

REVIEW

NADPH oxidases: an overview from structure to innate immunity-associated pathologies

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Oxygen-derived free radicals, collectively termed reactive oxygen species (ROS), play important roles in immunity, cell growth, and cell signaling. In excess, however, ROS are lethal to cells, and the overproduction of these molecules leads to a myriad of devastating diseases. The key producers of ROS in many cells are the NOX family of NADPH oxidases, of which there are seven members, with various tissue distributions and activation mechanisms. NADPH oxidase is a multisubunit enzyme comprising membrane and cytosolic components, which actively communicate during the host responses to a wide variety of stimuli, including viral and bacterial infections. This enzymatic complex has been implicated in many functions ranging from host defense to cellular signaling and the regulation of gene expression. NOX deficiency might lead to immunosuppression, while the intracellular accumulation of ROS results in the inhibition of viral propagation and apoptosis. However, excess ROS production causes cellular stress, leading to various lethal diseases, including autoimmune diseases and cancer. During the later stages of injury, NOX promotes tissue repair through the induction of angiogenesis and cell proliferation. Therefore, a complete understanding of the function of NOX is important to direct the role of this enzyme towards host defense and tissue repair or increase resistance to stress in a timely and disease-specific manner.

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INTRODUCTION

Reactive oxygen species (ROS) are small, oxygen-derived molecules that include oxygen radicals, such as superoxide and hydroxide, and certain non-radicals, such as hydrochlorous acid and ozone. ROS play dual, opposing roles dependent on the context. ROS-mediated stress has been implicated in various malfunctions and diseases, including cardiovascular pathology, immunodeficiency and pulmonary diseases.^{1–5} However, the NADPH oxidase (NOX)-mediated release of ROS, also called oxidative burst, leads to the elimination of invading microorganisms in macrophages and neutrophils and thereby serves as an inflammatory mediator.⁶ For many years, superoxide generation through NOX was thought to occur only in phagocytes; however, several enzymes responsible for ROS production have been recently identified in various tissues, which differ at the molecular level. These enzymes show similarities to phagocytic NOX (aka NOX2) and are

collectively referred to as the NOX family. The NOX genes produce the transmembrane proteins responsible for transporting electrons across biological membranes, which leads to the reduction of oxygen into superoxide.⁷ The common functions of NOX proteins has been attributed to the conserved structural properties of these enzymes including the NADPH-binding site at the C-terminus, the flavin adenine dinucleotide (FAD)-binding region located proximal to the C-terminal transmembrane domain, six conserved transmembrane domains, and four conserved heme-binding histidines.⁸ NOX enzymes mediate diverse functions in various organisms through redox signaling. The importance of ROS in host immunity has been clearly determined through the discovery of the genetic disorder, chronic granulomatous disease (CGD), which reflects defects in NOX2 (or associated subunits).⁹ CGD is characterized by defective neutrophil killing due to extremely low respiratory burst in these cells during phagocytosis.^{10,11}

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Patients with CGD are hypersensitive to various bacterial and fungal infections, and the accumulation of bacteria-containing phagocytes leads to the development of granulomas. This phagocyte accumulation reflects the inability of these cells to kill ingested pathogens or undergo apoptosis, due to defective NOX2 activity.^{12,13}

The role of NOX has also been well established under non-pathological conditions. Vascular NOX generate ROS important for maintaining normal cardiovascular health through the regulation of blood pressure, which is essential to health, as aberrations from normal levels can be lethal.^{14,15} The presence of ROS reduces the bioavailability of the endothelial-derived relaxation factor, nitric oxide (NO), which regulates blood pressure.^{16,17} In normal kidneys, ROS is produced through NOX3, and these molecules regulate renal function through the control of Na⁺ transport, tubuloglomerular feedback and renal oxygenation.^{18–20} Furthermore, oxygen radicals increase NaCl absorption in the loop of Henle, resulting in the modulation of Na⁺/H⁺ exchange.^{21,22} Pulmonary NOX2 has been implicated in airway and vascular remodeling.²² The p22^{phox}-dependent NOX2 regulates the proliferation²³ and differentiation²⁴ of smooth muscle cells through the activation of nuclear factor kappa B (NF-κB) and inducible nitric oxide synthase (iNOS). ROS generation through Duox and NOX1 in the colon mucosa promotes serotonin biosynthesis, which is essential in regulating secretion and motility.²⁵ NOX2 is involved in the normal functioning of the central nervous system through angiotensin II signaling in the nucleus tractus solitarius and the hypothalamic cardiovascular regulator nuclei.^{26,27} Moreover, microglial cells express NOX2 and p22^{phox}, and both of these enzymes participate in the regulation of microglial proliferation and neuronal apoptosis during nerve growth factor deprivation.²⁸ Thus, the importance of NOX in health and disease has been well established, and herein, we will discuss the structural and functional details of this enzyme.

HISTORY

Although respiratory burst was initially characterized in the early 20th century, the molecular mechanisms underlying this phenomenon were not deciphered until much later. In 1959, Sbarra and Karnovsky²⁹ demonstrated that hydrogen peroxide production is dependent upon glucose metabolism and oxidative phosphorylation and is an energy-requiring process. In 1961, Iyer *et al.*³⁰ observed the production of hydrogen peroxide during phagocytic respiratory burst, whereas in 1964, Rossi and Zatti³¹ demonstrated that NADPH is the primary substrate for generating oxidative burst-generating enzyme system. Almost a decade later, in 1973, Babior *et al.*³² reported that superoxide is the initial product of respiratory burst. These initial studies established the first clear picture of the biochemical role of the NOX system, and further studies deduced the intricate details of this pathway. In 1978, Segal *et al.*^{33,34} showed that cytochrome b₅₅₈ (a complex of gp91^{phox} and p22^{phox}) is responsible for the production of ROS. In addition, mutations in the CYBA, NCF1, NCF2, or NCF4 genes (which encode p22^{phox}, p47^{phox}, p67^{phox} or p40^{phox}, respectively) were

associated with the development of CGD.³⁴ Subsequently, gp91^{phox}, the catalytic subunit of the phagocyte NOX,³⁵ and other components of the enzyme complex, including the transmembrane protein p22^{phox} and the cytosolic subunits p40^{phox}, p47^{phox} and p67^{phox},^{36–38} were cloned and characterized. In 1970, Karnovsky *et al.*³⁹ was the first to report the expression of NOX in neutrophils. Since then, knowledge concerning leukocyte oxide production has extensively increased, and several isoforms of this enzyme have been identified and characterized in various tissues and cells (Table 1).

STRUCTURE AND ASSEMBLY OF NOX

NOX refers to the first characterized isoform NOX2, comprising six different subunits that interact to form an active enzyme complex responsible for the production of superoxide.⁴⁰ Two NOX subunits, gp91^{phox} (also known as the β subunit) and p22^{phox} (also known as the α subunit), are integral membrane proteins that together comprise the large heterodimeric subunit flavocytochrome b₅₅₈ (cyt b₅₅₈). Under unstimulated conditions, the multidomain regulatory subunits, p40^{phox}, p47^{phox} and p67^{phox}, exist in the cytosol as a complex.⁴¹ Upon stimulation, p47^{phox} undergoes phosphorylation, and the entire complex subsequently translocates to the membrane and associates with cyt b₅₅₈ to form the active oxidase (Figure 1). The activated complex transfers electrons from the substrate to oxygen through a prosthetic group, flavin, and a heme group(s), which carries electrons. The activation of the complex also requires two low-molecular-weight guanine nucleotide-binding proteins, Rac2 and Rap1A.⁴² Rac2 is localized in the cytosol in a dimeric complex with Rho-GDI (guanine nucleotide dissociation inhibitor), while Rap1A is a membrane protein.^{43,44} Upon activation, Rac2 binds guanosine triphosphate (GTP) and translocates to the membrane along with p40^{phox}, p47^{phox}, and p67^{phox} (cytosolic complex) (Figure 1).^{45,46} During phagocytosis, the plasma membrane is internalized and ultimately becomes the interior wall of the phagocytic vesicle. Subsequently, O₂⁻ is released into the vesicle through the enzyme complex, and upon conversion of O₂⁻ into its successor products, the internalized target becomes submerged in a toxic mixture of oxidants.⁴⁷ Rap1A and cyt b₅₅₈ are delivered to the plasma membrane through the fusion of secretory vesicles, thereby facilitating the release of these proteins to the exterior. ROS production is not restricted to phagocytic cells, and the discovery of gp91^{phox} homologs has significantly improved our understanding of free radical production. Collectively known as the NOX family, these gp91^{phox} homologs include several differentially expressed members: NOX1, NOX2 (formerly known as gp91^{phox}), NOX3, NOX4, NOX5, Dual oxidase Duox proteins (Duox1 and Duox2) (Table 1).

Some NOX family members possess additional features, including an additional N-terminal transmembrane domain and/or peroxide homology domain.⁴⁸ NOX2, commonly referred to as gp91^{phox}, possesses six transmembrane domains, with both the C- and N-terminus facing the cytoplasm. In addition, constitutive association with p22^{phox} stabilizes NOX2.^{49,50} The activation of NOX2 requires translocation

Table 1 Expression of NOX family members and their components in various tissues or cells

NOX isoforms	NOX type/component	Expression observed in	
		Tissue	Cells
1	NOX1	Colon and vascular smooth muscle, prostate, uterus ⁷⁹ Colon epithelia ¹²⁵ Uterus and placenta ⁵⁹ Rectum ⁶⁰	Endothelial cells ⁷⁹ Osteoclasts ⁷⁹ Retinal pericytes, neurons, astrocytes and microglia ²⁷²
2	NOX2 (gp91 ^{phox})		Phagocytes/granulocytes—induced through bacterial LPS ¹⁶⁵ Human umbilical vein endothelial cells ¹⁶⁶ Coronary microvascular endothelial cells ¹⁶⁸ Cardiomyocytes ¹⁷⁹ CNS, endothelium, VSMCs, fibroblasts, skeletal muscle, hepatocytes and hematopoietic stem cells ¹⁸⁰
3	NOX3	Fetal kidney ⁷⁹ Liver, lung and spleen. Low levels in the adult colon and kidney ²⁷³ Inner ear ¹²⁷	
4	NOX4	Kidney ¹²⁸ Liver ¹²⁹ Ovary and eye ⁹⁵	Mesangial cells ¹²⁵ Smooth muscle cells ⁷⁹ Fibroblasts ¹⁸² Keratinocytes ¹⁸⁰ Osteoclasts ^{180,274} Endothelial cells ⁴⁰ Neurons ¹⁵⁹ Hepatocytes ¹⁷⁰ VSMCs ²⁷⁶
5	NOX5	Spleen, testis, mammary glands and cerebrum ⁷⁹ Fetal brain, heart, kidney, liver, lung, skeletal muscle, spleen, thymus ²³⁹ Prostate ¹⁶⁹	
6	Duox1	Lymphatic tissue, endothelial cells ²⁷⁵ Thyroid, cerebellum and lungs ¹³⁹ Ileum, cecum, and floating colon ¹⁴⁶ Respiratory tract epithelium ²⁷⁸	Thyroid cells ⁷⁹
7	Duox2	Colon pancreatic islets and prostate ²⁷⁹ Stomach, duodenum, ileum, jejunum, cecum, sigmoidal colon, floating colon and rectum ²⁸⁰ Pancreas ²⁸¹ Tracheal and bronchial epithelium ²⁸² Thyroid ²⁸³	Thyroid cells in primary culture ⁹⁰
Components of NOX			
1	p67 ^{phox} (NCF-2)	Kidney ²⁸⁴	Neutrophil ^{37,38}
2	p47 ^{phox} (NCF-1)		Neutrophils ²⁸⁵
3	p40 ^{phox} (NCF-4)		Mononuclear cells ²⁸⁶
4	p22 ^{phox}	Human coronary arteries ²⁰⁸	
5	RAC1 (p21)		Neutrophils ²⁸⁷
6	RAC2		Hematopoietic cells ²⁸⁸
7	NOXO1, NOXA1	Colon ⁷⁹	

Abbreviations: NOX, NADPH oxidase; VSMC, vascular smooth muscle cell.

and association with phosphorylated p47^{phox} for the binding of other cytosolic components, including p67^{phox} and p40^{phox}, to the NOX2/p22^{phox} complex.^{51–53} Upon complex assembly, the GTPase Rac first interacts with NOX2 and subsequently interacts with p67^{phox}, resulting in an activated complex for the production of superoxide through electron transfer from cytosolic

NADPH to oxygen to the luminal or extracellular region.^{42,54} The subcellular distribution of NOX varies according to cell type and localization ranges from the plasma membrane to intracellular compartments, including secondary and tertiary granules. NOX1 was identified as the first homolog of NOX2 and shares 60% amino-acid identity.^{55,56} Data concerning the subcellular

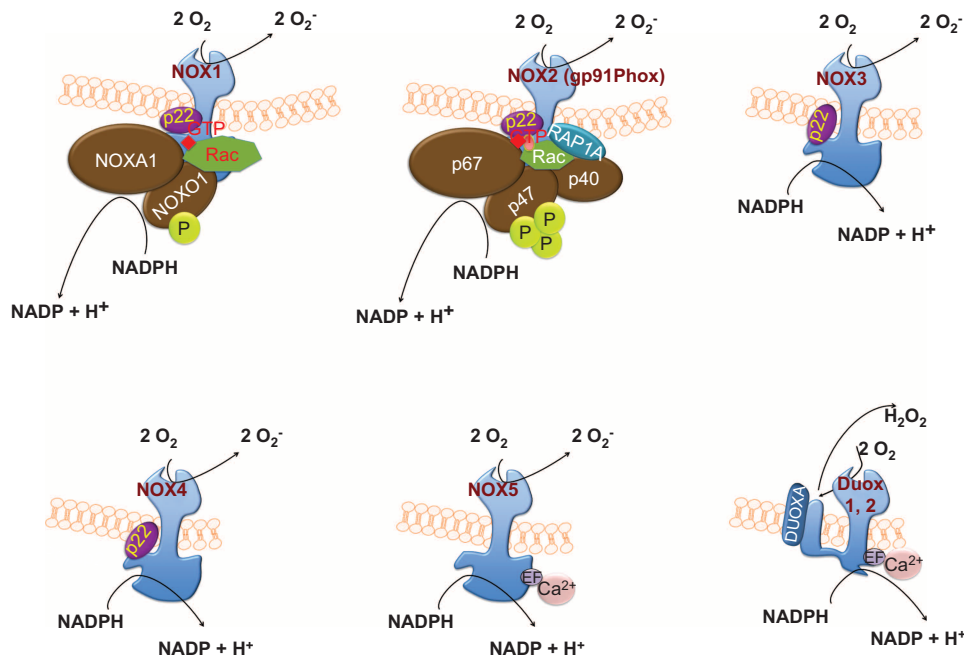


Figure 1 Assembly and activation of NOX enzymes: NOX1 and NOX2 activation involves the phosphorylation of NOXO1 and p47^{phox}, respectively, the translocation of the entire multidomain complex, including p40^{phox}, p67^{phox} and Rac from the cytosol to the membrane and the transfer of electrons from the substrate to oxygen. Similar to NOX1 and NOX2, NOX3 is p22^{phox}-dependent, but it does not bind to Rac. NOX4 activation involves p22^{phox} and Poldip2. In contrast, NOX5 and Duox activation is calcium-dependent. Duox proteins isolated from the thyroid gland possess a peroxidase-like domain. The mature form of Duox generates hydrogen peroxide, which may be the ultimate end product, likely reflecting the rapid dismutation of superoxide. NOX, NADPH oxidase.

localization of NOX1 are scarce; however, it has been suggested that this protein is membrane localized and potentially present within caveolar rafts.⁵⁷ NOX1 is widely expressed in various cell types (Table 1),^{58–60} with notably high expression in the colon epithelium.⁶¹ Evidence of the dependency of NOX1 activity on cytosolic subunits was supported through the discovery of NOX organizer 1 (NOXO1), a homolog of p47^{phox}, and NOX activator 1 (NOXA1), a homolog of p67^{phox}.^{62–64} For activation, NOX1 requires the membrane subunit (p22^{phox}), cytosolic subunits, and the Rac GTPase. The stringency of dependence on the cytosolic subunits varies between NOX2 and NOX3, which shares 56% amino acid identity with NOX2 and was first identified by Kikuchi *et al.* in 2000.^{65–69} Similar to NOX1 and NOX2, NOX3 is p22^{phox}-dependent; however the *in vivo* relevance of p22^{phox} for NOX3 function remains unclear.^{70,71} Furthermore, the requirement for NOXO1 and NOXA1 for the activation of NOX3 has been shown in some studies, although the involvement of p47^{phox} and p67^{phox} has not been demonstrated under physiological conditions, and the role for Rac in NOX3 activation remains controversial.^{66,70,72,73}

NOX4, identified by Geiszt *et al.*⁷⁴ in 2000, shares approximately 39% sequence homology with NOX2.^{48,74} The activity of NOX4 strongly depends on p22^{phox}⁷⁵ but not on cytosolic subunits.⁷⁶ Furthermore, the involvement of Rac in the activation of NOX4 remains questionable.⁷⁷ Poldip2, a polymerase (DNA-directed) delta-interacting protein, interacts with NOX4/p22^{phox} as a positive regulator in vascular smooth muscle

cells.⁷⁸ The association of Poldip2 with p22^{phox} has been demonstrated using GST pull-down assays.⁷⁸ The analysis of NOX4-rich tissues (e.g., aorta, lung and kidney) using western blotting, qRT-PCR and immunohistochemistry indicates increased Poldip2 expression.^{78,79} The role of Poldip2 as a positive regulator of NOX4 in association with p22^{phox} has been demonstrated using siRNA against Poldip2 in vascular smooth muscle cells.⁷⁸

Two independent research groups identified NOX5, and this protein comprises five isoforms. Banfi *et al.*⁸⁰ identified isoforms α , β , γ and δ , while Cheng *et al.*⁸¹ identified a fifth isoform, NOX5 ϵ or NOX5-S. NOX5 isoforms α – δ possess a long, intracellular N-terminal domain that contains a Ca²⁺-binding EF-hand region,^{80,82} whereas the fifth isoform lacks the EF-hand region and is structurally similar to NOX1–4.⁸¹ NOX5 isoforms α – δ do not require p22^{phox} or cytosolic subunits for activation, but rather, depend on cytosolic calcium for activation, reflecting the presence of a Ca²⁺-binding domain.⁷¹ In contrast, the NOX5 ϵ isoform, which lacks a Ca²⁺-binding domain, depends on the cAMP response element binding protein for activity.⁸³

The dual oxidases (Duox1 and Duox2, also known as thyroid oxidases) contain a NOX homology domain similar to that of NOX1–4, an EF-hand region like NOX5, and a seventh transmembrane at N-terminus which is a peroxidase-like domain but lacking the few functionally essential amino acids.^{84–86} The dual oxidases were first identified in the thyroid gland in

1999,⁸⁷ and these enzymes share approximately 50% amino acid identity with NOX2. Both Duox1 and Duox2 have two N-glycosylation states^{88,89} and have been identified as glycosylated proteins in the plasma membrane and endoplasmic reticulum.^{88,90,91} The retention of Duox proteins in the endoplasmic reticulum depends on maturation factors DuoxA1 and DuoxA2.⁹² The partially glycosylated, immature form of Duox2 generates O₂, whereas the mature form generates H₂O₂.⁹³ Although studies have shown the direct physical interaction of Duox proteins with p22^{phox}, there is no direct evidence regarding whether the activation of these proteins requires p22^{phox}.^{88,93,94} Furthermore, Duox proteins do not require cytosolic activator or organizer subunits for activation, but can be activated through Ca²⁺.⁹³

NOX family members not only regulate normal physiological functions, but also contribute to the pathogenesis associated with infections, vascular disorders and impaired immune responses due to environmental factors. In this review, we will highlight the structure and pathogenesis associated with NOX, including the role for this enzyme in bacterial/viral infections, vascular disorders and impaired immune responses due to cigarette smoke and alcohol exposure.

CYTOSOLIC COMPONENTS OF THE OXIDASE

The cytosolic components of NOX include p40^{phox}, p47^{phox} and p67^{phox}. p47^{phox} is phosphorylated in stimulated neutrophils, which increases the binding of this component to p67^{phox} and Rac approximately 100- and 50-fold, respectively, and these interactions lead to the translocation of the cytosolic complex to the membrane.⁹⁵ Neutrophils deficient in p47^{phox} do not show the efficient transfer of the cytosolic oxidase complex to the membrane; however, p67^{phox}-deficient neutrophils exhibited normal p47^{phox} translocation.⁹⁵ p47^{phox} stabilizes or facilitates the interaction of p67^{phox} and Rac1 with cytochrome b₅₅₈; however, at high molar concentrations of p67^{phox} and Rac1, p47^{phox} is not required for these interactions.^{96,97}

Electron flow from NADPH to oxygen through the redox centers is activated subsequent to the assembly of the cytosolic components with cyt b₅₅₈. The individual roles of p47^{phox} and p67^{phox} in electron flow have been further demonstrated using a cell-free system with samples from normal and CGD patients.⁹⁸ The results of this study showed that p67^{phox} alone can facilitate electron transfer from NADPH to the flavin center, whereas p47^{phox} is required for electron transfer from the flavin center to cytochrome b₅₅₈. Furthermore, p67^{phox} contains a catalytically essential binding site for NADPH; however, an NADPH-binding site is also present on cytochrome b₅₅₈.^{99–101} The relationship between these two NADPH-binding sites has not been determined, although notably, cyt b₅₅₈ alone catalyzes active O₂⁻ production using NADPH as a reductant.⁴⁰ The reduced interaction of mutated p67^{phox} with Rac and impaired translocation of the cytosolic oxidase complex in the activated phagocytes of CGD patients have recently been reported.^{46,102} These findings suggest that both p47^{phox} and p67^{phox} are important components of NOX2. Other NOX isoforms are either regulated through a

similar complex (NOX1, NOX3 and NOX4) or *via* Ca²⁺ (NOX5 and Duox).

MEMBRANE COMPONENTS OF THE OXIDASE

The NOX flavocytochrome b₅₅₈ mediates the terminal steps in electron transfer and generates O₂⁻ during respiratory burst. In phagocytes, flavocytochrome b₅₅₈ is a membrane-bound heterodimer comprising the large glycosylated subunit gp91^{phox} and the small non-glycosylated subunit, p22^{phox}, also known as β- and α-subunits, respectively. FAD and NADPH bind to the C-terminal cytoplasmic domain of gp91^{phox}, whereas the N-terminal 300 amino acids are predicted to form six transmembrane α-helices.¹⁰³ There are also two membrane-embedded non-identical heme groups per flavocytochrome b₅₅₈ heterodimer, which are non-covalently coordinated by histidines.^{104–106} The electrons are first transferred from NADPH to FAD, followed by transfer to the heme group, which reduces O₂ to O₂⁻.¹⁰⁷ These two electron transfer steps are accomplished through gp91^{phox} in complex with the necessary cofactors.

The α-subunit, p22^{phox}, comprising 195 amino acids, is another membrane-bound subunit of NOX.¹⁰⁸ p22^{phox} associates with gp91^{phox} in a 1:1 complex and contributes to both the maturation and stabilization of this protein. The N-terminal portion of p22^{phox} is predicted to contain three transmembrane α-helices, whereas the C-terminal cytoplasmic portion is devoid of predictable structure, but rather, is a proline-rich region that undergoes phosphorylation at a threonine residue. Notably, the physiological role for the addition of this phosphate group is not understood.^{109,110} The coexpression of the flavocytochrome b₅₅₈ subunits, gp91^{phox} and p22^{phox} in COS7 cells resulted in the expression of functional flavocytochrome, while the expression of either gp91^{phox} or p22^{phox} alone in COS7 membranes did not rescue the flavocytochrome b₅₅₈ function in cell-free NOX assays. Thus, both flavocytochrome subunits are required for the functional assembly of the oxidase.¹¹¹

THE GTPASE RAC

Rac is a member of the Rho-family of small GTPases, and this protein plays an indispensable role in the regulation of various signaling pathways, including cytoskeletal remodeling and chemotaxis.¹¹² There are two differentially expressed isoforms of Rac, Rac1 and Rac2. Rac1 is ubiquitously expressed and activates NOX in non-hematopoietic cells, while Rac2 expression is confined to hematopoietic cells, specifically neutrophils, where this protein acts as a major NOX2-activating Rac GTPase.^{113,114} However, both proteins comprise 192 amino acids and share 92% sequence homology. Most of the differences between Rac1 and Rac2 occur in the insertion helix and hypervariable C-terminus, although the mediation of O₂⁻ production through these enzymes is similar in reconstituted, cell-free systems using purified proteins.^{115,116} Guanosine diphosphate maintains Rac in an inactive state, while GTP induces the active state. The activation of Rac mediates the interaction of this enzyme with downstream effectors and the

propagation of the signaling responses, regulated through GTPase-activating proteins and guanine-nucleotide-exchange factors. GTPase-activating proteins mediates the hydrolysis of GTP into guanosine diphosphate, whereas guanine-nucleotide-exchange factors facilitate the detachment of guanosine diphosphate and binding of GTP.¹¹⁷

Rac proteins, similar to other small GTPases, contain an effector region in the N-terminus that mediates interactions with effector proteins, including p67^{phox}.^{97,118} Activated Rac recruits p67^{phox}, which associates with p47^{phox} and cytochrome.^{119–121} Specifically, a recent study showed the absolute requirement for Asp57 in this region of Rac2 based on a mutation in a CGD patient who showed severely impaired neutrophil responses, including adhesion, chemotaxis, and superoxide production.^{122–124} NOX1 activation is mediated through Rac1, and there is increasing evidence of an important role for the Rac-mediated activation of NOX proteins in various cardiovascular ailments, making these signaling complexes important therapeutic targets.¹²⁵

NOX, INNATE IMMUNITY AND BACTERIAL INFECTIONS

In addition to NOX, pattern recognition receptors (PRRs) have also been identified as key components of innate immunity. PRRs are germline-encoded proteins that recognize conserved structures on microorganisms called pathogen-associated molecular patterns (PAMPs). PAMPs include lipopolysaccharides, lipoteichoic acids and lipoproteins located on the pathogen surface, and PRRs include Toll-like (TLRs), nucleotide-binding and oligomerization domain (NOD)-like (NLRs), RIG-like and C-type lectin receptors.

TLRs are type 1 integral membrane glycoproteins that function as homodimers and possess extracellular lectin-like receptors and cytoplasmic signaling domains that recruit signaling molecules.¹²⁶ The stimulation of TLRs leads to downstream signaling events, ultimately resulting in the activation of the transcription factor NF- κ B and the production of pro-inflammatory cytokines and chemokines important for controlling infections. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface, whereas TLR3, TLR7 and TLR9 are localized to the endosomal membrane. TLR1/TLR2 and TLR2/TLR6 heterodimerize and recognize structures associated with bacteria, viruses and fungi. TLR2 activation leads to the recruitment of the Toll-interleukin receptor domain, containing adaptor protein and MyD88, which results in the activation of NF- κ B and the production of cytokines and chemokines.^{127,128} TLR4 recognizes lipopolysaccharides (LPS) on the surface of Gram-negative bacteria, and this receptor plays a role in establishing commensal colonization and maintaining tolerance to commensal bacteria.¹²⁹ TLR5 recognizes flagellin, a constituent of the flagella of motile bacteria and is important for the detection of invasive flagellated bacteria at the mucosal surface.¹²⁷ TLR5 plays an indispensable role in maintaining intestinal homeostasis through the regulation of host defenses against enterobacterial infections. TLRs 3, 7 and 8 recognize viral RNAs and synthetic viral RNA analogs, while TLR9, which is localized intracellularly in the endosomal compartment, recognizes bacterial

unmethylated CpG DNA, and TLR11 senses profilin and uropathogenic bacteria.¹³⁰

In addition, NLRs belong to a family of cytosolic PRRs that recognize intracellular PAMPs. The NLR family, also referred to as the NOD, NALP, or CATERPILLER protein family,¹³¹ comprises more than 20 cytosolic proteins¹³² and has been classified into two subfamilies, the CARD and Pyrin subfamilies, based on the presence of a specific death domain. The former is also known as the NLRC (NACHT, lectin-like receptor and CARD domains-containing proteins), whereas the latter is known as the NLRP (NACHT, lectin-like receptor and PYD domains-containing proteins). NOD1 recognizes peptidoglycan, a component of the bacterial cell wall, whereas NOD2 recognizes muramyl dipeptide, a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria (Figure 2).¹³³ A different set of NLRs, including Ipaf and Naip, are activated through bacterial flagellin. NLRP3 (cryopyrin) is activated through a variety of molecules, including bacterial RNA, pore-forming toxins and endogenous ligands, including uric acid crystals and ATP.^{134–137} NLRP1, NLRP3 and NLRC4 form inflammasomes that are involved in inflammatory caspase maturation.¹³⁸ In addition to direct activation through PAMPs, inflammasomes can also be activated *via* ROS.

Crosstalk between TLRs and NOX

NOX2-mediated respiratory burst plays an indispensable role in antimicrobial innate immune responses. Moreover, it has been shown that PRR receptor-mediated ROS generation is coupled with NOX isozymes in phagocytic cells.¹³⁹ However, the molecular mechanisms underlying ROS generation downstream of PRRs reportedly differ. In response to LPS, the cytoplasmic region of TLR4 recruits MyD88, linking TLR4 to IL-1R kinase associated with TRAF6. The sequential activation of IL-1R kinase and TRAF6 results in NF- κ B activation.¹⁴⁰ In 2004, Park *et al.* reported the direct interaction of NOX4 through TLR4 in kidney epithelial cells (HEK293T) and U937 monocytic cells.¹⁴¹ Specifically, the TIR region of TLR4 physically interacts with the C-terminus of NOX4, and this interaction is important for LPS-mediated NF- κ B activation in human aortic endothelial cells.¹⁴² NOX1, which is predominantly expressed in colon epithelial cells,⁵⁵ is activated through a TLR5-mediated pathway through p41NOXA and p51NOX homologs of p47^{phox} and p67^{phox}, respectively.^{143–145} Recombinant flagellin from *Salmonella enteritidis* stimulates ROS generation through TLR5 in T84 cells, and the cotransfection of p41NOXA and p51NOX significantly enhances ROS production.¹⁴⁶ In the nasal airway epithelium, ROS generation and IL-8 expression has been associated with interactions between TLR5 and another NOX isozyme, Duox2, in response to flagellin exposure.^{147,148} Furthermore, in response to flagellin, the TIR domain of TLR5 interacts with the C-terminal region of Duox2 for the activation of this protein in a calcium-dependent manner.¹⁴⁹ These studies reveal that ROS production is associated with

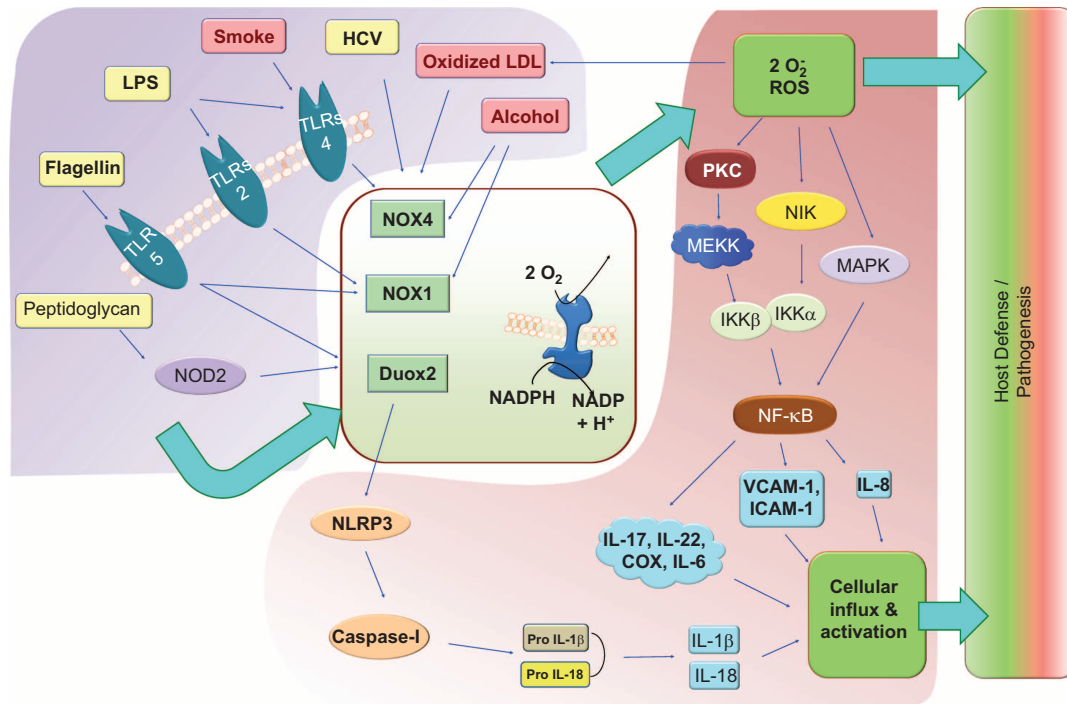


Figure 2 The NOX system is central to host defense. When induced through various triggering elements (top-left block), NOX family members become activated, producing ROS (central block). The block arrows represent the main signal pathway. While ROS directly kills microorganisms, additional downstream cellular factors work in an orchestrated manner to mount pressure against the invading pathogens or the cells harboring these microbes (right block). However, when non-pathogenic inducers, such as cigarette smoke or alcohol, induce the NOX system, ROS exerts damaging effects on the host, causing pathogenesis. The signaling mechanisms illustrated in this figure include the induction of TLR4-mediated signaling through cigarette smoke and LPS, leading to the activation of NOX4. TLR5 recognizes flagellin and activates NOX1 and Duox2. Virus-like HCV also activates NOX4, leading to ROS production. TLR5 and NOD2, when activated through flagellin or peptidoglycan, respectively, activate Duox2, leading to NLRP3 binding and activation. Furthermore, the NLRP3-mediated induction of caspase-1 activity leads to the maturation of pro-IL-1 β and pro-IL-18, promoting the influx and activation of lymphocytes. ROS, produced through NOX, activates NF- κ B through various intermediates, including PKC, MEKK and NIK, thereby inducing the upregulation of IL-8, ICAM-1 and VCAM-1 and promoting the lymphocyte influx important for host defense/pathogenesis. HCV, hepatitis C virus; ICAM-1, intercellular adhesion molecule-1; MEKK, MEK kinase; NF- κ B, nuclear factor kappa B; NIK, NF- κ B-inducing kinase; NOX, NADPH oxidase; PKC, protein kinase C; ROS, reactive oxygen species; TLR, Toll-like receptor; VCAM-1, vascular cell adhesion molecule-1.

TLR, and the induction mechanisms are different between TLR4 and TLR5.

CROSSTALK BETWEEN NLRs AND NOX

Studies using diphenyliodonium, a NOX inhibitor, have shown that ROS is generated in response to the activation of NOX2.¹⁵⁰ NLRP3 inflammasome activators, including silica and ATP, require ROS production for activation.¹⁵¹ Thus, these studies suggest that NOX functions upstream of NLR signaling.¹⁵² Furthermore, another NOX family member, Duox2, is involved in NOD2-dependent ROS production in response to muramyl dipeptide induction.¹⁵³

Hyperhomocysteinemia (hHcys) is a significant risk factor for many degenerative diseases and pathological processes, including cardiovascular disease, chronic obstructive pulmonary disease, pneumonia, and neurological and renal diseases. The lethal consequences of hHcys, including glomerular dysfunction, result from the activation of the NALP3 inflammasome. The ROS-mediated activation of inflammasomes in podocytes is

one of the putative underlying mechanisms of hHcys.^{154,155}

NOX4 is reportedly expressed in kidney cells and is a major producer of ROS.¹⁵⁶ In addition, normal renal function is restored after the inhibition of NOX,¹⁵⁷ and either the inhibition of NOX4 or silencing of the gp91^{phox} gene attenuates hHcys-induced inflammasome formation in podocytes.¹⁵⁸ As NALP3 activation leads to caspase-1 induction and IL-1 β production, it is reasonable that the inhibition of NOX also attenuates hHcys-induced caspase-1 activity and IL-1 β production in podocytes. Furthermore, apocynin, diphenyleneiodonium, gp91^{ds-tat} and gp91^{phox} siRNA are all inhibitors of NOX-mediated ROS production, and these proteins have been used to show the involvement of this complex in disease.¹⁵⁹ Therefore, we concluded that NOX activation leads to ROS production, which induces NLR signaling and the formation of inflammasomes, resulting in inflammation and tissue injury in hHcys.

Mutual tuning between chemokines and NOX

The NOX system is activated in pulmonary neutrophils in response to bacterial infection. This activation is regulated

through the chemokines CXCL1 and LTB₄, as demonstrated in CXCL1^{-/-} mice infected with *Klebsiella*. The intrapulmonary administration of LTB₄ reverses immune defects and leads to improved survival, cytokine/chemokine expression, NF-κB/MAPK activation and ROS/RNS production. Similarly, the chemokine MCP-1 causes lysosomal enzyme release and H₂O₂ production in monocytes.¹⁶⁰ Furthermore, decreased p47^{phox} expression in both neutrophils and macrophages from MCP-1 and CCR-2 gene-deficient mice suggests that the MCP-1/CCR-2 axis regulates the expression and activation of the NOX system.¹⁶¹

The silencing of NOX4 using short, interfering RNA leads to a reduction in ROS production, and intercellular adhesion molecule-1 (ICAM-1) and MCP-1 expression in response to LPS.¹⁴¹ Moreover, treatment with antioxidants or the inhibition of p22^{phox} using neutralizing antibodies or antisense oligonucleotides in vascular smooth muscle cells inhibited p38 MAP kinase activation and MCP-1 expression in response to thrombin.¹⁶² Similarly, treatment with antioxidants abolished angiotensin II-induced ROS production and MCP-1 expression in cultured human mesangial cells.¹⁶³ A recent study also demonstrated that the treatment of *Helicobacter pylori*-infected gastric epithelial cells with red ginseng extract inhibited MCP-1 and inducible NOS expression through the inhibition of NOX activity and the JAK2/STAT3 pathway.¹⁶⁴ Each of these studies highlights a direct link between chemokine signaling and NOX activation.

ROLE OF NOX IN VIRAL PATHOGENESIS

Acute respiratory tract infections resulting from influenza virus infection leads to cell death, reflecting an over-exuberant immune response that causes localized tissue damage and systemic illness via the induction of a cytokine storm.^{165,166} The production of superoxide downstream of NOX limits influenza virus propagation through the death of the infected cells;¹⁶⁷ however, ROS also acts as second messengers to activate redox-sensitive transcription factors, which elicit immune and subsequent inflammatory responses that eventually cause damage (Figure 2).¹⁶⁸ Reduced ROS generation through the genetic depletion or malfunction of NOX enzymes favors pulmonary influenza infection potentially through the promotion of professional phagocyte populations. Inhibitors of superoxide effectively improve lung pathology and survival during viral pneumonia, suggesting that superoxide contributes to the pathogenesis of viral disease.¹⁶⁹ Hepatitis C virus (HCV) causes mortality due to the induction of various liver diseases and liver cancer. The occurrence of chronic infection and liver injury likely reflects HCV-mediated oxidative stress. The HCV polyprotein undergoes proteolytic processing, giving rise to the viral core proteins, non-structural protein 3 (NS3), NS4 and NS5, which trigger ROS production in human monocytic cells.¹⁷⁰ The translocation of p47^{phox} and p67^{phox} from the cytoplasm to the membrane has been visualized in HCV-infected cells, confirming the involvement of NOX2 during infection. Furthermore, the role of calcium influx in the NS3-induced activation of NOX has been confirmed using

digital microscopy to visualize rapid calcium signaling. The calcium channel inhibitor, lanthanum chloride, not only reduces calcium signaling but also inhibits oxidative burst.¹⁷⁰ NOX4 is also responsible for superoxide and hydrogen peroxide generation in hepatocytes during HCV infection.^{171,172} Human hepatoma cells transfected with HCV cDNA exhibit increased NOX4 mRNA and protein expression and prominent NOX4 localization in the nuclear compartment.¹⁷¹ Furthermore, human hepatocytes (Huh-7) infected with HCV showed a five-fold increase in NOX4 mRNA expression compared with cells transfected with vector alone.¹⁷² Gene silencing studies using siRNA directed against NOX4 have revealed reduced levels of ROS and nitrotyrosine in HCV-infected liver tissue, confirming that NOX4 is a major source of oxidative stress during HCV infection.¹⁷¹ Interestingly, a role for transforming growth factor (TGF-β1) as a mediator in the upregulation of NOX4 during HCV infection has been established, as the HCV-induced over-expression of NOX4 was restricted using antibodies against TGF-β1.¹⁷¹ Moreover, it has been reported that TGF-β1 induces NOX4 expression, and the concentration of TGF-β1 is elevated in hepatitis C patients.^{173,174}

CIGARETTE SMOKE EXPOSURE/COPD AND NOX

Cigarette smoke is one of the most potent risk factors for peripheral cardiovascular-related and cerebrovascular diseases. The cigarette smoke contributes to cellular oxidative stress, leading to pulmonary inflammation, asthma, and tumors.^{175,176} Human alveolar epithelial NOX is activated through ligand binding to the kinin B1 receptor, and this interaction contributes to superoxide anion production and leads to downstream detrimental effects.¹⁷⁷ The principal ligand of this receptor is bradykinin, which is involved in various neurological and cardiovascular diseases, the chronic phase of inflammation, trauma, burns, shock and allergy.¹⁷⁸ Cigarette smoke also induces p47^{phox} translocation and TLR4/MyD88 complex formation. The direct effects of smoking on cardiac remodeling include alterations in ventricular mass, volume and geometry, and heart function. In addition, smoking not only induces morphological alterations and systolic dysfunction but also augments heart stress, which is characterized by an increase in NOX activity. Furthermore, a reduction in the activity of antioxidant enzymes, such as catalase and superoxide dismutase, has also been observed following exposure to cigarette smoke. These perturbations in the oxidant and anti-oxidant balance lead to lung and airway inflammation. Several studies have reported that cyclooxygenase-2 and prostaglandin E-2 play a critical role in respiratory inflammation. Prostaglandin E-2 increases both IL-6 production and leukocyte counts in bronchoalveolar lavage (BAL) fluid.¹⁷⁹ In human tracheal smooth muscle cells, the induction of cyclooxygenase-2 and prostaglandin E-2 is dependent on NADPH signaling.¹⁸⁰ In addition, leukocyte influx into BAL fluid was also reduced using an NOX inhibitor following exposure to cigarette smoke.¹⁸⁰ Thus, these studies have revealed that cigarette smoke-induced ROS generation is mediated through NOX, and these events

leads to the eventual induction of cyclooxygenase and IL-6-dependent airway inflammation (Figure 2).¹⁸⁰

Compared with non-smokers, the neutrophil count in COPD patients is higher in both BAL fluid and in the sputum, a hallmark of COPD.¹⁸¹ Furthermore, circulating neutrophils from patients with stable COPD produce a larger respiratory burst than neutrophils from non-smokers.¹⁸² Mac-1 is an integrin molecule expressed on neutrophils, and this molecule plays a role in neutrophil emigration and recruitment and the adhesion of these cells to the endothelium. COPD patients exhibit the significant upregulation of Mac-1 and ROS, thus causing excessive neutrophil recruitment.¹⁸² The *in vitro* phosphorylation of Mac-1 leads to NOX activation, and the cross-linking of Mac-1 induces neutrophil respiratory burst.^{183–185} In COPD patients, increased oxidants present in cigarette smoke or excessive ROS production in leukocytes enhance inflammation through the activation of NF- κ B,¹⁸⁶ which regulates genes for many inflammatory mediators, such as the cytokines IL-8, TNF- α and nitric oxide.¹⁸⁷ Furthermore, the increased oxidant burden causes the upregulation of antioxidant genes that play protective roles. For example, the induction of the GSH gene increased the accumulation of GSH in the epithelial lining fluid in the airspaces, which is important for prevention against oxidative injury.¹⁸⁸

CHRONIC ALCOHOL EXPOSURE AND OXIDATIVE STRESS

Chronic alcohol abuse is one of the most important risk factors for a type of lung injury called acute respiratory distress syndrome. Previous studies have shown that alcohol abuse causes significant oxidative stress within the alveolar space and impairs both alveolar epithelial cells and macrophages.¹⁸⁹ This alcohol-induced lung dysfunction is attenuated through treatment with glutathione precursors,¹⁹⁰ and glutathione depletion in the alveolar space is associated with oxidative stress and alveolar macrophage dysfunction.¹⁹¹ Alveolar macrophages play an indispensable role in innate and acquired immunity, and the ability of alveolar macrophages to bind and internalize pathogens is impaired through chronic alcohol ingestion.¹⁹² The expression of NOX2 in alveolar macrophages is the main source of ROS generation in the lungs.¹⁹³ The ligation of peroxisome proliferator-activated receptor gamma regulates NOX2 expression in the lungs following chronic alcohol ingestion.¹⁹⁴ In addition, chronic alcohol ingestion reduces the level of the antioxidant glutathione in BAL fluid through the augmentation of the activity of the renin-angiotensin system.¹⁹⁵ Of the two classes of angiotensin, angiotensin II stimulates NOX2 expression and induces intracellular oxidative stress. Because renin-angiotensin system regulates the level of antioxidant glutathione after chronic alcohol ingestion, the use of renin-angiotensin system inhibitors, such as lisinopril, prevents ethanol-induced glutathione depletion and provides protection against epithelial dysfunction.¹⁹⁶ Alcohol abuse is also correlated with bacterial pneumonia, as alcoholic patients are more prone to *Klebsiella pneumoniae* infections than non-alcoholic patients.¹⁹⁷ In addition, chronic alcohol abuse

augments the colonization of the mouth and pharynx by Gram-negative bacteria.¹⁹⁸ Considering the deleterious effects of alcohol abuse on the entire airway from the mouth to the alveolus, the overlapping risk for pneumonia and lung injury is not surprising.

Alcohol exposure also negatively affects the liver, with fibrosis and cirrhosis being major histological features.¹⁹⁹ Alcohol increases the gut permeability to bacterial endotoxins, leading to the activation of resident hepatic macrophages and Kupffer cells through the induction of NOX1 and NOX2 ROS production in response to endotoxins.^{200,201} The chronic ethanol treatment of wild-type or p47^{phox} knockout mice with severe liver injury resulted in detectable free radical production and NF- κ B activation only in the wild-type group, therefore, p47^{phox} might be a potential therapeutic target for alcohol-induced liver injury. These findings suggest that the oxidants generated through NOX, most likely derived from Kupffer cells, play an important role in alcohol-induced liver injury.¹⁹⁹

VASCULAR DISORDERS: ROLE OF NOXS/INNATE IMMUNITY/RAC

Although several sources, including xanthine-xanthine oxidase, lipoxygenase, cyclooxygenase and NOS contribute to the generation of vascular superoxide, the NOX-mediated generation of superoxide has been clinically associated with atherosclerosis, a chronic inflammatory disease characterized by lipid retention and atherosclerotic lesions.²⁰² The expression of NOX1 and NOX4 in human vascular smooth muscles and NOX4 in endothelial cells has been associated with the development of cardiovascular disorders.^{57,203} Oxidized LDL induces NOX4 expression in the endothelium, thereby increasing oxidant production.²⁰⁴ The formation of compromised atherosclerotic lesions has been reported in cases of reduced monocyte infiltration into the blood vessel wall and in LDL receptor-deficient mice.^{205–207} Expression of adhesion molecules (such as selectins, VCAM-1 and ICAM-1) induced by NF- κ B downstream of ROS in the vascular endothelium play indispensable roles in atherogenesis. The activation of NF- κ B downstream of ROS induces the expression of adhesion molecules in the vascular endothelium, such as selectins, vascular cell adhesion molecule-1 and ICAM-1, which play indispensable roles in atherogenesis.^{209,210} Additionally, ROS induces the inactivation of endothelial NO through superoxide anions, which reduce the bioavailability of NO, an important vasodilator, and promote vasoconstriction.^{206,207,211–213}

Interestingly, the exposure of macrophages to LPS from *Chlamydia pneumoniae* stimulates the over-expression of the fatty acid-binding protein, adipocyte protein 2 (ap2), and augments the uptake of LDL and foam cell formation.^{214,215} Activated macrophages dramatically affect vascular cell function through the release of bioactive molecules, such as pro-inflammatory cytokines and ROS.²¹²

Innate immune cells provide the first line of defense against invading pathogens.²⁰⁵ In contrast, these cells play a detrimental role in atherosclerosis. TLR ligands, such as LPS and aldehyde-derivatized proteins, are not only involved in microbial pathogenesis but also in atherogenesis, thereby establishing

a close link between innate immunity and atherosclerosis.²¹⁶ Hektoen and Osler reported the co-existence of tuberculosis and atherosclerosis characterized by aberrant changes in the arterial wall.²¹⁷ Moreover, *in vivo* studies in rabbits and serological evidence have shown an association between *Chlamydia pneumoniae* and the development of atherogenesis.²¹⁸ The studies also suggest that TLR-mediated signaling plays a mechanistic role in atherosclerosis. TLRs recruit adaptor molecules to the intracellular signaling domain, leading to the activation of NF- κ B and mediators of innate immunity, such as cytokines, vasoactive peptides, histamine, and eicosanoids, which are also involved in atherogenesis.²¹⁹ The TLR-mediated activation of cytokines and growth factors, such as platelet-derived growth factor and heparin-binding growth factor, promote the proliferation of vascular cells.²²⁰ Intriguingly, a direct link between TLRs and NOX has also been proposed. LPS activates NOX4 through TLR4, and the siRNA-mediated silencing of NOX4 in the U937 monocytic cell line expressing NOX4 attenuates LPS-induced superoxide production.¹⁴¹ This evidence suggests a correlation between atherosclerosis, innate immunity and NOX.

Furthermore, calcification, an important step in the progression of atherosclerosis, is strengthened through TGF- β .²²⁰ In addition, immunogenic cross-reactions between bacterial and human heat shock-protein 60 have also been reported in atherogenesis.^{221,222} Moreover, it has been reported that the proliferation of human vascular smooth muscle cells through Chlamydial heat shock-protein 60 is driven in a TLR4-dependent manner.²²³

As a key signaling molecule, Rac plays an indispensable role in both healthy and diseased cardiovascular systems. In mice, Rac is responsible for the progression of hypercholesterolemia, which includes increased NADPH-derived superoxide production, impaired endothelium-dependent vasorelaxation, increased macrophage infiltration into the vasculature and enhanced plaque rupture.²²⁴ In the atherogenic mouse, Rac1–NOX-mediated ROS generation inhibits NO synthesis and promotes the formation of LDL and various inflammatory mediators, such as ICAM-1 and vascular cell adhesion molecule-1, which contribute to the progressive development of atherosclerosis.²²⁵ A transgenic mouse model constitutively expressing Rac v12 under the control of the α -actin promoter for smooth muscle selective expression exhibited hypertension and enhanced superoxide production.²²⁶ Furthermore, angiotensin II-induced hypertension has been directly associated with elevated vascular NOX-mediated superoxide production and high blood pressure, which is regulated through the translocation of Rac1-GTPase to the cell membrane.^{227,228} High blood pressure induces biochemical stress, which causes NOX1/NOX4-mediated superoxide generation, resulting in vascular dysfunction.²²⁹ Moreover, Rac is responsible for the development of thrombogenicity of the vascular wall, which attributes to vascular remodeling through the activation of NF- κ B and ROS.²³⁰ Thus, these studies suggest a direct role for Rac activation in cardiovascular diseases. Thus, it is apparent that Rac and mediators of innate immunity/NOX play important roles in vascular diseases.

NOX INHIBITORS

Previous studies have focused on strategies to enhance the removal of ROS using either antioxidants or drugs that enhance endogenous antioxidants. Recent studies have focused on proteins further upstream to reduce oxidative stress using blocking enzymes that promote ROS production. Unfortunately, the currently available NOX inhibitors that target the catalytic site lack specificity. Because the structure of NOX2 enzymes has been elucidated and it has been shown that the correct assembly of NOX2 enzymes is critical for function, the inhibition of assembly represents a novel therapeutic approach. For example, the membrane association of the GTPase Rac 1, which is essential for NOX activation, is prevented using HMG-CoA reductase inhibitors.²³¹ Similarly, apocynin (4-hydroxy-3-methoxyacetophenone) is an orally active agent that also blocks NOX assembly.²³² Originally isolated from the medicinal plant *Picrorhiza kurroa*, apocynin inhibits both intracellular and extracellular ROS production through the inhibition of the phagosomal association of the cytosolic protein p47^{phox}.^{233,234} However, a limitation of apocynin is that this protein requires peroxidase for activation, and therefore, apocynin activity is not instantaneous.^{235,236} The nitration and nitrosylation also lead to the inhibition of NOX. For example, the NO donor, DETA-NONOate, suppresses basal NOX-dependent superoxide production.²³⁷ In addition, ethyl pyruvate plays a significant role in oxidative decarboxylation and is an effective inhibitor of NOX.²³⁸ Furthermore, there are inhibitors that perturb the signal transduction of NOX-related pathways. Aqueous solutions of pyruvate rapidly undergo an aldol-like condensation reaction to form 2-hydroxy-2-methyl-4-ketoglutarate (parapyruvate), a potent inhibitor of both the extracellular and intracellular production of ROS through transferrin receptor targeting for the recognition of LPS in microglia cells.²³⁹ Membrane channel blockers also act as NOX inhibitors.¹⁶⁵ Angiotensin II stimulates NOX-derived ROS production in smooth muscle cells through the activation of calcium channels.²⁴⁰ Therefore, calcium channel antagonism is an important approach to reduce NOX activity, specifically NOX5 or Duox proteins. Felodipine and amlodipine are two commonly used calcium channel blockers that significantly suppress NOX activity.²⁴¹ Gomicin C, a lignin extract from *Schizandra chinensis*, decreases cytosolic calcium levels in intracellular stores and exhibits weak inhibitory action against NOX2.²⁴² Compounds, such as 6-aminonicotinamide, deplete the availability of electron donors, such as NADPH, and inhibit the pentose phosphate pathway, which precludes NOX activation.^{243,244} Furthermore, NOX activation is also regulated through angiotensin II, platelet-derived growth factor and TGF, all of which are blocked through valsartan.^{245,246}

Small molecule inhibitors also offer a powerful tool for NOX inhibition due to their high affinity towards targets. For example, sulfhydryl-modifying reagents, such as N-ethylmaleimide or p-chloromercuribenzoate, inhibit the assembly and activation of NOX2.^{247,248} Honokiol, isolated from the herb *Magnolia officinalis*, blocks oxidative burst in both intact cells

and particulate neutrophil fractions. This molecule is potentially useful in pathological conditions because it completely inhibits superoxide production at a low concentration, even after the enzyme is activated.^{242,249} Another plant-derived compound, Plumbagin, inhibits NOX4 oxidase activity and exerts anticarcinogenic and anti-atherosclerosis effects in animals.²⁵⁰ The decreased activity of NOX4 has been demonstrated in plumbagin-treated human embryonic kidney 293 (HEK293) and brain tumor LN229 cells.²⁵⁰ Prodigiosins are natural red pigments produced from the bacteria *Serratia marcescens* that inhibit NOX2 through membrane binding and the inhibition of the interaction between Rac and p47^{phox}.²⁵¹ ten Freyhaus *et al.* characterized a novel NOX inhibitor, 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-d]pyrimidine (VAS2870) in vascular smooth muscle cells.²⁵² The pre-treatment of smooth muscle cells with VAS2870 completely abolished NOX activity and platelet-derived growth factor-dependent intracellular ROS production.²⁵² Furthermore, VAS2870 treatment inhibited ROS production and restored endothelium-dependent aorta relaxation in spontaneously hypertensive rats.²⁵³ Pyrazolopyridine derivatives, such as GK-136901, which are structurally related to VAS2870, were recently identified as potent inhibitors of NOX1 and NOX4.²⁵⁴ Although the mechanisms underlying the effects of these compounds have not been deciphered, the structural resemblance to NADPH suggests that these inhibitors might act as competitive substrates.²⁵⁵

Peptide inhibitors have many benefits over other oxidase inhibitors, such as good tissue penetration, a high degree of purity, low toxicity of degradation products and well-developed strategies for the synthesis of inhibitors with domain selectivity.²⁵⁶ However, the greatest challenge is to design peptides that specifically target individual NOX isoforms. Peptides corresponding to domains in the oxidase components involved in NOX assembly, such as NOX2, p22^{phox}, p47^{phox} and Rac, are inhibitory when added prior to complex assembly.²⁵⁶ Small peptides of 7–20 amino acid residues, designed to bind natural whole protein targets, can serve as inhibitors through the inhibition of intermolecular or intramolecular protein–protein or protein–lipid interactions during the assembly of the oxidase complex through competitive mechanisms.²⁵⁷ Two such NOX2 peptides, spanning 559–565 and 78–94 amino acids have been examined.^{258,259} Furthermore, NOX2 peptide 86–94 possesses a NOX2 B loop sequence essential for the activity and translocation of cytosolic components.^{260,261} NOX2 peptide 86–94 has been well studied and demonstrated as an oxidase inhibitor in cells, organs and whole animals. Endogenous proline-arginine (PR)-rich antibacterial peptide (PR39) is another peptide inhibitor produced in neutrophils, which blocks interactions between the Src homology 3 domain of the p47^{phox} subunit and proline-rich regions and inhibits NOX.^{262–264}

The catalytic subunit of NOX differs between each NOX isoform. Therefore, NOX1 and NOX2 are the best candidates for drug targeting to achieve isoform selectivity.²⁵⁵ However, the functional domains of the different NOX catalytic subunits

are highly conserved, which prevents selective pharmacological targeting.⁸¹ For example, diphenyleiodonium, which acts on the electron transport chain and prevents electron flow through interactions with FAD, does not discriminate between NOXs and other FAD-containing enzymes, including NOS, xanthine oxidase and cytochrome P450.^{255,265,266} Thus, this compound inhibits all NOX and Duox isoforms at low micromolar concentrations.²⁶⁷

Due to cytosolic and membrane-associated components, the optimal functioning of NOX depends on post-translational modifications, including phosphorylation and lipidation. Compactin and lovastatin block NOX activation through the inhibition of post-translational Rac1 isoprenylation. Studies using differentiated HL-60 cells have shown that lovastatin and compactin-sensitive components of NOX localize to the cytosolic fraction. These effects were reversed upon the administration of rRac1 to the system.²⁶⁸ Gliotoxin (mycotoxin), produced in *A. fumigatus*, significantly inhibits PMA-induced O₂ generation in PMNs,²⁶⁹ reflecting a reduction in the phosphorylation and incorporation of p47^{phox} into the cytoskeleton. Gliotoxin also inhibits the membrane translocation of p67^{phox}, p47^{phox} and p40^{phox}, however, there are no reports regarding the inhibition of Rac2 using this compound. The SUMO-1 and SUMO-specific conjugating enzyme, UBC-9, also significantly suppresses the activity of several NOX isoforms.²⁶⁸ The irreversible serine protease inhibitor, 4-(2-aminoethyl)-benzenesulfonyl fluoride, reduces the production of ROS through the inhibition of NOX2 assembly with p47^{phox} in both intact, stimulated macrophages and cell-free systems.²⁷⁰ 4-(2-aminoethyl)-benzenesulfonyl fluoride interferes with the activation of NOX, but not with electron flow. NOX activation and electron flow are two separate steps in the NOX pathway, and the effect of 4-(2-aminoethyl)-benzenesulfonyl fluoride is likely mediated through the chemical modification of cytochrome b₅₅₈.^{270,271}

Taken together, these data suggest that NOX inhibitors might reduce ROS-mediated stress in various malfunctions and diseases; however, these compounds must first be assessed for safety, selectivity, toxicity, bioavailability and the absence of significant side effects.

CONCLUSIONS

The primary function of NOX proteins in the immune system is to produce a respiratory burst that facilitates the killing of bacteria and fungi. This function is underscored by the fact that people with an inherited deficiency in NOX2 develop CGD and are unable to fight off common infections. However, while NOX isoforms play important roles in immunity, the short-term or incomplete inhibition of this pathway could potentially have therapeutic effects without disabling antibactericidal activity. Considering that the currently available pharmacological and natural compounds targeting NOX show encouraging results against ROS production, these compounds might also be effective against tissue damage and inflammation.

SUMMARY

ROS-mediated stress contributes to a number of diseases, including pneumonia, COPD, emphysema, and cardiovascular pathology. The activation of various PRRs, including TLRs and NLRs, leads to ROS production and inflammation, mediated through the NOX system. This system plays a significant role in the protection against both exogenous and endogenous pathogens, and excess ROS might cause tissue damage and many other diseases. Currently, researchers are focusing on various strategies to remove excess ROS, including the use of antioxidants and drugs that enhance endogenous antioxidants. One interesting approach is to block ROS production using either small molecules or peptide inhibitors, plant-based compounds or inhibitors of post-translational modifications. Therefore, it is important to fully characterize the mechanisms of ROS production and determine the effects of ROS in various tissues. Moreover, it is critical to understand the NOX system and the roles of other NOX family enzymes as major sources of ROS production at specific sites of injury/inflammation.

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