysteine, *cPLA2α* cytosolic phospholipase A2, *COX-2* Cyclooxygenase-2, *AP-1* Activator protein 1, *α-SMA* α-smooth muscle actin, *CREB* cAMP response element-binding protein, *PKA* Protein Kinase A, *G6PD* glucose-6-phosphate dehydrogenase, *CCL3* Chemokine (C-C motif) ligand 3, *CCR1* chemokine (C-C motif) receptor 1, *LC3* light chain 3.

Table 3. Role of NOX-inhibitors in cardiovascular diseases. Section 1: in vitro studies. Section 2: in vivo and clinical studies

Disease	Study Model	Type of treatment/ disease Model	NOX inhibitor/Cardio protective agent	Major Findings	References
Hypertension	HUVECs	Ang II (100 nmol/L for 24 h)	TMEM16A	Deletion of TMEM16A reduced blood pressure and improved endothelial dysfunction in angiotensin II– induced hypertension. Furthermore, TMEM16A bound directly to NOX2 and inhibited its degradation through the proteasome-dependent degradation pathway.	[128]
Hypertension	HUVECs	Ang II (1 μmol/L for 24 h)	p38 inhibitor-SB203580 (10 μmol/L for 6 h)	Ang II treatment increased ClC-3 expression, ROS production, NADPH activity. While ClC-3 silencing abolished Ang II-induced effects. Overexpression of ClC-3 increased Ang II-induced phosphorylation of $p47^{phox}$ and p38 MAPK in HUVECs, the effects were abolished pretreatment with a p38 inhibitor SB203580.	[131]
Atherosclerosis	Human THP-1 monocyte derived macrophages	LPS (0.1 to 1 μg/mL for 24 h)	HAT inhibitors (CPTH2, C646)	In cultured human macrophages, LPS increased the levels of HAT1, H3K27ac, H3K9ac, and Nox5, as well as the recruitment of p300 and HAT1 at active transcription sites within the NOX5 gene promoter. Inhibition of histone acetyl transferase reduced the NOX5 expression.	[148]
Atherosclerosis	HUVECs	ox-LDL (80 μg/mL for 2 h)	Salvianic acid A (SAA) (pretreatment with 10^{-5} and 3×10^{-5} M for 3 h)	Increased expression of Lectin-like oxidized low-density lipoprotein receptor (LOX)-1, p-NF-κB (p65), VCAM-1, ICAM-1, ROS and NOX4 was observed in ox-LDL-induced HUVECs. SAA treatment significantly inhibited these patterns.	[115]
Atherosclerosis	RAW264.7 (murine Macrophages)	ox-LDL (50 μg/ml for 12 h)	PYR-41 (UBA1 inhibitor) (pretreatment with 5 μM for 2 h)	PYR-41 reduced the aortic expression of IL-1β, IL-6, NOX1, NOX2, and NOX4.	[146]
Atherosclerosis	Mouse monocyte derived macrophages	LPS (100 ng/ml) +INFγ (20 ng); IL-4 (20 ng/ml)	Suberoylanilide hydroxamic acid (SAHA) (5 μM)	In M1-like macrophages increased mRNA levels of MCP-1, TNF-α, TLR4, TLR2, HDAC1, HDAC2, HDAC3,HDAC4, HDAC6, and HDAC11, whereas M2-like macrophages increased CD206 and IL-10. Treatment with SAHA reduced the expression of NOX1, NOX2, NOX4, SOD1 and SOD2.	[147]
Atherosclerosis	HUVECs	ox-LDL; miR-29b mimic	miR-29b antagomir	Both miR-29b mimic and ox-LDL increased the expression of miR-29b, TNF-α, ROS, NOX activity. These effects were augmented upon treatment with miR-29b antagomir.	[116]
Atherosclerosis	HUVECs; rabbit aortic rings	H_2O_2 (1 mM)	Scutellarin (12.5, 50, 200 μM)	Pretreatment with scutellarin increased NO concentration, SOD, GPx, and CAT with decrease in NADPH activity in H_2O_2 challenged HUVECs.	[138]
Atherosclerosis	Human aortic endothelial cells (HAECs)	Flagellin (100 ng/ml)	NOX4 knock-out	Flagellin-induced interaction between NOX4 and TLR5 led to H_2O_2 generation, which in turn induced IL-8 secretion and ICAM-1 expression. Knockdown of the NOX4 resulted in attenuated IL-8 and ICAM-1 expression and reduced adhesion and trans-endothelial migration of monocytes.	[139]

Disease	Sample size	Study Model	Type of treatment/ disease model	NOX inhibitor/Cardio protective agent	Major Findings	References
Hypertension	$n = 47$	Male Wistar rats	Ethanol (5, 10, 20% (v/v) in drinking water for 5 weeks)	Apocynin (10 mg/kg/day, i.p)	Ethanol increased SBP, superoxide anion ($O_2^{\cdot-}$) generation, lipid peroxidation, NOX1, PKCδ, nNOS, SAPK/JNK and SOD; decreased plasma and vascular nitrate/nitrite (NOx) levels. These responses were prevented by Apocynin	[124]
Hypertension	$n = 1768$	Both genders Han Chinese population	None	None	After multiple testing correction, three novel $P67^{phox}$ SNPs were found to be significantly correlated with longitudinal SBP changes. Gene-based studies also showed an association of the $p67^{phox}$ gene with BP changes over time, which is consistent with these results.	[113]

Table 3. continued

Disease	Sample size	Study Model	Type of treatment/ disease model	NOX inhibitor/Cardio protective agent	Major Findings	References
Hypertension	$n = 40–50$	Male C57BL/6 mice	Ang II infusion (1000 ng/kg/min for 1, 2, 3 weeks)	Apocynin (10 mg/kg/day)	Ang II induced BP, aortic wall thickness, collagen deposition, inflammation, oxidative stress, vascular function; and the activation of p38 MAPK, JNK1/2, STAT3 and NF-κB pathway in mouse aorta. Apocynin treatment diminished these deleterious effects.	[122]
Hypertension	$n = 32$	Male Wistar rats	Ang II infusion using mini osmotic pumps (0.4 mg/kg/day for 4 weeks)	Eicosapentaenoic acid (EPA): Docosahexaenoic acid (DHA) 6:1 (daily gavage 500 mg/kg/day for 5 weeks)	Ang II infusion increased the expression of $p47^{phox}$, $p22^{phox}$, COX-1, COX-2, endothelial NO synthase and AT1R; while it resulted in decreased NO-mediated relaxations, Sk_{ca} and connexin 37. Intake of EPA:DHA 6:1 prevented Ang-II induced effects.	[127]
Hypertension	$n = 38$	Male Wistar rats	Ethanol (5, 10, 20% v/v for 6 weeks)	Apocynin ((30 mg/kg/day; p.o. gavage)	Ethanol increased BP, $O_2^{\cdot-}$ generation, IL-10, SAPK/JNK; while NOx levels, eNOS expression were found reduced. These responses were abrogated by Apocynin.	[117]
Hypertension	$n = 16$	Male SD rats	Ang II infusion using mini osmotic pumps (0.7 mg/kg/day for 5 days)	Puerarin (100 mg/kg/day, i.p. once a day for 15 days)	Ang II aborgated phospho-eNOS (Ser 1177) levels; increased SBP, aortic and left ventricular weight, expression of $gp91^{phox}$, $p22^{phox}$, TGF-β and VCAM-1. Puerarin improved EDR and reversed Ang-II-induced effects.	[130]
Hypertension	$n = 32$	Male C57BL/6 J mice	Ang II infusion (400 ng/ kg/min for 4 weeks)	Chloroquine (autophagy inhibitor) (10 mg/kg/day, i.p. for 2 weeks); Treg (T-regulatory) cells (200,000 cells, ip single dose)	Treatment with chloroquine or administration of Treg cells decreased the expression of NOX2, NOX4, phosphorylated eNOS, Akt, AMPK, ATG5, Beclin1, ATG7, mTOR, IL-6 and TNF-α in Ang-II infused mice.	[134]
Hypertension	$n = 42$	Male Dahl rats	High salt diet (8% NaCl)	Hydroxylamine hydrochloride (HA) or GYY4137 (a slow-releasing H_2S compound) injected into bilateral PVN at 9 nmol/h or 2 nmol/h respectively	In high salt-induced hypertensive rats- increased levels of IL-1β, ROS, NOX2, NOX4, mean arterial pressure (MAP), heart rate (HR), and plasma norepinephrine (NE) were observed. GYY4137 treated group showed reduced levels in the observed readouts, compared to HA group.	[123]
Hypertension	$n = 49$	Male SD rats	Chronic immobilization stress and nicotine	Lancemaside A (LMA) (1, 20, or 40 mg/kg for 3 weeks)	LMA reduced aortic expression of NOX2, eNOS, MDA, NF-κB, and MAPK; while increase in serum nitrite levels was reported.	[118]
Hypertension	$n = 56$	Male Wistar rats	High salt diet (8% NaCl for 8 weeks)	Alpha-lipoic acid (ALA) (60 mg/kg by gastric perfusion for 8 weeks)	High salt fed rats showed increase in MAP, plasma NE, cardiac hypertrophy, NOX2, NOX4, MDA and decreased Cu/Zn SOD, and GSH levels. ALA supplementation attenuated hypertension response and cardiac hypertrophy by decreasing NOX2 and NOX4 expression.	[125]
Hypertension	$n = 20–24$	Male C57BL/6 J mice	Ang II infusion using osmotic mini pumps (1.2 mg/kg/day)	Sodium nitrite (50 mg/L for 2 weeks in drinking water)	In ang-II infused mice, sodium nitrite treatment reduced SBP, nitrotyrosine, and NOX4 levels; while increase in plasma nitrite and vascular cGMP was observed.	[132]
Hypertension	$n = 40$	Male SD rats	Chronic intermittent hypoxia (CIH)	GKT137831 (40 mg/kg/day i.p., NAC (30 mg/kg/day, i.p), or Y27632 (10 mg/kg/day i.p)	Hypertension induced by CIH was mediated through NOX4-derived ROS/RhoA/ROCK pathway.	[114]

Table 3. continued

Disease	Sample size	Study Model	Type of treatment/ disease model	NOX inhibitor/Cardio protective agent	Major Findings	References
Hypertension	$n = 36–42$	Male SD rats	Ang II infusion (0.3 nM, PVN microinjection); high fat diet	Intermedin (50 pM), AM22-52 (1 nM).	Intermedin, AM22-52 decreased Ang-II induced NOX activity, ROS levels, protein expression of AT1R, NOX2, NOX4 and ERK activation in PVN.	[133]
Hypertension	$n = 28$	Male Dahl salt-sensitive rats	High salt diet (8% NaCl for 6 weeks)	Pyrrolidine dithiocarbamate (PDTC) (NF-κB inhibitor) (bilateral PVN infusion via osmotic minipump 5 μg/h)	Increase in MAPK, NE, p-IKKβ, NF-κB p65, Fra-like (Fra-LI) activity, NOX4, NLRP3, IL-1β and decreased IL-10 levels was reported in the study model. PDTC delayed the progression of hypertension by attenuating the effects induced by high-salt diet.	[126]
Atherosclerosis	$n = 50$	Male SD rats	High-fat diet (typical diet supplemented with 40% saturated fatty oil and 5% cholesterol for 6 weeks and a single i.p. injection of Vitamin D3 600,000 IU/kg)	Salvianic acid A (SAA) (3, 10 mg/kg/d for 6 weeks)	SAA treatment increased Nrf2 and OH-1 expression; whereas decreased IL-1β, IL-6, TNF-α, $p22^{phox}$, $p47^{phox}$, MDA in high fat diet induced atherosclerotic rats.	[115]
Atherosclerosis	$n = 48$	Male C57BL/6 J mice	$ApoE^{-/-}$ mice; High-fat diet (21% fat and 0.21% cholesterol for 12 weeks)	Polydatin (50 and 100 mg/kg/day intragastric gavage daily for 12 weeks)	Polydatin increased SOD, CAT levels and decreased MDA, NOX2, NOX4, CD68, IL-6 and TNF-α in $ApoE^{-/-}$ mice.	[143]
Atherosclerosis	$n = 12$	Male C57BL/6 J mice	$ApoE^{-/-}$ mice; High-fat diet (0.5% cholesterol and 20% fat for 8 weeks)	PYR-41 (UBA1 inhibitor) (10 mg/kg 2 times/week i.p for 8 weeks)	UBA1 was observed to colocalize with CD68. PYR-41 reduced the aortic expression of MCP-1, VCAM-1, ICAM-1, IL-1β, IL-6, NOX1, NOX2, and NOX4.	[146]
Atherosclerosis	$n = 36$	Male C57BL/6 J mice	$ApoE^{-/-}$ mice; High-fat diet for 10 weeks	suberoylanilide hydroxamic acid (SAHA) (10 mg/kg, i.p every other day for 4 weeks)	In $ApoE^{-/-}$ mice, Class I (HDAC1, HDAC2, HDAC3), Class IIa (HDAC4), Class IIb (HDAC6), and Class IV (HDAC11) expression levels were upregulated. Treatment with SAHA reduced the expression of NOX1, NOX4, CD46, CD68, NOS2 and MMP-9.	[147]
Atherosclerosis	$n = 24$	Male C57BL/6 J	$ApoE^{-/-}$ mice; High-fat diet (21% fat and 0.15% cholesterol for 12 weeks)	NOX2ds-tat peptide (10 mg/kg/day i.p. for 4 weeks)	In $ApoE^{-/-}$ mice (with and without high-fat diet for 12 weeks), increased mRNA levels of IL-1β, NOX2 levels and DNA repair enzymes was observed. While decrease in GPx1, GPx4), peroxiredoxin 1 (PRDX1), SOD-1 was reported. Pharmacological inhibition of NOX2 by NOX2ds-tat peptide decreased ROS production and oxidative modifications of proteins in $ApoE^{-/-}$ mice.	[142]
Atherosclerosis	$n = 240$	Male $ApoE^{-/-}$ $Fgf2^{lmw-}$ mice	High-fat diet (15% cocoa butter and 0.25% cholesterol for 8, 12 and 16 weeks)	$Fgf2^{lmw-}$ mice	Expression MOMA-2, VCAM-1, NOX4, $p47^{phox}$ and MCP-1 was significantly reduced in DKO ($ApoE^{-/-}$ $Fgf2^{lmw-}$) mice compared to $ApoE^{-/-}$ mice	[141]
Atherosclerosis	$n = 20-40$	C57BL/6 mice	$ApoE^{-/-}$ mice; High-fat diet; recombinant FliC (rFliC) (2 h of i.v. injection 25 μg/mouse)	NOX4/ApoE double knockout mice	The expression of ICAM-1, VCAM-1, MIP-2 and keratinocytes-derived chemokine (KC) was induced in rFliC-injected ApoE KO mice. These expressions were abrogated in rFliC-injected Nox4ApoE DKO mice.	[139]

MAPK Mitogen activated protein kinase, *MCP-1* monocyte chemoattractant protein-1, *NF-κB* nuclear factor kappa B, *TGF-β1* Transforming growth factor beta 1, *COX-2* Cyclooxygenase-2, *TNF-α* Tumor necrosis factor α, *MDA* malondialdehyde, *JNK- c* Jun N-terminal kinase, *VCAM-1* Vascular cell adhesion protein 1, *SBP* Systolic blood pressure, *SNP* Single nucleotide polymorphism, *AT1R* Angiotensin II receptor type 1, *HUVECs* Human umbilical vein endothelial cells.

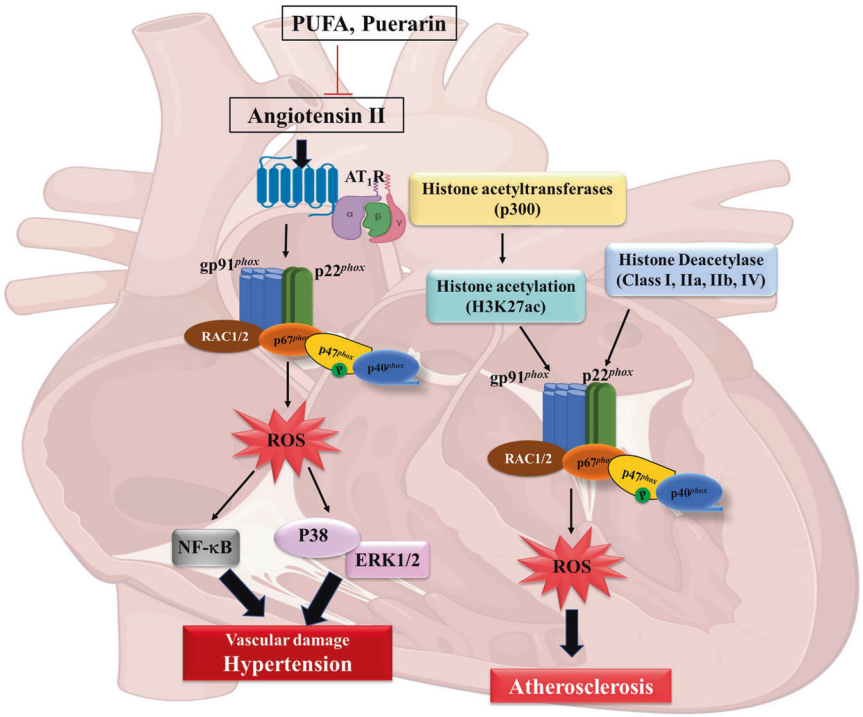

Fig. 9 Role of NOX family proteins in cardiovascular diseases. Angiotensin II activates NADPH oxidase, leading to increased activation of downstream mediators. These events result in vascular damage and hypertension. Furthermore, HDAC inhibition by puerarin or PUFAs reduces ROS production and atherosclerotic lesions

of the NOX family, NOX1, 2, 4, and 5 are known to be expressed in both cardiomyocytes and cardiovascular cells [119–121]. In Table 3, we summarized some of the studies demonstrating that NOX inhibitors can be used to treat cardiovascular diseases.

Hypertension

The renin-angiotensin system (RAS) plays a vital role in hypertension and other cardiovascular diseases. Angiotensin II (Ang II) is a central factor in inducing vasoconstriction, salt retention, and inflammation [122–128]. Upon binding to its type 1 receptor (AT1R), Ang II stimulates ROS production in the vasculature by activating NOX enzymes. The increase in ROS production further leads to the activation of multiple signaling pathways (MAPKs, STAT, and NF-κB), promoting vascular injury and inflammation during hypertension (Fig. 9) [122, 129–133]. In a study conducted by Li and colleagues, increased expression of gp91^{phox} and p22^{phox} was observed in the aortas of SD rats treated with Ang II (0.7 mg/kg/day) [130]. Similarly, Ang II infusion (1000 ng/kg/min) has been shown to increase NOX1 and p22^{phox} expression in male C57BL/6 mice [122].

Radwan and colleagues reported increased oxidative stress, as indicated by enhanced expression of NOX2, NOX4, and NOX activity in the mesenteric resistance artery (MRA) of C57BL/6 J mice infused with Ang II (400 ng/kg/min). These effects were significantly reduced in mice that were administered a single dose of Treg cells (CD4$^+$ CD25$^+$ T regulatory cells, i.p. 200,000 cells) or chloroquine (autophagy inhibitor, 10 mg/kg/day, i.p.) [134].

In fact, ROS modulate cellular signaling by oxidative posttranslational modification of signaling molecules. Under diseased conditions, high concentrations of ROS can lead to irreversible oxidation, such as protein carbonylation (modification of amino acid side chains to carbonyl derivatives) or the formation of sulfinic and sulfonic acids on cysteine residues (SO_2H, SO_3H), resulting in protein damage, degradation, and cell death. Camargo and his group reported an increase in protein sulfenylation and oxidation in spontaneously hypertensive rats (SHRs). Their findings showed an increase in NOX1 expression in membranes and the nuclear/ER fraction of vascular smooth muscle cells (VSMCs) obtained from SHRs [135]. During cellular stress, ROS have been shown to significantly modulate autophagy (eliminate damaged proteins and organelles) [136]. An increase in autophagic flux may also be one of the primary cellular responses during hypertension [137].

Atherosclerosis

Atherosclerosis is a predominant underlying contributor to most fatal conditions, such as myocardial infarction, unstable angina, sudden cardiac death, and stroke [138, 139]. To study the roles of NOX family proteins in atherosclerosis, apolipoprotein E-knockout (ApoE$^{-/-}$) mice is the commonly used study model [140–142]. In a study by Yi and colleagues using ApoE$^{-/-}$ mice, it was reported that the livers of high-fat diet-fed animals showed increases in (a) ROS production and (b) the expression of NOX2 and NOX4 [143].

Another study showed that deficiencies in ubiquitin-like modifier activating enzyme 1 (UBA1) expression or activity correlated with cardiovascular disorders [144]. The ubiquitin–proteasome system (UPS) is the major pathway in eukaryotic cells for intracellular protein degradation. Any disturbance in protein homeostasis can lead to a rapid and transient burst of ROS [52, 145, 146]. Liao and colleagues showed that i.p. administration of PYR-41 (a UBA1 inhibitor) reduced macrophage infiltration and altered plaque composition and necrosis in murine atherosclerotic plaques. Furthermore, PYR-41 suppressed aortic mRNA expression of NOXs (NOX1, NOX2, and NOX4) in ApoE$^{-/-}$ mice fed an atherogenic diet, which correlated with reduced macrophage mortality and necrotic core development [146]. These reports demonstrate that UBA1 is a potential therapeutic target for the treatment of atherosclerosis.

Epigenetic mechanisms play important roles in the expression of NOX isoforms in atherosclerosis. A report by Manea and colleagues provided evidence for the upregulation of multiple isoforms of histone deacetylases [Class I (HDAC1, HDAC2, and HDAC3), Class IIa (HDAC4), Class IIb (HDAC6), and Class IV (HDAC11)] in human carotid arteries and the atherosclerotic

aortas of hypercholesterolemic ApoE$^{-/-}$ mice. HDAC inhibition by suberoylanilide hydroxamic acid (SAHA) (10 mg/kg i.p.) effectively suppressed the expression of NOX1 and NOX4 but not NOX2 in the aortas of ApoE$^{-/-}$ mice. In this study, subtypes of class I, IIa, IIb, and IV HDACs and the NOX isoforms NOX1, NOX2, and NOX4 were shown to be significantly elevated in monocyte-derived proinflammatory M1 macrophages obtained from the spleens of C57BL/6 J mice fed a normal diet. (Fig. 9) [147]. However, another report demonstrated significant upregulation of p300, histone acetyltransferase (HAT)1, histone 3 lysine 27 acetylation, and NOX5 expression in the human atherosclerotic carotid artery and human atherosclerotic plaques. Interestingly, inhibiting HATs (CPT2, C6H6) in LPS-challenged macrophages significantly reduced NOX5 gene and protein expression, while overexpression of p300 or HAT1 improved the function of the NOX5 gene promoter [148].

Intriguingly, crosstalk between miRNAs and NOX4 has also been reported. MicroRNAs (miRNAs) are small noncoding RNA molecules that regulate gene expression in living organisms at the posttranscriptional level. NOX4 inhibition reduced the effects of miRNA-30e on oxidative stress in human umbilical vein endothelial cells (HUVECs) [149]. Furthermore, in patients with atherosclerotic plaques, telomere length was shortened and correlated with an increase in NOX activity and elevated 8-hydroxy-2-deoxyguanosine (8-OHdG) levels (an index of oxidative stress) [150]. Telomere length (the protective caps of chromosomes) is increasingly being used as a marker of health because it has been shown to predict survival chances in a range of endothermic species, including humans. Oxidative stress is thought to be a major cause of telomere shortening. This evidence suggests the involvement of epigenetic changes in the NOX-mediated oxidative stress response in atherosclerosis and warrants further detailed investigation.

ROLE OF NOX ISOFORMS IN RENAL DISEASES

NOX4 is the most abundant NOX isoform in the kidney and has been reported to have a central role in oxidative stress [151–154]. Of the seven NOX isoforms, NOX1, NOX2, NOX4, and recently NOX5 have been identified in glomerular cells (mesangial and podocytes), glomerular endothelial cells, VSMCs, and tubulointerstitial cells [155, 156]. A review of the literature suggests a crucial role of NOX enzymes in the pathophysiology of renal diseases, a brief account of which is given in the following paragraphs.

Acute kidney injury (AKI)

AKI is defined as an abrupt (within 48 h) reduction in kidney function [157]. There are multiple causes of AKI, of which ischemia or nonischemic kidney tissue hypoxia are the most prevalent [158]. Hypoxia not only leads to energy shortages in tissues but also changes intracellular signaling and gene expression. Various mechanisms have been introduced as mediators of hypoxia-induced AKI, including calcium overload, endoplasmic reticulum stress, complement system activation, and ROS generation. Excessive ROS production promotes hypoxia-induced AKI by affecting the function of cellular DNA, proteins, and lipids. Furthermore, alterations in NOX4 expression during hypoxia are thought to affect the progression of AKI. Sung and colleagues demonstrated a significant increase in the expression of NOX4 at both the transcriptional and translational levels in human renal proximal tubular epithelial cells (HK-2) after hypoxic stimulation with $CoCl_2$ (300 μM). Additionally, NOX4-siRNA or pharmacologic inhibition with GKT137831 reduced the production of ROS and attenuated the apoptotic pathway in an ischemia/reperfusion SD rat model (Fig. 10) [159]. Hence, therapies targeting NOX4 can serve as potential strategies in the treatment of AKI.

Chronic Kidney Disease (CKD)

CKD is characterized by a reduction in kidney structure and function over a period of time [160]. The progression of CKD to its advanced stages is associated with a significant increase in the generation of free radicals and other oxidants [161–165]. Impaired mitochondrial function or enhanced mitochondrial ROS production has been suggested to be causes of elevated oxidative stress in CKD [161].

According to previous reports, TGF-β1 upregulates NOX2 and NOX4 and plays a key role in the phenotypic transition of fibroblasts to myofibroblasts and fibrogenesis [166, 167]. However, the role of NOX4 in the development of renal fibrosis is not clear. In an experimental model of unilateral ureteral obstruction (a well-established model of renal tubular stress leading to renal fibrosis), NOX4 deletion in C57BL/6 J mice correlated with a) TGF-β1-mediated tubular cell apoptosis; b) defective hypoxia-inducible factor-1α (HIF-1α) oxygen sensing; c) Nrf2 antioxidant pathways; and d) increased renal fibrosis in obstructed kidneys. Thus, NOX4-mediated regulation of the Nrf2 pathway was responsible for regulating antioxidant function in renal tubular cells (Fig. 10) [168]. These findings provide evidence about the critical role of NOX enzymes in kidney diseases. Hence, it is paramount to understand the role of these enzymes in each disease pathology to identify the specific targets to effectively reverse disease symptoms without causing any side effects.

Diabetic nephropathy (DN) or diabetic kidney disease (DKD)

Increased ROS levels due to hyperglycemia-mediated NOX activation results in oxidative stress that causes kidney tissue damage, leading to diabetic nephropathy (DN) [169–172]. DN is clinically characterized by a progressive increase in albuminuria and a subsequent decrease in the glomerular filtration rate, podocyte apoptosis, and tubulointerstitial fibrosis, which eventually results in end-stage renal disease (ESRD) [173, 174]. Several reports have shown that the upregulation of NOX1, NOX2, NOX4, NOX5, and regulatory subunits ($p22^{phox}$, $p47^{phox}$, and $p67^{phox}$) contributes to mesangial cell hypertrophy, tissue expansion, ECM protein accumulation, and podocyte apoptosis in the glomerulus [155, 174, 175].

The excessive production of ROS caused by hyperglycemia activates several pathways associated with diabetic tissue damage, including advanced glycation end products (AGEs), which serve as ligands for the cellular receptor RAGE [176, 177]. Additionally, the activation of signaling networks by AGEs stimulates NF-κB activity, which induces the generation of proinflammatory cytokines, including IL-6 and TNF-α [178]. AGEs are also considered potent stimulators of VEGF expression and PKC activation, leading to the upregulation of TGF-β1 expression, which in turn is causally linked to the expression of ECM proteins in mesangial expansion [174].

Yao and colleagues demonstrated that NOX4 mediates high glucose-induced apoptosis in the HK-2 cell line via the Notch pathway. Both the protein and mRNA expression of NOX4 and Notch-1 were induced when HK-2 cells were stimulated with high glucose. The inhibition of NOX4 by NAC and diphenylene iodonium (DPI) inhibited Notch signaling and ROS generation, consequently preventing HK-2 cell apoptosis (Table 4) [179, 180].

It should also be noted that chronic hyperglycemia in diabetes stimulates the expression and activation of NOX isozymes in renal tissues, leading to sustained oxidative stress. Various pathways may contribute to the stimulation of NOX-dependent ROS generation, as shown in Fig. 10 and discussed in Table 4.

NOX INHIBITORS

Considering the universal nature of NOX enzymes and their crucial roles in modulating most signaling pathways, these enzymes are being extensively studied as drug targets for therapeutic development in various disease states. In this context, the term “NOX inhibitor” is now used as a generic umbrella term that refers to NOX binders, ROS scavengers, and antioxidant molecules [181]. In

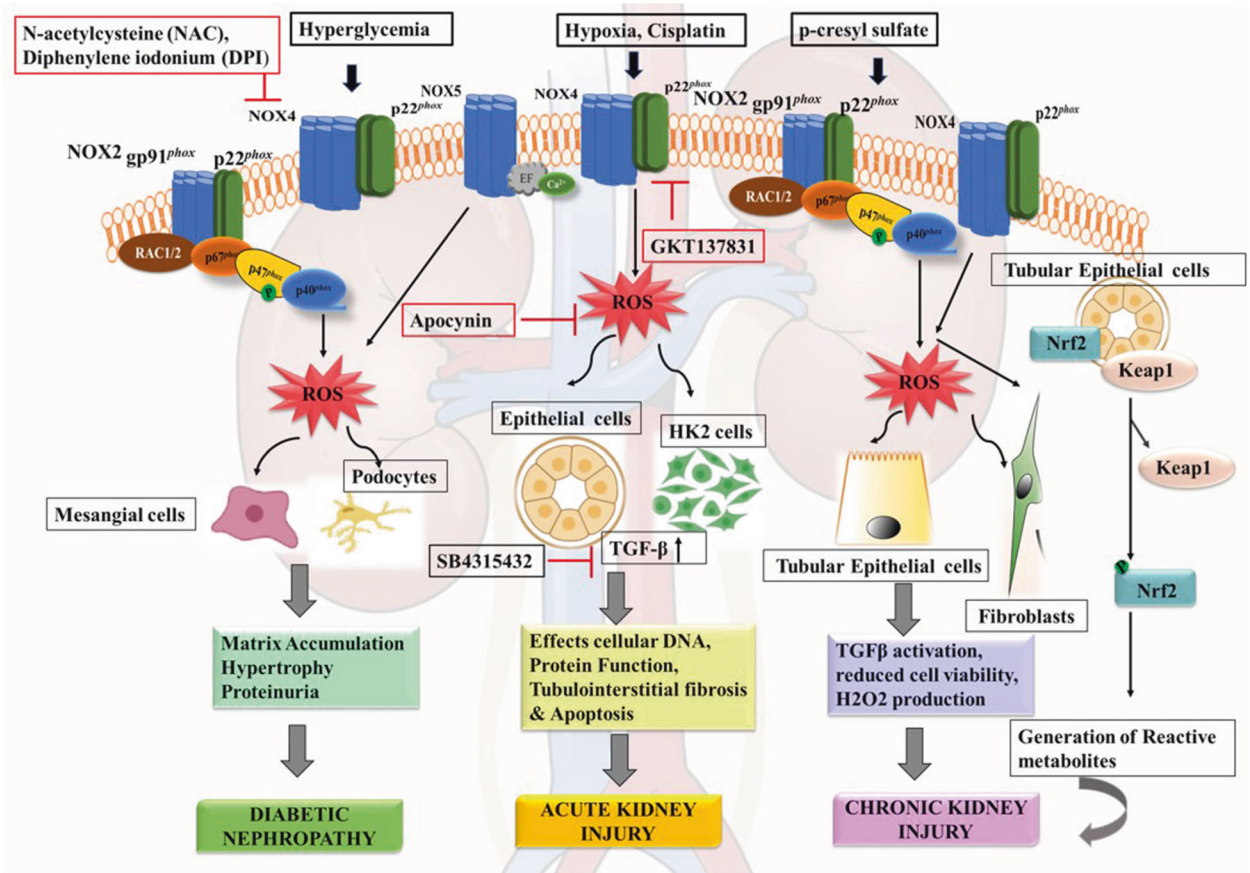

Fig. 10 Role of NOX family proteins in the development of renal complications. Hyperglycemic conditions and inducing agents such as cisplatin, hypoxia, and p-cresyl sulfate activate NOX4 and NOX5 isoforms, which enhances ROS production. The excessive production of NOX-derived ROS contributes to cell and tissue injury and activation of the TGF-β and Nrf2 pathways. These events ultimately lead to renal complications such as diabetic nephropathy, acute and chronic kidney injury. Inhibiting NOX family proteins abrogates these responses

the last 5 years, research focusing on NOX inhibition has received considerable attention, with over 5000 publications referenced from PubMed.gov alone. NOX binders, also referred to as bonified NOX inhibitors, are compounds that bind to a NOX subunit and inhibit ROS production [181–184].

The multi-functional roles of NOXs includes their involvement in several pathways associated with pharmacological reactions, such as inhibiting enzyme assembly or competition with NADPH and O_2 substrates [181]. In this context, particular focus has been paid to the $p47^{phox}$, NOXO1 subunits, as well as the $p67^{phox}$ and NOXA1 activators in combination with the Rac subunit, and the main objective is to develop an enzyme with improved potency and specificity for binding to protein subunits [185]. However, some of the challenges in this area have been the complexity of NADPH oxidase and the low specificity, selectivity, and high toxicity of most NOX inhibitors [5, 182]. The difficulty in detecting and quantifying ROS due to its extremely short half-life, in a biological context has also played a role in slowing down the progress in this field. Thus, our identification of the related mechanism of these inhibitors remains an ongoing project [181].

Currently, only GKT137831 (a specific inhibitor of NOX1 and NOX4) is in human clinical trials (phase 2) for pulmonary fibrosis and cirrhosis [182, 186, 187]. In this section, we will review some of the pharmacologically active NOX enzyme inhibitors that have been recently reported to be useful as therapies.

Apocynin is the most popular and widely investigated nonspecific NOX inhibitor [183]. This drug acts as an antioxidant and a scavenger of nonradical oxidant species [188], and suppresses superoxide release via NOX by preventing $p47^{phox}$ translocation to the membrane, thus interfering with the formation of the functional NOX complex. However, the compound's inhibitory activity occurs only after a time lapse and apocynin does not suppress NOX or the formation of superoxide in cells that lack MPO. Together MPO and H_2O_2 can promote apocynin dimerization (diapocynin), and these dimers can impair the formation of an active enzyme complex [5, 15, 183]. Several studies have shown that treatment of ischemic stroke with apocynin can minimize brain hemorrhage as well as lesion size and restore neurological function. Apocynin has also been shown to cross the blood–brain barrier (BBB) and to exert its effects without hindrance, thus making it a promising drug candidate [5]. However, several off-target effects, such as interference with assays (Amplex Red/HRP interference) and acute cytotoxicity are reason for concern [189].

The triazolo pyrimidines, including the commercially available VAS2870 and its derivatives, such as VAS3947, are selective NOX inhibitors [190, 191]. VAS2870 inhibits NOX2 by preventing the formation of the enzymatic complex and NOX4 by reducing its expression levels. VAS3947 is a more soluble derivative of VAS2870. Both VAS2870 and VAS3947 inhibit NOXs through the covalent alkylation of a conserved active-site cysteine residue, thus affecting the first step of the reaction (NADPH binding and FAD reduction) and thereby blocking NOXs at the beginning of their catalytic cycle [181]. However, the mechanism by which NOX1 and NOX5 isoforms are inhibited remains unclear [183, 192, 193]. Preclinical experiments showed a neuroprotective effect of VAS2870 during stroke caused by transient middle cerebral artery occlusion (tMCAO) in C57BL/6 $NOX4^{-/-}$ mice by inhibiting NOX-induced apoptosis [194]. Furthermore, the

Table 4. NADPH oxidase family proteins as potential therapeutic targets in renal diseases. Section 1: in vitro studies. Section 2: in vivo and clinical studies

Disease	Study Model	Type of treatment/ disease model	NOX Inhibitor/protective agent	Major Findings	References
Diabetic nephropathy	Normal human mesangial cells	High glucose (25 mmol/L), TGF-β1 (5 ng/mL) or mannitol (25 mmol/L for 4 h and 2 days)	NOX5 knockdown	High glucose alone or in combination with TGF-β1 increased the expression of profibrotic and proinflammatory mediators including TRPC6, PKC- α and PKC- β expression. These effects were attenuated by knocking down NOX5.	[174]
Diabetic nephropathy	Podocytes	High glucose (30 mM for 24 h)	Naringin (10 μM for 2 h)	In high glucose-treated podocytes, naringin inhibited NOX4 expression thereby suppressing apoptosis and ROS levels.	[170]
Diabetic nephropathy	Primary vascular smooth muscle cells (VSMC)	High glucose (30 mmol/L for 24 h)	Apocynin (3 mmol/L for 90 min), VAS2870 (5 μmol/L for 90 min) suppressors of cytokine signaling (SOCS1) (100 μg/mL for 2 and 24 h)	SOCS1 and apocynin caused significant reduction in ROS production.	[171]
Diabetic nephropathy	Podocytes	High glucose (30 mM for 72 h)	APX-115 (5 μ M for 60 min before high glucose)	Pretreatment with APX-115 abrogated high glucose-induced expression of NF-κB p65, NOX2, NOX4, MCP-1, and profibrotic molecules including TGF-β1, PAI-1, and collagen IV.	[155]
Diabetic nephropathy	Human proximal tubular epithelial (HK-2) cells	High glucose (5, 15, 30, ad 45 mM and 30 mM for 12, 24, 48 h)	Enzastaurin (3×10^{-4} M)	$p22^{phox}$, NOX4 and PKC-β gradually increased with the increasing concentrations of glucose. These effects were reversed with an inhibitor of PKC-β enzastaurin.	[172]
Acute kidney injury	HK-2 and mouse renal tubular epithelial cells (mTECs)	Cisplatin (20 μM for 24 h)	Apocynin (100 μM) or NAC (50 μM) for 12 h	NOX4 knockdown alleviated Cisplatin-induced cell death and inflammatory response, while opposite effects were observed with NOX4 overexpression. Moreover, N-acetyl-L-cysteine -mediated inhibition of ROS suppressed the cell injury initiated by NOX4-overexpression	[154]
Acute kidney injury	HK-2 cells	LPS (1 μg/ml for 3, 6, 12 h)	RIPK3-deletion by targeted guide RNA (gRNA)	Reduced NOX1 and NOX4 expression was observed in RIPK3-deficient HK-2 cells following LPS-exposure.	[156]
Acute kidney injury	HK-2 cells	Colistin (50 and 100 μg/mL for 10 min, 30 min, 1, 3, 6, 12, and 24 h)	GKT137831 (20 μg/mL), SB4315432 (TGF-β1 receptor I inhibitor)	NOX4 knockdown decreased ROS production, reduced MAPKs activation implicated in colistin-induced nephrotoxicity, and attenuated apoptosis by altering Bax and caspase-3/7 activity. These results were confirmed by pretreatment with GKT137831.	[153]
Acute kidney injury	HK-2 cells	$CoCl_2$ (Hypoxia) (300 μM for 24 h)	GKT137831	Induced ROS levels, caspase-3/7 activation, apoptosis by hypoxia were significantly reduced by NOX4 knockdown or GKT137831 pretreatment.	[159]

Disease	Sample size	Study Model	Type of treatment/ disease Model	NOX inhibitor/ protective agent	Major findings	References
Diabetic nephropathy	$n = 20–24$	Male $SM22^{+}Nox5^{+}$ and $SM22^{+}Nox5^{-}$ mice (FVB/N background)	Streptozotocin (55 mg/kg i.p. 5 times a day for 10 weeks), $SM22^{+}Nox5^{+}$	$SM22^{+}Nox5^{-}$ mice	NADPH-dependent glomerular superoxide production, glomerular intensity of DHE fluorescence and nitrotyrosine accumulation was found in $SM22^{+}Nox5^{+}$ diabetic mice compared with $SM22^{+}Nox5^{-}$	[174]
Diabetic nephropathy	$n = 69$	Male Wistar rat	Streptozotocin	RAGE-aptamer	Continuous infusion of RAGE-aptamer attenuated the development and progression of experimental diabetic nephropathy by blocking AGE-RAGE axis.	[177]
Diabetic nephropathy	$n = 50$	Male SD rat	Streptozotocin (60 mg/kg body weight, tail vein injection for 5 days)	Naringin (20, 40, 80 mg/kg tail vein injection daily for 12 weeks)	Naringin attenuated NOX4 expression, kidney injury and oxidative stress in STZ-induced rats. Down-regulation of NOX4 reduced apoptosis and ROS levels.	[170]
Diabetic nephropathy	$n = 40$	Male ApoE-deficient mice	Streptozotocin (125 mg/kg/day two i.p. injections on consecutive days)	SOCS1-targeted therapies (3 mg/kg/day i.p every second day for 6 weeks)	SOCS1 blocked ROS generation by inhibiting the expression of NOX subunits and the assembly of NOX complex. This effect was mediated by regulated JAK2, STAT1, and PI3K signaling pathways.	[171]

Table 4. continued

Disease	Sample size	Study Model	Type of treatment/ disease Model	NOX inhibitor/ protective agent	Major findings	References
Diabetic nephropathy	$n = 44$	Male mice (C57BLKS/ J-leprdb/leprdb)	Diabetic db/db	APX-115 and GKT137831 (60 mg/kg/day oral gavage for 12 weeks)	APX-115 significantly attenuated NOX1, NOX2, and NOX4 protein expression, whereas GKT137831 attenuated NOX1 and NOX4 expression. Both, TLR4 and NF-κB p65 expression was reduced by APX-115- and GKT137831 in kidney tissues.	[155]
Diabetic nephropathy	$n = 50$	Male C57BL/ KsJ db/db mice	db/db obesity	Zingerone (50 mg/kg/day, i.p for 10 weeks)	In db/db mice, zingerone decreased the MDA levels, NOX4 expression with increase in the content of GSH	[180]
Acute Kidney Injury	N.S	Male Mice	Cisplatin (20 mg/kg, i.p.)	Apocynin (100 mg/kg, i.p)	Apocynin suppressed cisplatin-induced NOX4 expression, downregulated mRNA levels of TNF-α, IL-1β and IL-6 and reduced the protein levels of RIPK1, RIPK3, P-MLKL and cleaved caspase-3.	[154]
Acute Kidney Injury	$n = 16$	Male C57BL/6 N mice	Cecal ligation and puncture (CLP)	$Ripk3^{-/-}$	Elevated NOX4 expression in the kidney tissues of $Ripk3^{+}/^{+}$ mice compared to $Ripk3^{-/-}$ mice. Suggests that RIPK3 regulates the NOX4 expression in the context of sepsis-induced AKI.	[156]
Acute Kidney Injury	$n = 21$	Male C57BL/ 6 mice	Iohexol (i.p 4000 mg/kg)	GKT137831 (40 mg/kg orally once a day for 4 days)	In an iohexol-induced acute kidney injury model, GKT137831 retained structure, reduced the expression of 8-hydroxy-2′-deoxyguanosine (8OHdG) and kidney injury molecule-1 (KIM-1), and reduced TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive cells.	[153]
Acute Kidney Injury	$n = 20$	Female SD rats	Renal ischemia/ reperfusion injury	GKT137831 (10 mg/kg)	GKT137831 pretreatment reduced hypoxia-induced acute renal damage and oxidative stress in rats.	[159]
Acute Kidney Injury	$n = 32–40$	Male BALB/c mice	LPS (10 mg/kg, i.p.)	Spleen tyrosine kinase inhibitor (R406) (10 mg/kg, i.p. 1 h and 12 h after LPS injection)	R406 resulted in reduced IL-6/MCP-1 production by dendritic cells; and iNOS, NOX2 and nitrotyrosine in neutrophils during sepsis-induced AKI	[157]
Chronic Kidney disease	$n = 33$	Female Spontaneously hypertensive rats (SHR)	Adriamycin (2 mg/kg b.w. twice in a 21-day interval, intravenously (femoral vein) under anesthesia)	Losartan (10 mg/kg/day b.w), tempol (100 mg/kg/day b.w)	Losartan reduced and anti-inflammatory defense by restoring NOX2 expression in kidneys. Tempol, on the other hand, was more effective in reducing systemic oxidative stress, proteinuria, MMP-1, and glomerulosclerosis. The combined treatment, however, failed in slowing the progression of ADR-induced nephropathy in SHR.	[162]
Chronic Kidney disease	$n = 344$ rats and $n =$ 24–40 mice	Male Fisher rats and C57BL/6 J mice	Cyclosporine A (15 mg/kg/day, i.p. for 1 month in rats, for mice 30 mg/kg/day, i.p for 4–8 weeks)	Diphenyleneiodonium (0.5 mg/kg/day, i.p. for 1 month) and Apocynin (16 mg/kg/day, p.o. for 1 month)	In rats and mice, chemical and genetic inhibition of NOX2 resulted in prevention of CsA-induced hypoxia independent of regional perfusion (BOLD and DCE MRI, pimonidazole, HIF1-α).	[163]

TRPC6 Transient receptor potential cation channel, *PKC* Protein kinase C, *TGF-β1* Transforming growth factor beta 1, *PAI-1* Plasminogen activator inhibitor-1, *MCP-1* monocyte chemoattractant protein-1, *NF-κB* nuclear factor kappa B, *RIPK3* Receptor-interacting serine/threonine-protein kinase 3, *MAPK* Mitogen activated protein kinase, *LPS* Lipopolysaccharide, *RAGE* Receptor for advanced glycation endproducts, *MLKL* mixed lineage kinase domain-like pseudokinase.

neurotoxicity induced by oxidative stress in uric acid-, indoxyl sulfate-, or methyl guanidine-stimulated bulbospinal rostral ventrolateral medulla (RVLM) neurons is suppressed by VAS2870-mediated inhibition of NOX2 and NOX4 [5]. However, this is still being assessed in preclinical studies and further testing and approval will be needed for clinical application.

DPI is a nonspecific inhibitor of NOXs and has been shown to inhibit nitric oxide synthases, cytochrome P450, flavoenzymes, and xanthine oxidase [181, 183, 195–197]. During ischemia-induced stroke, DPI is thought to protect the brain by actively suppressing cerebral immunological responses and preserving the stability of the BBB. Several in vitro and in vivo studies have been conducted to test the effects of DPI administration in various disease states. For example, DPI administration in a Wistar rat stroke model reduced inflammatory responses and ROS development and decreased the expression of MMP-2 and MMP-9 [198]. DPI administration also reduced lesion frequency and postinjury inflammation in SD rats with spinal cord injury [199] and decreased LPS-induced preoligodendrocyte loss in neonatal rat brain tissue sections [5, 200]. In Parkinson's disease, DPI pretreatment reduced neurodegeneration by attenuating microglia-induced chronic neuroinflammation via NOX inhibition in both LPS- and MPTP-treated C57BL/6 J mice [201]. Similarly, in a recent in vivo study, DPI was shown to attenuate NOX activation during LPS-induced ALI in the lung tissue of rats [202].

Another inhibitor with specificity for multiple isoforms being considered for treatment of neurodegenerative diseases is ebselen. Classified as an antioxidant (PZ 51) and a potent peroxynitrite scavenger [183, 203], the NOX inhibitory mechanism of ebselen mainly involves blocking the interaction between $p47^{phox}$ and $p22^{phox}$, thereby preventing the membrane recruitment of $p47^{phox}$ and $p67^{phox}$ [183, 204, 205]. Currently, ebselen is the drug of choice for the treatment of stroke in Japan [5]. Furthermore, preclinical and phase 3 clinical trials have shown that ebselen may be effective for treatment of acute ischemic stroke [203]. In a streptozotocin (STZ)-induced AD mouse model, ebselen significantly reduced the number of apoptotic neurons by reducing the level of oxidative stress [206]. In addition, in spinal cord injury, the use of ebselen showed a neuroprotective effect by increasing mitochondrial ATP levels and Na^+/K^+-ATPase activity and decreasing the mitochondrial membrane potential (MMP) and mitochondrial apoptosis [5, 207].

Honokiol is another NOX inhibitor used in neurodegenerative therapy. A naturally occurring plant quinoid, honokiol is of interest because it inhibits superoxide production after the respiratory burst. In mouse C2CL2 skeletal myoblasts, honokiol substantially attenuated H_2O_2-induced DNA damage and apoptosis by inhibiting ROS generation [208]. Additionally, honokiol was shown to reduce ROS production and mitochondrial membrane potential and improve hippocampal neuronal viability, spatial learning, and memory in Aβ oligomer-treated C57BL/6 mice [209]. However, the molecular process by which honokiol counteracts the deleterious effects of β-amyloid's in AD is still unclear [5].

Another plant-based inhibitor of interest is plumbagin, a naphthoquinone metabolite found in the roots of black walnut. Plumbagin is believed to act through different cell signaling mechanisms, including NF-κB, Bcl-2, Akt, topoisomerase, STAT-3, nuclear factor of activated T-cells (NFAT), and MMPs [210]. It has been shown to inhibit NOX activity in HEK293 and LN229 cells, which mainly express NOX4 [211], and to protect neurons during focal brain ischemic injury [5]. Treatment of the STZ-induced mouse-AD model with plumbagin improves cognitive function by suppressing astrogliosis and the function of the β-secretase enzyme in an Nrf2/ARE-dependent manner. Plumbagin inhibits NOX4 activation in VSMCs, which explains the antiatherosclerotic effect of this dominant NOX homolog in the study model [5].

Some of the most precise and potent inhibitors for NOX enzymes are peptides such as the NOX2 docking sequence-tat (NOX2ds-tat/gp91 ds-tat), an 18-amino acid chimeric peptide that exclusively inhibits NOX2 [204]. This synthetic, chimeric peptide contains nine amino acids from the HIV-tat sequence (for internalization) and nine amino acids from the NOX2 intracellular B-loop sequence for binding to $p47^{phox}$. This peptide is known to prevent the assembly and activation of NOX2 [183, 212]. Treatment with NOX2ds-tat decreases oxidative neuronal damage and shifts the equilibrium of macrophages toward the M2 type to provide neuroprotection in Traumatic Brain Injury [213]. In this context, NOX2 inhibition by NOX2ds-tat was shown to reduce free radical formation and proinflammatory cytokine production in a rat spinal cord injury model and helped to restore function [211]. Furthermore, NOX2ds-tat was also shown to improve cellular defense by inhibiting rotenone-dependent apoptosis in human neuroblastoma (SHSY-5Y) cells [214].

Several chemical compounds, such as the sulfonylation agent 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), can also be used as NOX inhibitors [215]. AEBSF is an irreversible serine protease inhibitor that inhibits ROS formation from NOXs in macrophages and cell-free systems. AEBSF does not interfere with electron transport or scavenge oxygen radicals during the NOX inhibition process. Instead, it impacts NOX components in the plasma membrane by interfering with the binding of the cytosolic components-$p47^{phox}$ and $p67^{phox}$ [5].

Statins are often used to prevent cardiovascular disease and mortality in high-risk patients by lowering cholesterol levels by inhibiting the enzyme 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) [216, 217]. Recently, the therapeutic effects of statins on the pulmonary system have been the focus of several pulmonary hypertension (PH) studies. In an aortic-banded rat PH model, simvastatin lowered brain plasma ROS levels and the expression of NOX2 [218]. Similarly, the administration of simvastatin attenuated pulmonary expression of NOX2 regulatory subunits ($p47^{Phox}$ and $p67^{Phox}$) and the generation of ROS [218]. Moreover, simvastatin inhibited the NOX/MAPK pathway and downregulated NF-κB transcription in 6-OHDA-treated SH-SY5Y cells. Furthermore, this treatment upregulated the expression of SOD, heme oxygenase-1 (HO-1), peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), and glutamate-cysteine ligase modifier subunit (GCLM) [219].

IN SILICO STUDIES OF NOX

Enhancing our understanding of NOX family proteins and their roles in the progression of various diseases is important. To this end, in silico modeling may be a beneficial approach to understanding protein–protein interactions and affinities [220]. The in silico modeling of biological interactions depends on the prediction of noncovalent interactions among chemical groups through hydrogen bonds, salt bridges, and electrostatic forces. This approach has been used to determine the physicochemical properties (molecular weight, isoelectric point, total number of positively and negatively charged residues, half-life, extinction coefficient, instability and aliphatic index, grand average of hydropathicity (GRAVY), amino acid percentage) of NOXs [221]. In this regard, an in silico computational study was used to elucidate the 3-dimensional model of human $p22^{phox}$ based on published protein sequence data and using transmembrane-specific protein prediction algorithms. The structure showed that $p22^{phox}$ is composed of two domains, an N-terminal transmembrane domain (124 a.a.) and a C-terminal cytoplasmic domain (71 a.a.) [222]. A recent study aimed at estimating the anti-melanoma activity of soft jet cold atmospheric plasma (CAP) by using N_2 gases and other treatments and understanding the role of ROS and NOS conducted in silico simulation studies. The investigators were able to establish the role of H_2O_2 and NOX in regulating the apoptotic pathway and identified the possible

interactions of the apoptotic proteins: apoptosis signal-regulating kinase 1 (ASK1) and thioredoxin 1 (TRX1) [223].

Furthermore, a broader use of in silico techniques would focus on the application of rapid virtual screenings to identify possible NOX inhibitor molecules [224]. Recent studies include the use of in silico techniques to elucidate the mechanism by which CPP11G and CPP11H inhibit NOX2 via the suppression of $p47^{Phox}$ cytosol-to-membrane translocation, thereby inhibiting NOX2 complex formation [184]. Similarly, Macías-Pérez and colleagues used computational modeling to study the interactions of NOX2-$p47^{phox}$ subunits with $p22^{phox}$ in the presence of apocynin derivatives. The presence of apocynin derivatives can inhibit $p22^{phox}$-$p47^{phox}$ binding through interactions with the SH3A and SH3B domains, preventing the complex formation with $p22^{phox}$ [225]. Similar docking studies were used during in silico screening to identify the inhibitors of the Rac1-$p67^{phox}$ interaction in the NOX2 signaling axis [224]. This approach was shown to facilitate the identification of a list of inhibitors that are pathologically relevant to the inflammatory Rac signaling pathway based on a structure/function relationship [224].

CONCLUSION

The expression of NOX subunits and the activation potential of NOX isoforms are regulated in a variety of disorders including-neurological, cardiovascular, and pulmonary. Pharmacological inhibition and genetic ablation studies support the idea of targeting NOX subunits for therapeutic benefit in these disorders. However, there are still several obstacles to overcome in order to achieve effective NOX-specific targeting that would benefit patients while reducing negative side effects. While addressing these issues remains difficult, the use of in silico approaches and technological advancements provide hope that the hybrid approaches can be employed to achieve future goals.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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