

FORUM REVIEW ARTICLE

NOX2 As a Target for Drug Development: Indications, Possible Complications, and Progress

Becky A. Diebold,¹ Susan M.E. Smith,² Yang Li,¹ and J. David Lambeth¹

Abstract

Significance: NOX2 is important for host defense, and yet is implicated in a large number of diseases in which inflammation plays a role in pathogenesis. These include acute and chronic lung inflammatory diseases, stroke, traumatic brain injury, and neurodegenerative diseases, including Alzheimer's and Parkinson's Diseases. **Recent Advances:** Recent drug development programs have targeted several NOX isoforms that are implicated in a variety of diseases. The focus has been primarily on NOX4 and NOX1 rather than on NOX2, due, in part, to concerns about possible immunosuppressive side effects. Nevertheless, NOX2 clearly contributes to the pathogenesis of many inflammatory diseases, and its inhibition is predicted to provide a novel therapeutic approach. **Critical Issues:** Possible side effects that might arise from targeting NOX2 are discussed, including the possibility that such inhibition will contribute to increased infections and/or autoimmune disorders. The state of the field with regard to existing NOX2 inhibitors and targeted development of novel inhibitors is also summarized. **Future Directions:** NOX2 inhibitors show particular promise for the treatment of inflammatory diseases, both acute and chronic. Theoretical side effects include pro-inflammatory and autoimmune complications and should be considered in any therapeutic program, but in our opinion, available data do not indicate that they are sufficiently likely to eliminate NOX2 as a drug target, particularly when weighed against the seriousness of many NOX2-related indications. Model studies demonstrating efficacy with minimal side effects are needed to encourage future development of NOX2 inhibitors as therapeutic agents. *Antioxid. Redox Signal.* 23, 375–405.

General Roles of Reactive Oxygen Species and Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form Oxidase Enzymes

REACTIVE OXYGEN SPECIES (ROS) are produced by the partial reduction of oxygen to form superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$). Other reactive molecules are also formed both enzymatically and non-enzymatically through the reaction of ROS with other species: peroxynitrite ($ONOO^-$) is produced by the spontaneous reaction of $O_2^{\bullet-}$ with nitric oxide (NO), and hypochlorous acid (HOCl) is formed by the myeloperoxidase-catalyzed reaction of H_2O_2 with chloride. While $O_2^{\bullet-}$ is weakly reactive and H_2O_2 is a moderately potent oxidant,

$ONOO^-$, HOCl, and $\bullet OH$ are highly reactive and produce molecular damage in DNA, protein, and lipids, resulting, for example, in DNA strand breaks, chlorination of protein tyrosine residues, and loss of membrane integrity (79, 80).

Phagocytic cells have capitalized on this chemical reactivity, generating microbicidal ROS within the phagosome as a part of innate immune mechanisms. In addition to their microbicidal functions, ROS, especially H_2O_2 , act as signaling molecules, impacting the function of signal transduction proteins, ion channels, and transcription factors (91, 327, 328). ROS are, thus, increasingly recognized as central players in a range of normal physiological processes. Early studies showed that H_2O_2 is produced under normal physiological conditions, for example, in response to the growth

¹Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia.

²Department of Biology and Physics, Kennesaw State University, Kennesaw, Georgia.

factors platelet-derived growth factor (PDGF) (291) and epidermal growth factor (12), and that it is overproduced in transformed cells expressing oncogenically activated Ras (115). Signaling pathways impacted by ROS include ERK1/2, JNK, nuclear factor-kappa B (NF-kappa B), focal adhesion kinase, AP-1, Akt, Ras, Rac, JAK-STAT, and many others (31).

The best characterized molecular mechanism by which ROS regulate signaling involves oxidation of low pKa cysteine residues that exist as thiolate anions (Cys-S⁻) at physiological pH, rendering them susceptible to oxidation by H₂O₂ (237, 328). This oxidation may occur directly or may require an additional protein such as a thioredoxin (312). Redox-sensitive thiols are often located in specialized protein environments such as active sites, where their oxidation typically inhibits enzymatic activity. Examples of such “oxidant-sensor” proteins include protein phosphatases (*e.g.*, protein tyrosine phosphatases [PTPs], low-molecular-weight protein tyrosine phosphatases, and MAP kinase phosphatases), the lipid phosphatase PTEN, and regulatory enzymes of ubiquitin and ubiquitin-like proteins such as SUMO and Nedd8 (237, 250, 312). As one example, PTP is oxidized in response to growth factor activation of receptor tyrosine kinases, thus simultaneously triggering protein phosphorylation and inhibiting the means of removing tyrosine phosphates from target proteins. The net result is to markedly increase tyrosine phosphate levels over those seen in the absence of oxidative mechanisms (12). Physiological stimuli that increase H₂O₂ may also result in the oxidation of protein thiols that can be reversed by, for example, thioredoxin or glutathione. This serves as an “off/on” switch analogous to protein phosphorylation/dephosphorylation and enables rapid regulation of downstream signaling pathways.

In addition to their normal signaling roles, ROS are recognized as a double-edged sword, implicated by virtue of their reactivity and pro-inflammatory properties in the pathogenesis of a long list of diseases, many of them inflammatory and/or chronic in nature (17, 154). Due to this association, antioxidant therapy has been investigated both in animals and in a large number of human clinical trials. Unfortunately, this approach has been largely unsuccessful, probably as a result of the common use of vitamins and/or dietary compounds that are generally very weak antioxidants *in vivo*. In addition, efficient cellular enzymatic antioxidant systems (superoxide dismutase [SOD], catalase, peroxidases, *etc.*) probably render the added effect of exogenous antioxidants rather small (156). The disappointing results with antioxidant therapy clinical trials have turned attention in recent years to eliminating the production of ROS at its source.

Cellular sources of ROS

While cellular ROS have classically been described as arising from a variety of redox-active enzymes (xanthine oxidase, cyclooxygenases, lipoxygenases, myeloperoxidase, heme oxygenase, monoamine oxidases, aldehyde oxidase, nitric oxide synthases [NOS], and cytochrome P450) as well as from the mitochondrial respiratory chain, ROS production from these sources is largely an “accidental” byproduct of catalysis involving redox-active coenzymes that have a low but finite reactivity with molecular oxygen (225). Generally, these sources generate low amounts of ROS, but levels can increase under pathological conditions, as occurs, for

example, in genetically mutated mitochondria (318) or in NOS that has been exposed to oxidants (the so-called “kindling reaction”) (157). NOX enzymes, on the other hand, efficiently produce O₂^{•-} or H₂O₂ as their primary catalytic function, thus earning the status of “professional” ROS-generating enzymes, and cellular mechanisms are in place to tightly regulate NOX activity (*e.g.*, phosphorylation, transcription) for cells to regulate their ROS levels both acutely and chronically (17, 156).

NOX enzymes are a family of NADPH-dependent oxygen reductases that are widely expressed in eukaryotes from plants to fungi to vertebrates. The catalytic NOX or dual oxidase (DUOX) subunit, represented in humans by seven paralogous genes (NOX1-5 and DUOX1 and 2), contains both flavin adenine dinucleotide (FAD) and two heme groups. The FAD, bound within a cytoplasm-facing flavo-protein dehydrogenase domain, oxidizes NADPH in a two-electron hydride transfer reaction; single electrons then pass in sequence from the FAD through the two non-identical heme groups located within a transmembrane domain, and, finally, to oxygen on the other side of the membrane, to form O₂^{•-} (Fig. 1). In some cases (*e.g.*, NOX4, DUOX1 and 2), the major detectable product is H₂O₂ rather than O₂^{•-} (294). Except perhaps for NOX5, NOX family members require interactions with other membrane-associated partner proteins

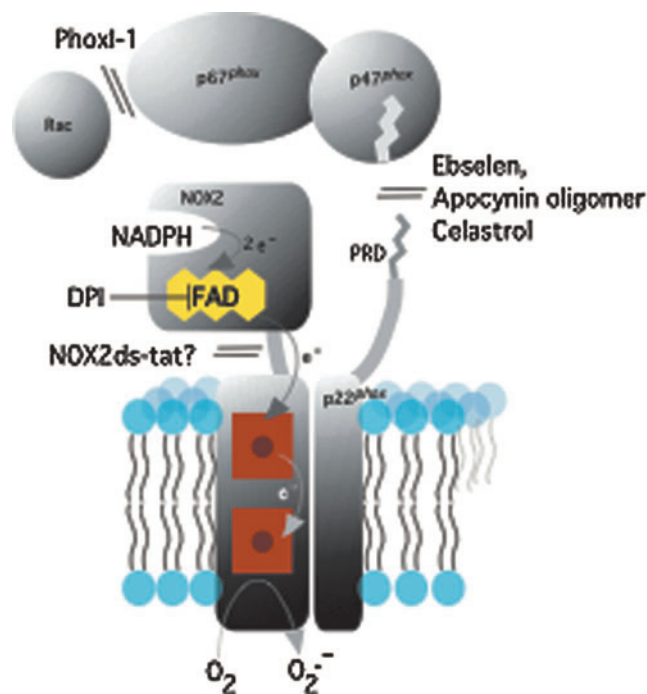


FIG. 1. Schematic diagram of NOX2 and NOX2 regulatory subunits, along with sites of inhibitor action. NOX2 and p22phox are shown in the membrane, along with NOX2 regulating cytosolic subunits. PRD refers to the proline-rich domain of p22phox. Shown also is the pathway of electron flow from FAD through the two heme groups (represented by red squares). FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NOX, NADPH oxidase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

for stability and/or localization; these include p22*phox* for NOX1–4 (9, 62, 134, 178, 308), and DUOX1 and DUOX2 for DUOX1 and DUOX2, respectively (90, 188). NOX1–3 require assembly with regulatory subunits for full catalytic activity, while NOX4 is constitutively active.

While NOX2 and its regulation were discovered and characterized first, the recognition of NOX enzymes as a family of ROS-generators has focused a great deal of attention on the other members of the family, especially with regard to their biochemistry, physiological, and pathophysiological roles. NOX4, for example, is frequently associated with fibrotic diseases, and has attracted a great deal of attention as a target for pharmaceutical development. Nevertheless, it has become apparent (*vide infra*) in recent years that the over-activity of NOX2 is also associated with a large number of diseases, particularly those with an inflammatory component. Here, we focus on NOX2 as a target for drug development, discussing its normal physiology and pathobiology, as well as possible complications that might arise from drug targeting of this enzyme.

Overview of the NOX2 enzyme

NOX2 and its regulatory partner proteins have been extensively characterized since their molecular cloning in the 1990s (4, 142, 163, 212, 243, 300, 317, 324). The catalytically dormant NOX2 in its membrane complex with p22*phox* becomes activated as a result of assembly with cytosolic regulatory partner proteins p40*phox*, p47*phox*, p67*phox*, and Rac1/2, a process triggered by phosphorylation of p47*phox* and probably other components, and by guanine nucleotide exchange on Rac. The structure and function of NOX enzymes has been extensively reviewed (17, 141, 153, 155, 287). For the present purpose, we point out that the presence of multiple specialized domains that mediate protein–protein interactions during the assembly process provide, in addition to the NADPH-binding site on NOX2, a number of candidate binding sites through which inhibitors might target the NOX2 system by disrupting assembly.

Physiological roles of NOX2

The known or proposed physiological roles and mechanisms of action of NOX2 are summarized in Table 1, as prologue to considering the possible complicating effects of drugs that target the NOX2 enzyme system. While levels of NOX2 are highest in phagocytes, NOX2 mRNA and/or protein have been detected at low levels in a large number of other tissues [(17), and Table 1]. In many cases, the co-expression and possible redundant function of other NOX isoforms complicates the interpretation of specific roles for NOX2. Likewise, the use of non-selective NOX inhibitors as tools (see next) also complicates interpretations. The use of genetic methods, including RNA interference and gene ablation, can be considered to be more definitive. Table 1 should, therefore, be considered in this context.

Professional phagocytes

Neutrophils and macrophages. NOX2 functions in neutrophils and macrophages in host defense against invading microorganisms (Table 1). Pursuing a chemical trail of microbial products (*e.g.*, formylated peptides) and host-derived

inflammatory factors (cytokines, lipid mediators), phagocytes locate and engulf invading microbes into phagosomes, in which a high concentration of NOX2-derived $O_2^{\bullet-}$ is generated. SODs form H_2O_2 , which reacts with chloride in a myeloperoxidase-catalyzed reaction and forms the highly microbicidal HOCl (17, 152, 194, 195, 265). The central role of the NOX2 system in host defense is demonstrated by the inherited condition chronic granulomatous disease (CGD) in which phagocytes genetically defective in NOX2 or one of its interacting regulatory subunits fail to kill ingested microorganisms, despite normal phagocytosis, resulting in frequent and chronic infections in affected individuals. Definitive evidence for the microbicidal role of ROS was provided by experiments which showed that exogenous H_2O_2 could restore the ability of defective neutrophils from CGD neutrophils to kill microorganisms (87). Neutrophils from mice in which NOX2 or one of its regulating subunits is genetically deleted show identical microbicidal defects (119, 223).

Similar to neutrophils, some populations of monocytes and macrophages respond to chemotactic signals to gather at sites of inflammation. Monocytes take up residence in specific tissues, becoming macrophages that are specialized for their role as “first responders” within the local environment. Macrophages can be activated both by pathogens themselves and by damage-associated molecular patterns (58, 278), to which they respond by phagocytosis of the offending material, producing cytokines and other signaling molecules, and presenting antigens. Macrophages express relatively high levels of NOX2 components, although they are also reported to express other NOX isoforms (17, 160, 309). The NOX2-derived respiratory burst in microbicidal killing in macrophages is similar to, but less robust than, that of neutrophils (17, 58, 278).

In addition to direct damage to microbial molecules, NOX2-derived ROS promote the formation of neutrophil extracellular traps (NETs), which also participate in innate immunity (28). NETs are composed of chromatin strands to which antimicrobial proteins are attached. Neutrophils release NETs in novel cell-death pathways that are triggered by various stimuli; the NET response to some stimuli depends on ROS-initiated breakdown of the phagocyte nuclear envelope (82, 133, 152). Neutrophils from CGD patients and mouse NOX2 knockouts fail to make NETs under stimulation conditions that usually promote NET formation, and NET formation in neutrophils from various mouse strains correlates with the amount of ROS produced (74, 82). It is important to note that certain stimuli promote NETs without ROS involvement (35, 41) and that the role of NOX2-derived ROS in NETS *in vivo* is unclear (196, 211).

NOX2-mediated ROS generation in phagocytes, especially macrophages, leads to the elaboration of immune mediators from various cell types, including phagocytes: inflammatory cytokines (*e.g.*, interleukin [IL]-1, IL-6 and tumor necrosis factor [TNF]-alpha), phagocyte-attracting chemokines (*e.g.*, CXCL8, CCL3, and CCL4), and bioactive lipids (*e.g.*, prostaglandins, leukotrienes, *etc.*) (58, 278). Such mediators are important in both innate and adaptive immune responses, recruiting inflammatory cells to sites of infection or inflammation. In macrophages of the vessel wall, low-density lipoprotein triggered TLR receptors to activate NOX2-dependent ROS generation, leading to the production of pro-inflammatory cytokines (13, 234) that are implicated in the development of atherosclerotic plaques; macrophages from CGD patients

TABLE 1. PHYSIOLOGICAL ROLES OF NOX2

<i>NOX2 tissue expression</i>	<i>Proposed function</i>	<i>Proposed mechanism</i>	<i>Evidence for NOX2 role</i>	<i>References</i>
Neutrophil	Host defense	ROS damage to macromolecules	CGD, <i>NOX2</i> KO mouse	(87)
		ROS-dependent NET generation	CGD, <i>NOX2</i> KO mouse	(74, 82)
		ROS signaling	<i>NOX2</i> KO mouse	(105, 149)
Macrophage	Host defense	ROS damage to macromolecules	CGD	(259)
		ROS-dependent cytokine production	CGD	(13, 234)
		ROS control of antigen processing	<i>NOX2</i> KO mouse	(248)
	Resolution of inflammation	ROS-dependent mediator production	CGD	(76)
	T-cell activation and proliferation	ROS oxidation of T-cell surface Cys thiols	p47 <i>phox</i> mutant rat	(85)
Dendritic cells	Host defense	ROS control of antigen processing	Ebselen; <i>NOX2</i> KO mouse	(180, 256)
Microglial cells	Host defense	ROS damage to macromolecules	<i>NOX2</i> KO mouse	(64)
		ROS-regulated cytokine production, signaling, and transcription	<i>NOX2</i> KO mouse	(136, 214)
Endothelial cells	Cell proliferation and survival Endothelial permeability	ROS-regulated transcription	<i>NOX2</i> siRNA	(78, 221)
		ROS-regulated signaling and transcription	p47 <i>phox</i> siRNA	(164)
	Vascular tone	O ₂ ^{•-} depletion of NO	<i>NOX2</i> KO mouse	(89)
Vascular smooth muscle	Vascular tone, growth and development	ROS-regulated signaling and transcription	<i>NOX2</i> siRNA	(29)
Skeletal muscle	Contractility	ROS regulation of Ca ²⁺ channels, transcription	<i>NOX2</i> KO mouse	(168)
Heart muscle	Contractility	ROS regulation of Ca ²⁺ channels, transcription	<i>NOX2</i> KO mouse	(231)
Neurons	Neuronal plasticity, memory	ROS regulated signaling, ion channels, and transcription	<i>NOX2</i> , p47 <i>phox</i> KO mice	(139)
	Neuronal development	ROS regulation of signaling and transcription	<i>NOX2</i> KO mouse	(61)
Pancreatic beta cells	Insulin secretion	ROS regulation of signaling and transcription	<i>NOX2</i> siRNA and <i>NOX2</i> KO mouse	(165, 337)
Hepatocytes	Apoptosis	ROS regulation of transcription	<i>NOX2</i> expression	(191)
Hematopoietic cells	Development, mitosis	ROS regulation of signaling and transcription	<i>NOX2</i> KO mouse	(311)
Adipocytes	Differentiation	ROS regulation of signaling and transcription	Inhibitors, translocation of p47 <i>phox</i> and p67 <i>phox</i>	(255)
Pulmonary neuroepithelial bodies	O ₂ sensing	ROS activation of O ₂ ^{•-} sensitive K ⁺ channels	<i>NOX2</i> siRNA	(34)
Lens epithelial cells	Cell proliferation	ROS regulation of signaling	p22 <i>phox</i> siRNA, translocation of p47 <i>phox</i> and p67 <i>phox</i>	(320)

CGD, chronic granulomatous disease; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NO, nitric oxide; NOX, NADPH oxidase; NET, neutrophil extracellular trap; ROS, reactive oxygen species.

showed greatly reduced levels of pro-inflammatory cytokines (13, 234) and increased anti-inflammatory cytokine IL-10. To our knowledge, a decreased propensity to develop atherosclerosis has not been evaluated in CGD patients, but a protective effect has been noted in *NOX2* gene-deleted mice (125).

Paradoxically, macrophages also participate in the resolution of inflammation by phagocytosis of apoptotic neutrophils, and in the regulation of the adaptive immune response *via* the elaboration of anti-inflammatory mediators. *NOX2*-deficient neutrophils and macrophages produce significantly lower levels

of the anti-inflammatory mediators cyclopentenone prostaglandin D₂ and transforming growth factor beta (32).

NOX2 also plays a role in non-canonical autophagy, such as that associated with phagocytosis in which the cell engulfs intracellular debris and helps prevent escape of a phagocytized organism. In neutrophils, Toll-like and Fc γ receptors activate NOX2, and the ROS produced participate in a signaling cascade that recruits the autophagy protein LC3 to phagosomes (105).

Microglia. Microglia are macrophage-like phagocytic cells that reside in the brain where they function in host defense and repair after tissue damage. As with other phagocytes, their effects may be mediated directly by ROS or indirectly *via* ROS-dependent inflammatory mediators. Spinal cord microglia from NOX2 knockout mice made less ROS and produced less pro-inflammatory cytokines in response to agonists (214) or injury (136) than did wild-type microglia, demonstrating an essential role for NOX2-derived ROS in up-regulating microglial pro-inflammatory cytokines.

Dendritic cells. Nox2-generated ROS also play a critical role in antigen processing before presentation by dendritic cells to naïve T lymphocytes [reviewed in ref. (145)]. Some of the evidence for a role of NOX2 in this area comes from studies which showed that the presentation of antigens such as ovalbumin by mouse bone marrow-derived dendritic cells to CD4+ T lymphocytes was decreased by the NOX2 inhibitor ebselen (180) and defective in dendritic cells isolated from NOX2 knockout mice (256). Observations that the pH within the phagosomes of dendritic cells isolated from wild-type mice, but not NOX2-deficient mice, rises slightly above pH 7 after internalization of beads or bacteria led to the model that NOX2-generated ROS consumes incoming protons, causing an increase in pH that is suboptimal for phagosomal (and also endosomal/lysosomal) proteases which prefer acidic environments. This process would serve to prevent complete proteolysis of antigens, enabling proper processing and presentation to occur. However, another study does not support this pH-based model and suggests a redox-based model instead (247). Besides the regulation of phagosomal pH, NOX2-generated ROS within phagosomes may directly oxidize and inactivate proteins of the phagosome, such as vacuolar H(+)-ATPase (75) and cysteine proteases (cathepsins B, L,S) (192), and also directly oxidize the antigens themselves to facilitate antigen presentation (44, 229).

Vascular cells

The physiological effects of NOX-generated ROS on various vascular cells, most notably endothelial and vascular smooth muscle cells (VSMCs), have been a focus of intense interest (27, 141, 159, 174, 260). Understanding specific roles for NOX2 is complicated by the presence of several NOX family members in vascular cell types, and different isoform expression in different anatomical regions of the vasculature (venous *vs.* arterial, lung *vs.* general circulation, microvasculature, *etc.*).

An important function of NOX enzymes in blood vessels is control of vascular tone, a process that involves both VSMC and endothelial cells. The endothelium produces NO, which potently mediates the relaxation of vascular smooth muscle (111). O₂^{•-} reacts rapidly with NO, eliminating its vaso-

relaxing effects. Angiotensin II (Ang II) also activates NOX-dependent ROS production in VSMC, signaling kinases and transcription pathways that increase vascular tone (159). While this is usually attributed to NOX1 (173) and/or NOX4 (264), siRNA depletion of NOX2 inhibited both basal and Ang II-induced ROS production in primary VSMC isolated from spontaneously hypertensive rats (29). NOX2 knockout mice displayed significantly less endothelial ROS production and significantly higher endothelium-dependent relaxation than wild-type mice (89). Human CGD patients manifest increased vascular NO and increased vasodilation, providing a direct link between NOX2 and endothelial function (314).

The endothelial NOX2 system also appears to play a role in regulating interactions between endothelial cells and neutrophils that lead to neutrophil migration through the endothelial layer. Coronary microvascular endothelial cells from p47phox^{-/-} mice stimulated with TNF-alpha failed to initiate normal ROS-dependent kinase and transcription factor cascades, and, consequently, also failed to express ICAM-1, which is necessary for neutrophil adhesion (164). It should be cautioned, however, that p47phox and its homologue NOXO1, while usually assumed to be specific for NOX2 and NOX1, respectively, have the *in vitro* ability to activate the other NOX isoform (42); that is, p47phox can activate NOX1, and NOXO1 can activate NOX2. If such cross-activation occurs *in vivo*, this might lead to an incorrect interpretation of the role of NOX2 (153).

NOX-derived ROS also functions in vascular growth, proliferation, and apoptosis in both VSMCs and endothelial cells (78, 173). Various agonists differentially stimulate ROS production from various NOX isoforms through several receptor, kinase, and transcription pathways that lead to altered developmental programs (49, 78). In one study, siRNA against NOX2 inhibited both ROS production and p38-MAP kinase-dependent proliferation in an endothelial cell line and in primary endothelial cells, while overexpression of NOX2 increased both responses (221). Using the same methods, NOX4-derived ROS was also shown to contribute to both responses, indicating a degree of isoform functional redundancy.

Muscle cells

ROS modulate functions of both cardiac and skeletal muscle, notably calcium signaling and contractility (7, 118). In particular, ROS sensitize ryanodine receptors, increasing calcium release (98, 251). NOX2 is expressed in sarcolemma and t-tubule membranes (98, 186), and studies in knockout mice pinpointed the source of stretch-activated ROS in cardiac muscle cells as NOX2 (231). In addition, gp91ds-tat, a peptide inhibitor of NOX2, inhibited the normal ROS-induced intracellular calcium release. Skeletal muscle fibers from NOX2 knockout mice did not produce ROS in response to muscle activity, and they also failed to release specific histone deacetylases that usually control gene expression (168). Despite these associations, we are not aware of any reports of functional defects in cardiac or skeletal muscle function in CGD patients, suggesting that if such effects exist, they are subclinical or compensated.

Other cell types

Along with the cell types discussed earlier, ROS produced by NOX enzymes are implicated in signaling in a large number of tissues and cell types (17, 31, 225, 232), although little

TABLE 2. DISEASES IN WHICH NOX2 IS IMPLICATED

Disease	Type of evidence for NOX2 role	References
Hypertension	NOX2 siRNA in mice	(220)
	Apocynin in mice	(303)
Acute lung inflammation	NOX2 KO mice	(216, 217, 315, 316, 340)
	p47 $phox$ KO mice	(114, 340)
Chronic obstructive pulmonary disease	P47 $phox$ KO mice	(150)
	Apocynin effects on H ₂ O ₂ in patients	(283)
Asthma	Steroids lower NOX2 in patients	(203)
	Apocynin effects on H ₂ O ₂ in patients	(284)
	Ebselen effects in guinea pigs	(338)
Cystic fibrosis	Neutrophil infiltration seen in patients	(95)
Pulmonary hypertension	NOX2 KO mice	(198)
	NOX2 activation and apocynin effects in lamb model	(302)
Ischemia-reperfusion injury in lung	p47 $phox$ KO mice	(333)
	Apocynin effects in mice, rats, and sheep	(43, 215, 333)
Ischemic stroke	NOX2 KO mice	(116, 167, 297)
	Apocynin effects in mice	(116, 295)
	VAS3870 effects in mice	(140)
Traumatic brain injury	NOX2 KO mice	(64)
	Apocynin effects in mice	(45, 279, 339)
	gp91(NOX2)ds-tat peptide in mice	(339)
Alzheimer's disease	A β peptides activate ROS and cytokine production <i>in vitro</i>	(23)
	gp91 (NOX2) activation in human AD brain	(10, 270)
	NOX2 KO mice	(209)
	gp91(NOX2)ds-tat inhibitor peptide in mice	(210)
Parkinson's disease	NOX2 KO mice	(331)
	NOX2 increased in human PD brain	(331)
Amyotrophic lateral sclerosis	NOX2 increased in ALS patients	(330)
	NOX2 KO mice	(175, 330)
	Apocynin effects in mice	(94)

(continued)

TABLE 2. (Continued)

Disease	Type of evidence for NOX2 role	References
Schizophrenia	NOX2 increased in neurons in mouse models	(19, 280)
	Apocynin effects in rodents	(19, 258, 280)
	NOX2 KO mouse	(280)
	p47 $phox$ mutated rat	(258)
Muscle disorders	NOX2 activated by stretch in rodent <i>in vitro</i> models	(231)
	NOX2 in muscle activated in mdx mice	(135)
	DPI inhibitor effects <i>in vitro</i>	(231)
	gp91(NOX2)ds-tat peptide inhibitor <i>in vitro</i>	(135, 231)
	NOX2 KO mice	(231)

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; DPI, diphenylene iodonium H₂O₂, hydrogen peroxide; PD, Parkinson's disease.

evidence specifically implicates NOX2. For example, NOX2 has been reported as a signal generator in hepatocytes (191) and adipocytes (255), based on tissue expression of NOX2 and the use of inhibitors and ROS scavenging. Over-expression of p22 $phox$ and membrane translocation of NOX2 cytosolic regulatory subunits correlates with ROS-mediated mitogenic signaling in lens epithelial cells (320). Evidence for the participation of NOX2-derived ROS in signaling pathways controlling beta cell insulin secretion has been obtained using siRNA (337) and NOX2-deficient mice (165). Neurons from NOX2-deficient mice showed impaired *N*-methyl-D-aspartate (NMDA) receptor-dependent long term potentiation (139) and also dysregulation of signaling pathways that are essential to proliferation (61). A study using NOX2-deficient mice also suggests that NOX2-derived ROS control release of the neurotransmitter glutamate in response to specific agonists (280). In hematopoietic cells, low oxygen tension in the bone marrow maintains quiescence and stem cell potential. The higher pO₂ in the vasculature leads to ROS production that signals cell division and migration (72, 112); other signals also induce NOX2-derived ROS that promote hematopoietic cell differentiation, migration, and senescence (261, 268). Studies using knockout mice report that NOX2 is a major source of the ROS which control cell division in myeloid precursor populations (311), although the physiological significance is unclear as human CGD patients are not reported to have myeloid cell deficiencies. In addition to controlling the general types of physiological and developmental processes in the cell types discussed earlier, NOX2 is implicated as an oxygen sensor in pulmonary neuroepithelial bodies (34).

Diseases in Which NOX2 Is Implicated

Throughout the eukaryotic domain, NOX enzymes function to trigger adaptive mechanisms in response to stresses, environmental assaults, or other noxious stimuli (5). In this

context, a role of NOX enzymes in causing direct damage to invading microbes can be considered to be one subclass of a stress response that exploits the ability of ROS to damage macromolecules of invading microbes. Other adaptive roles of ROS in innate immunity also conform to this paradigm; for example, ROS activate immune signaling pathways that trigger cytokine release, differentiation, apoptosis, *etc.* Paradoxically, NOX-generated ROS can damage host tissues directly, can initiate a hyper-inflammatory response that results in further tissue damage, and can cause longer-term changes, including alterations to cell apoptotic and differentiation programs, for example, differentiation of myofibroblasts which then deposit fibrotic material. While disease-triggering events are legion and may be unknown, a variety of diseases seem to have in common the central role of NOX-generated ROS in the pathogenic process. This section focuses on those conditions, summarized in Table 2, in which NOX2-derived ROS are considered to play an important role in pathogenesis, and which, therefore, represent candidate indications for NOX2-targeted drugs.

Vascular diseases

Hypertension. Hypertension is a major risk factor for stroke, heart failure, aneurysms, and peripheral artery disease, and it is a cause of chronic kidney disease. Even moderate elevation of arterial blood pressure predicts a shortened life. While multiple factors contribute to hypertension, oxidative stress is a unifying theme (264). In addition to vasculature, tissues involved in the pathogenesis of hypertension include the central nervous system (220, 342) and the kidney (249).

Evidence supports a role for ROS, particularly from NOX1, 2, and 4 in hypertension (264). In an acute model of Ang II-induced hypertension in mice, adenoviral-mediated delivery of either NOX2 siRNA or NOX4 siRNA to the brain subfornical organ prevented increases in mean arterial pressure and in heart rate, and simultaneous delivery of both siRNAs resulted in even greater suppression. The Ang II-induced increase in ROS was significantly inhibited in cultured forebrain neurons from NOX2- or NOX4-siRNA treated animals, and abolished in neurons from animals treated with both (220).

In another study (303), Dahl salt-sensitive rats were maintained on high-sodium drinking water, with or without the NOX inhibitor apocynin. By day 35, mRNA expression of renal cortical NOX2 and regulatory subunits markedly increased in high-salt rats but not in apocynin-treated rats. In apocynin-treated animals: (i) renal cortex showed a less oxidizing environment, based on reduced glutathione-to-oxidized glutathione (GSH:GSSG) ratios; (ii) renal cortical $O_2^{\bullet-}$ decreased; and (iii) renal glomerular and interstitial damage were markedly improved. Apocynin also decreased renal cortical monocyte/macrophage infiltration, improved renal hemodynamics, and decreased arterial pressure. Due to ambiguities about the mechanism of action of apocynin detailed next, definitive identification of NOX2 as the source of ROS in these studies should be confirmed using other approaches.

In contrast, eliminating NOX2-associated ROS production was ineffective in some chronic models of hypertension. In one study, transgenic mice overexpressing human renin (TTRhRen) exhibited hypertension and cardiac hypertrophy by age 10–12 weeks. Although TTRhRen/NOX2^{-/-} mice had significantly lower ROS levels in heart and aorta, these

mice still developed hypertension and cardiac hypertrophy (305). It is possible that other NOX isoforms might compensate for NOX2 loss in this model, or that other non-NOX mechanisms are involved. Consistent with roles for other NOX isoforms in hypertension, aortic media of spontaneously hypertensive rats showed ~2.5-fold increased NOX4 mRNA and ~10-fold increased NOX1 mRNA compared with control rats, whereas NOX2 and p22*phox* mRNA levels were similar (6).

Pulmonary hypertension. Pulmonary hypertension (PH) is a complex disease in which increased blood pressure develops in the lung vasculature, leading to extreme exertion symptoms during normal activity, exercise intolerance, and, in some cases, heart failure. PH has been classified into several types (273); this discussion is limited to hypoxia-related PH. Chronic hypoxia arising from obstructive sleep apnea, high altitude environment, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis can lead to PH. Currently, there is no known cure for PH, and current treatments aim at controlling symptoms and preventing additional lung damage.

While much of the evidence supports a role for NOX4 in the pathogenesis of PH (183, 184), evidence suggests that NOX2 is also involved. Obstructive sleep apnea, characterized by intermittent periods of hypoxia, is a risk factor for the development of PH. Experimentally, chronic intermittent hypoxia (CIH) induces PH, by increasing expression of NOX2 as well as NOX4, both of which may contribute to pulmonary vascular remodeling and hypertension (77, 198). Male mice exposed to CIH had increased right ventricular systolic pressure, right ventricle hypertrophy, and increased thickness of the right ventricular anterior wall and evidence of pulmonary vascular remodeling. Pathological changes were attenuated in NOX2 knockout mice that were subjected to CIH, consistent with a role for NOX2 in CIH-induced PH (198).

NOX2 may also contribute to PH indirectly *via* autophagy (302). In a study in which PH was induced surgically in fetal lambs, isolated pulmonary artery endothelial cells showed increased ROS production and translocation of p47*phox*, pointing to NOX2 activation. In addition, autophagy was increased compared with sham-operated fetal lambs. Inhibition of autophagy using 3-methyl adenine or chloroquine decreased NOX2 activation and $O_2^{\bullet-}$ generation, while administration of the antioxidant *N*-acetyl cysteine or the NOX2 inhibitor apocynin starting at birth improved lung oxygenation. Thus, autophagy may contribute to PH by increasing NOX2 activity. Collectively, these studies suggest that in addition to NOX4, NOX2 may be a target for drug development of PH.

Lung diseases

Acute lung injury and acute respiratory distress syndrome. NOX2-generated ROS are associated with a range of respiratory inflammatory diseases/injuries, including acute lung inflammation (ALI) and its more severe form, acute respiratory distress syndrome (ARDS). ALI/ARDS can result from an over-reaction of the host immune system to certain infections (certain influenza strains such as the 1918 flu, avian flu, sepsis, *etc.*) and also from physical trauma, blood loss, transfusion, hyperoxia, ventilator-induced lung injury, aspiration, and pancreatitis (181). Conventional anti-inflammatory drugs are ineffective in ARDS, which affects 200,000 U.S.

patients yearly and is fatal in approximately 40% of cases (244). The NOX2 system and other NOX enzymes have been implicated in ALI/ARDS in several studies. NOX2 and other NOX isoforms are expressed in endothelial and epithelial cells of the lung, where they may participate in early signaling events preceding ALI/ARDS (39, 298). Neutrophil infiltration into the alveolar spaces of the lung during the acute phase of ALI/ARDS is clearly visible in postmortem lung sections from ARDS patients as well as in bronchoalveolar lavage fluid (181). In mouse models, the effects on inflammatory responses observed in NOX2 knockout mice or p47phox-deficient mice depend on the model system [reviewed in ref. (39)]. When TNF-alpha was used to induce ALI, mice deficient in NOX2 or p47phox exhibited markedly diminished inflammatory responses (340). However, in a sepsis-induced ALI mouse model, the inflammatory response in p47phox knockout mice was not significantly different compared with wild-type mice (143). The role of NOX2 in the development of hyperoxic lung injury is also unclear. In one set of studies (216, 217), NOX2-deficient mice exposed to acute hyperoxia had less severe pulmonary edema and neutrophil influx into the lung, but in another study, NOX1- but not NOX2-deficient mice were protected from lung injury (38).

Several studies have focused on the role of NOX2 in lung inflammation resulting from viral infection. When challenged with inactivated H5N1 influenza virus, mice deficient in p47phox showed less severe lung pathologies and decreased virus titers compared with control mice (114). NOX2-deleted mice infected with influenza A viruses displayed significant reductions in viral titers, peri-bronchial inflammation, BALF macrophages, BALF inflammatory cell $O_2^{\bullet-}$, lung ONOO⁻, monocyte chemoattractant protein-1 (MCP-1), and alveolar epithelial cell apoptosis compared with wild-type mice. Lung levels of the anti-inflammatory factor IL-1beta were ~3-fold higher in NOX2-deleted mice. *In vivo* administration of apocynin to infected wild-type mice decreased viral titer, airway inflammation, and inflammatory cell $O_2^{\bullet-}$ production after infection. These findings suggest that NOX2-selective inhibitors may have therapeutic potential for control of lung inflammation and damage in viral infections (315, 316).

Chronic obstructive pulmonary disease. COPD is projected to become the fourth leading cause of death worldwide by 2030 (170). COPD is characterized by progressive lung inflammation and irreversible narrowing of the airways. Three risk factors are associated with COPD: (i) cigarette smoking; (ii) heavy exposure to occupational and indoor air pollution; and (iii) alpha-1 antitrypsin deficiency (69, 110). Available therapies for COPD include long-acting bronchodilators and at late stages, glucocorticoids. These treatments are largely ineffective at attenuating the inflammation or reversing the airflow obstruction associated with the disease, highlighting the need for new therapies.

In patients with COPD, there is an accumulation of neutrophils and macrophages in the lungs of smokers *versus* non-smokers (228, 246, 322). Phagocyte-generated ROS can contribute to diminished enzymatic activity of proteinase inhibitor enzymes (24) such as secretory leukocyte proteinase inhibitor (40) and tissue inhibitors of matrix metalloproteinases (321), and also can increase the activity of proteinases such as matrix metalloproteinase (81). As in other lung

inflammatory diseases, elevated ROS lead to increased pro-inflammatory cytokines. Oxidative stress may also damage the hypoxia response element within the vascular endothelial growth factor promoter of COPD patients (213), causing defective responses to hypoxia.

There are conflicting reports as to whether the deletion of p47phox reduces inflammation in the cigarette smoke-induced COPD mouse model. In one study (150), the number of macrophages and neutrophils and levels of IL-6, keratinocyte-derived chemokine (KC/CXCL1), and MCP1/CCL2 in BALF were lower in p47phox^{-/-} mice exposed to cigarette smoke compared with mice exposed to air. However, in another study (334), while ROS production was decreased in BALF cells of p47phox^{-/-} mice and NOX2^{-/-} mice, the knockout mice showed increased lung inflammation with development of distal airspace enlargement and alveolar destruction. Inflammation was associated with activation of the TLR4-NF-kappa B pathway in the gene-deleted animals. The authors concluded that genetic ablation of components of NADPH-oxidase enhances susceptibility to the proinflammatory and lung-damaging effects of cigarette smoke. This finding may relate to pro-inflammatory effects seen in humans in which components of the NOX2 system are mutated, as discussed in the section "CGD, hyperinflammation, and autoimmune disease." or may highlight the inadequacies of mouse models. While it is not clear from earlier descriptions as to whether NOX2 represents a promising target for human drug development, apocynin administered to COPD patients was effective in decreasing H₂O₂ levels in exhaled breath condensates (283).

Asthma. Asthma is a chronic inflammatory disorder of the airways that is characterized by episodic and reversible airflow obstruction and airway hyper-responsiveness (26). An estimated 300 million people worldwide suffer from asthma, with 250,000 annual deaths attributed to the disease (1). Emphasizing the need for new therapeutic approaches, current therapies are unsatisfactory in about half of the cases, and a subgroup of patients are refractory to current anti-inflammatory and bronchodilator therapies (100).

Oxidative stress and NOX enzymes appear to participate in the pathobiology of asthma, but it is not yet clear which isoform is likely to represent the best target for drug development. Blood of asthmatic children showed elevated biomarkers of oxidative stress (203). Consistent with the involvement of NOX2, inhaled corticosteroids that relieve symptoms also diminished NOX2 mRNA expression in circulating leukocytes from asthmatics. Administration of the NOX2 inhibitor apocynin to asthmatic patients led to decreased H₂O₂ levels in exhaled breath condensates (284). In guinea pigs, ebselen, a potent NOX2 inhibitor (276, 338), was able to improve the ovalbumin-induced asthmatic inflammatory responses, consistent with a role for NOX2 (276, 338). However, other evidence suggests that NOX2 may have a protective role in asthma, at least in mice. In the ovalbumin asthma model, NOX2 gene-deleted mice showed increased inflammatory responses and airway hyper-reactivity compared with wild-type mice. Based on co-culture experiments, the authors proposed that this resulted from increased interaction between Th2 cells and macrophages in the absence of NOX2 (14, 15).

Other NOX isoforms have also been associated with the pathobiology of asthma. In the ovalbumin asthma mouse

model, increased expression of NOX1, 2, 3, and 4 was seen, and symptoms were improved using artesunate, an antimalarial drug with antioxidant properties (99). Primary airway smooth muscle cells isolated from biopsies from individuals with asthma *versus* healthy controls showed increased oxidative DNA damage along with increased ROS production that was attributed to increased NOX4 expression. Airway smooth muscle cells isolated from individuals with asthma exhibited increased bradykinin-induced contractility compared with non-asthmatic control cells. This was abrogated by NOX4 siRNA, diphenylene iodonium (DPI), or apocynin (93, 293). In addition to NOX4, epithelial DUOX1 was induced by the Th2 cytokines IL-4 and IL-13, which are commonly elevated within asthmatic airways (93). Thus, while oxidative stress appears to play a pathogenic role, a distinct role for NOX2 in asthma will require additional investigation.

Cystic fibrosis. Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene resulting in misfolding of the CFTR protein and defective regulation of chloride transport by epithelial cells in several tissues; respiratory failure is the main cause of mortality and morbidity (95). CF, diagnosed in 1 out of 3000 births, is characterized by sustained neutrophil recruitment to lung and neutrophil-dominated inflammation from a very young age. Neutrophil NOX2-derived H₂O₂ can also fuel myeloperoxidase-dependent HOCl generation, which has been suggested to correlate with the severity of the disease (236, 329). DUOX1/2 may also contribute to CF (224). Thus, inhibitors of NOX2 and/or myeloperoxidase may decrease inflammation and diminish lung tissue damage in this condition.

Ischemic conditions

Ischemia-reperfusion injury after lung transplantation. Patients undergoing lung transplantation risk graft dysfunction secondary to reperfusion injury during which ROS are formed, leading to tissue destruction. In addition, patients with pulmonary artery blood clots, receiving cardiopulmonary bypass, or recovering from some form of pulmonary crisis are also likely to incur reperfusion injury. Currently, proven preventative or treatment drugs are unavailable, although clinical trials of promising therapies are under way (323).

ROS play an important role in lung ischemia-reperfusion injury (LIRI). In sheep subjected to LIRI, apocynin attenuated LIRI-induced increases in vascular permeability and pulmonary arterial hypertension (215). Apocynin also alleviated lung pathologies in a rat model of LIRI (43). In a mouse model of LIRI, wild-type mice, p47phox^{-/-} mice, or chimeras created by bone marrow transplantation between p47phox^{-/-} and wild-type mice were subjected to LIRI to investigate whether neutrophils deficient in p47phox would decrease the severity of LIRI (333). Both wild-type mice treated with apocynin and p47phox^{-/-} mice displayed markedly decreased pulmonary dysfunction and injury (vascular permeability, edema, neutrophil infiltration, and lipid peroxidation) compared with untreated wild-type mice. In addition, in this study, pulmonary dysfunction and injury occurring after LIRI were significantly decreased in the p47phox^{-/-}/wild-type (donor/recipient) chimeric mice, but not in wild-type/p47phox^{-/-} donor/recipient chimeras.

Moreover, the induction of TNF-alpha, IL-17, IL-6, RANTES (CCL5), KC (CXCL1), MIP-2 (CXCL2), and MCP-1 (CCL2) was significantly lower after LIRI in p47phox^{-/-} mice and p47phox^{-/-}/wild-type chimeras but not wild-type/p47phox^{-/-} chimeras. These results suggest that NOX2 contributes to LIRI. Thus, NOX2 inhibitors may provide a novel therapeutic approach for ischemia-reperfusion in the lung.

Ischemic stroke. Ischemic stroke is a leading cause of death (171), and the repeated failure of promising experimental stroke treatments in human clinical trials (127) makes it likely that this situation will not change soon. Both NOX2 and NOX4 have been implicated in stroke pathogenesis (128, 140, 182). Most animal studies have used the transient middle cerebral artery occlusion model, measuring infarct volume and blood-brain barrier permeability as parameters that increase after occlusion and reperfusion. These two parameters were improved in ischemic NOX2 knockout mice, and apocynin also attenuated blood-brain barrier permeability in wild-type mice (130). Other studies report similar findings and provide further support for a role for NOX2 in the pathogenesis of ischemic stroke (167, 296, 341).

On the other hand, another study (140) showed that there was substantial protection from induced ischemic stroke in NOX4-deficient mice, but not in NOX1- or NOX2-deficient mice. Still other studies have shown that NOX1 may exert a protective effect in stroke (129). NOX1-deficient mice showed no difference in sub-cortical cerebral infarct volume, but a four-fold greater cortical infarct volume. Apocynin (116, 167, 296, 341) and the small molecule NOX inhibitor, VAS2870 (140) improved outcome in the mouse models of ischemic stroke. The reported discrepancies among studies may relate to the duration of ischemia before reperfusion, and, in general, suggest that NOX2 and/or NOX4 inhibition is likely to be most effective when administered early after stroke. These studies also emphasize the need for isoform-selective inhibitors.

Neuroinflammatory diseases

Traumatic brain injury. The number of traumatic brain injury (TBI)-associated deaths continues to increase worldwide. While acute care of head injuries has improved, extension of the primary lesion due to oxidative damage and inflammation, disruption of the blood-brain barrier, excessive release of the neurotransmitter glutamate, and other events leading to neuronal death can exacerbate the injuries of brain trauma patients, who, as a result, may die days or weeks later (210, 254).

Evidence for the contribution of NOX2-generated ROS to neuroinflammation and neuronal death comes from studies using the rodent cortical impact model of TBI. In one study, unilateral TBI was induced in NOX2 knockout and wild-type mice (64). After injury, NOX2 expression increased mainly in microglial cells of the ipsilateral hemisphere of the wild-type mice. The contusion area, number of TUNEL-positive cells, and amount of O₂⁻ and ONOO⁻ metabolites produced were decreased in NOX2^{-/-} mice. In other studies (45, 138, 279, 339), NOX activity in the cerebral cortex and hippocampal regions increased rapidly after impact, and pre- or post-treatment with apocynin or the NOX2 inhibitory peptide NOX2ds-tat markedly decreased

$O_2^{\bullet-}$ levels in hippocampal neurons, oxidized lipid biomarker levels, blood–brain barrier disruption, microglial activation, and neuronal death. Thus, TBI may be an attractive indication for NOX2-directed drugs.

Alzheimer's disease. In 2010, 35.6 million people were estimated to be living with dementia, with an estimated 7.7 million new cases each year. The yearly cost of dementia health care in the United States is estimated at US\$ 604 billion. Alzheimer's disease (AD) is the most common form of dementia and is estimated to account for 60–70% of cases. Current treatments fail to cure and only minimally impact the progression of the disease, although new approaches to treatment are being investigated in clinical trials (2).

Amyloid beta protein is found in plaques of brains from AD patients. Beta-amyloid peptides $A\beta(1-40)$ and $A\beta(1-42)$ are generated by proteolytic cleavage from amyloid precursor protein, a transmembrane protein that is important for neuron growth, survival, and repair (227, 267). $A\beta(1-42)$ and $A\beta(1-40)$ oligomers accumulate in fibrils in the extracellular spaces of the brain in AD patients (102, 202). Beta-amyloid fibrils directly activate NOX2 in primary rat microglial cells as well as in human neutrophils and monocytes (23), leading to the production of ROS and pro-inflammatory cytokines that participate in inflammatory tissue damage. Biochemical studies showed increased *p47phox* and *p67phox* in membrane fractions from human AD postmortem cortices as well as increased NOX2 activity in brain cortex homogenates compared with age-matched non-diseased brains (10, 270).

In addition to microglial NOX2, endothelial NOX2 may also contribute to the pathogenesis of AD due to effects on blood flow. In wild-type mice, agents that release NO or stimulate its *in vivo* production caused increased cerebral blood flow which was attenuated in Tg2576 transgenic mice that overexpress $A\beta$. This impairment was not observed, however, in Tg2576 mice lacking NOX2, implicating NOX2 in the vascular dysfunction induced by $A\beta$ fibrils (209). Direct application of $A\beta(1-40)$ onto the cortex increased ROS production in wild-type mice; this increase was abrogated by gp91(NOX2)ds-tat and also in the $NOX2^{-/-}$ mice. A NOS inhibitor prevented $A\beta$ -induced modulation of blood flow, which was consistent with the idea that NOX2-generated $O_2^{\bullet-}$ scavenges NO, thus decreasing its bioavailability. While plaque load and brain $A\beta$ levels did not differ between Tg2576 and Tg2576/ $NOX2^{-/-}$ mice, Tg2576 mice lacking NOX2 were protected from behavioral dysfunction (210). These data point to AD as an indication for NOX2-targeted drugs.

Parkinson's disease. Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disease, with physiological manifestations that include tremor, rigidity, slowness of movement, and postural instability, along with impairments in speech, cognition, mood, and behavior. Pathologically, PD is characterized by the loss of dopaminergic neurons in the substantia nigra and the appearance in neurons of Lewy bodies composed of misfolded alpha-synuclein protein (277). Although the etiology of PD has been intensively pursued for decades, biochemical mechanisms, genetic and epigenetic factors leading to initiation and progression of the disease remain elusive. Only 10–15% of PD is due to known genetic mutations. Environmental

exposure has been proposed to account for a subset of PD, and exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat, and rotenone increases the risk of PD in humans. To date, no drug therapy alters the progression of PD. Levodopa improves motor impairments, but dyskinesia can be an unsettling side effect. While a number of new therapeutics [reviewed in ref. (222)] are in the pipeline, whether they are able to alter disease progression remains to be determined. To date, drug candidates that have gone through clinical trials have proved disappointing, and new treatment approaches are being explored, which include gene transfer, cell-based therapies, and deep brain stimulation. Thus, disease-modifying drugs remain an unmet medical need.

Regardless of the initiating cause, oxidative stress remains a leading theory for explaining the progression of PD. Studies with cell and animal models reveal oxidative and inflammatory properties of PD-inducing toxins and their ability to activate glial cells. Activated microglia produce a host of factors that are toxic to neighboring dopaminergic neurons. In particular, the microglial NOX2 system exerts pathological effects both by direct ROS damage to neighboring neurons and also by triggering inflammatory cytokine signaling that results in a vicious cycle of sustained microglial activation and neuronal damage (292, 325). In a PD mouse model (331), MPTP induced overexpression of microglial NOX2, elevated ROS, and increased biomarkers of oxidative damage in the substantia nigra pars compacta. ROS production, oxidative damage, and neurodegeneration were substantially reduced in MPTP-treated NOX2-deleted mice. SOD infusion into the left striata attenuated the lesion on this side, but not on the contralateral, non-SOD infused side. NOX2 protein expression in six human PD midbrains was increased twofold in comparison to age-matched control brains, pointing to human relevance of the mouse model. Other NOX enzymes may also contribute to PD under some conditions or model systems; for example, similar approaches implicate neuronal NOX1 in PD pathogenesis in the rat 6-hydroxydopamine PD model (46) and rat paraquat PD model (51). Therefore, NOX2 and perhaps other NOX isoforms are attractive targets for slowing the progression of PD.

Amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis (ALS), a.k.a. Lou Gehrig's disease, results in loss of motor neurons, leading to progressive muscle paralysis (226). ALS is relatively rare, with a reported incidence of 1–2 per 100,000 per year. However, during the 1990s, clusters of cases were seen in Japan, Micronesia, and Indonesia with a local incidence at least 50 times higher than that seen worldwide (18). The average life expectancy of ALS patients ranges from 2 to 10 years after diagnosis. Mutations in SOD1 account for ~20% of familial cases, which is about 2% of total cases (55, 59). The drug Riluzole (Rilutek) improves survival but does not reverse existing motor neuron damage, and patients should be monitored for liver damage, which occurs in ~10% of treated individuals. Oxidative stress has been proposed to function in the progression of ALS, and several antioxidants, including vitamin E, *N*-acetyl cysteine, and selenium, have been investigated in clinical trials; none has made a significant impact on disease progression (207).

While the initiating causes and pathogenesis of ALS in humans is poorly understood, evidence indicates that NOX enzymes contribute to the neurodegenerative process (238).

A transgenic mouse bearing the SOD1G93A mutation has been extensively used as a model of ALS; the mutation does not affect SOD enzymatic activity but rather affects its binding to some other proteins. NOX2 overexpression and increased biomarkers of oxidative stress have been observed in spinal cords of both ALS patients and SOD1G93A mice. In SOD1G93A mice in which NOX2 was deleted, neurodegeneration was delayed and survival was extended (330). The ability of NOX2 deletion to improve the life span of ALS mice was supported in another study in SOD1G93A mice (175) in which both NOX1 and NOX2 were induced; NOX2 deletion in these mice resulted in a dramatically increased life span. Deletion of NOX1 also improved life span, but less remarkably. Apocynin also dramatically increased the life-span of SOD1G93A mice (94), but this result could not be repeated in two independent studies (166, 306). This may indicate either that the SOD1G93A mice provide an inadequate model human ALS, or that a more potent and/or selective NOX inhibitor may be needed.

Schizophrenia. Schizophrenia, affecting around 1% of the population worldwide (241), is a complex and disabling neuropsychiatric disorder. Despite a long history of antipsychotic drug development, approximately 30% of patients with severe schizophrenia are refractory to existing medications (200), emphasizing the need for new therapeutics and novel targets. The disease is marked by dysregulation of several neurotransmitter systems, including dopamine, glutamate, and gamma-aminobutyric acid. The behavioral and physiological characteristics of the disease can be mimicked by drugs that cause dopamine overproduction or block NMDA receptors. Oxidative stress increases in schizophrenia as evidenced by reports of changes in oxidative stress biomarkers such as increased oxidized-to-reduced glutathione ratios in the blood of patients (48, 233, 245) and decreased reduced glutathione levels in cerebrospinal fluid and postmortem prefrontal cortices of schizophrenic patients (63). Limited studies have also reported amelioration of symptoms after treatment of patients with the antioxidant *N*-acetyl cysteine (21, 33).

The ketamine rodent model mimics many of the cognitive, behavioral, and social deficits seen in human schizophrenia (190). Sub-anesthetic concentrations of ketamine produce psychotic-like symptoms in human volunteers, as well as impairments in memory and sustained attention performance that mimic the cognitive deficits observed in schizophrenia patients. In mice, the activation of NOX2 contributes to the dysfunction of GABAergic interneurons after subchronic ketamine exposure (19, 20). Ketamine increases oxidative stress in rodent brain through the activation of neuronal NOX2, which initiates a cascade of events that, ultimately, leads to altered function in parvalbumin-expressing (PV+) neurons that are believed to control cognitive function impairments associated with schizophrenia. Significant increases in the expression of NOX2 and *p22phox* (but not NOX4) were observed in membrane preparations from brain cortex of ketamine-injected mice. This increase in NOX2 was accompanied by an increase in synaptosomal NADPH-oxidase activity and paralleled a loss of PV+ neurons (19). In another study (280), ketamine caused rapid behavioral alterations, release of neurotransmitters, and brain oxidative stress in wild-type mice; whereas NOX2-deficient mice did not display such alterations. Wild-type mice showed

decreased expression of subunit 2A of the NMDA receptor after repeated ketamine exposure, which did not occur in NOX2-deficient mice, implicating NOX2 in down-regulation of NMDA receptor subunits.

Social isolation of rodents provides an alternative model of schizophrenia, leading to behavioral and histopathological alterations that are similar to those seen in the human disease (162). While NOX2 mRNA was not detected in the brains of control non-isolated animals, it was highly expressed in specific regions in the brains of socially isolated rats, which also showed increased brain biomarkers of oxidative stress. The treatment of isolated rats with apocynin prevented the behavioral and histopathological alterations. Moreover, rats with a functional mutation in *p47phox* (109) were protected from behavioral changes, loss of PV protein, and decrease in NMDA receptor subunit 2A (258). Thus, several lines of inquiry point to NOX2 as a novel and promising target for the treatment of schizophrenia.

Muscle disorders

The dysregulation of signal transduction from mechanical stretch to muscle contraction contributes to heart failure and muscle myopathies (230). In cardiac muscle cells, mechanical stretch depolarizes the plasma membrane as a result of a small influx of Ca^{2+} via L-type voltage-gated calcium channels. This influx stimulates opening of ryanodine receptors (RyR2 in the heart), which are calcium channels on the sarcoplasmic reticulum that release transient bursts of Ca^{2+} into the cytoplasm (30, 299). These “calcium sparks” induce shortening of myofibrils and contraction, which ends with relaxation when intracellular Ca^{2+} returns to resting levels and the RyR close. However, oxidative stress targets several cysteine residues on ryanodine receptors (11, 65, 98, 176), causing the receptor to become over-sensitized to Ca^{2+} levels, and to remain open longer than normal (231). Ultimately, the sarcoplasmic reticulum stores of Ca^{2+} fall to inadequate levels that cannot support contraction, leading to heart failure or skeletal muscle myopathies.

Recently, it was demonstrated that excessive Ca^{2+} release from over-sensitized RyR2 results from rapid and reversible ROS signaling originating in an intact microtubule network. These observations were made using single cardiomyocytes from *mdx* mice, which have a genetic mutation in the gene coding for the cytoskeletal protein, dystrophin. Myocytes from *mdx* mice had elevated ROS, and moderate stretching produced excessive Ca^{2+} release from over-sensitized RyR2 receptors (231). NOX2 and its subunits are localized in the membrane of the transverse tubules of rodent cardiomyocytes, and moderate stretching of isolated single myocytes activates ROS generation and translocation of NOX2 regulatory subunits to the membrane. Stretch-induced Ca^{2+} spark production was inhibited by gp91(NOX2)ds-tat, as well as by DPI. Moreover, in myocytes isolated from NOX2-deleted mice, stretch-induced ROS generation was absent (135, 231). Earlier observations (251) showed that NOX2 is present in skeletal muscle fibers, and its activation contributes to Ca^{2+} release from sarcoplasmic reticulum. ROS is also elevated in skeletal muscle from *mdx* mice or dysferlinopathy, another muscular dystrophy (230). It should be noted that NOX4-generated ROS and oxidation of RyR1 have also been implicated in muscular dystrophies (289).

Possible Complications Resulting from Inhibition of NOX2

When considering clinical drug development programs targeting NOX2, it is important to bear in mind possible complications that might arise from inhibiting the normal functions of this enzyme and to weigh these against the hypothetical benefits of treatment. The human genetic disorder CGD as well as animal models provide insights into this question.

CGD as a model for possible complications of NOX2 inhibition

CGD is a genetic disease that is characterized by severe bacterial and fungal infections and abscesses in the lung and liver (266). Before the advent of antibiotics, affected individuals frequently succumbed to infections during childhood, but with modern antibiotics and other therapies (131), individuals often survive into the fourth decade and beyond. Neutrophils from CGD patients are defective in the respiratory burst, the process by which molecular oxygen is reduced by the phagocyte NOX2 system to generate microbicidal ROS (see section “General roles of reactive oxygen species and NADPH oxidase enzymes” I, above). Genetically, the condition results from mutations in or deletion of any of the genes encoding subunits of the respiratory burst oxidase (97, 148) and is described in Table 3.

While targeting of NOX2 or its regulatory subunits with small-molecular-weight inhibitors raises concerns about the suppression of innate immunity resulting in increased infections, infection-related symptoms are seen only when the NADPH-oxidase activity is <15–20% of normal (97, 148); individuals with ROS-generating activity greater than this value are asymptomatic and are, therefore, never diagnosed with CGD. Moreover, an extensive analysis of deaths in a study population of 287 CGD patients followed for more than two decades (148) revealed that survival was independent of the particular gene affected, but depended solely on the extent of residual NADPH-oxidase activity. Significant mortality was manifested only when residual activity fell below ~2%: a single death occurred among patients classified as having relatively high residual ROS generation, whereas around a third of those with severe ROS deficiency had died by age 40. It should be pointed out that today’s patients are generally treated with prophylactic antibiotics, and that conclusions are likely to differ in naïve patients. Nevertheless, these studies point out that there is a considerable reservoir of excess NOX activity and that even a modest residual activity confers benefit. Dosing and scheduling of a NOX2-targeting drug can, in principle, be managed so as to

avoid a high level of continuous inhibition, and the cyclic nature of dosing should result in intervals during which oxidase activity returns to levels that allow adequate microbicidal function. In addition, it should be noted that even in CGD patients with complete loss-of-oxidase function, the decreased survival is seen over the time scale of decades, and mortality from suppression of innate immunity is not likely to be an acute problem.

CGD, hyperinflammation, and autoimmune disease

In addition to impaired host defense, CGD is a disease of excessive inflammation and increased risk of autoimmune disorders (266). A hallmark of the disease is formation of granuloma, foci in which macrophages and other immune cells concentrate. Granuloma may occur in many organs, including the gastrointestinal tract, where they contribute to enteritis resembling Crohn’s disease, and in the genitourinary tract, where they may result in blockages.

The pathogenesis of granulomatous lesions is not entirely clear, but it has been suggested to result, in part, from failure to kill and clear microbes after minor infections. However, recent studies indicate that this may be an over-simplification, and that excessive inflammation may be explained by an important role for the NOX2-derived ROS in regulating the inflammatory response through redox-sensitive signaling and transcription pathways (108, 266). Consistent with this interpretation, phagocytic cells in CGD are markedly perturbed in their gene expression, including increases in pro-inflammatory genes, decreases in anti-inflammatory genes, and alterations in apoptotic genes (144). The net result of the latter renders phagocytes less susceptible to apoptosis, perhaps accounting for their accumulation in granuloma. Another theory is that tryptophan catabolism *via* the O₂^{•-}-dependent enzyme indoleamine 2,3-dioxygenase is defective in CGD, leading to a deficiency in production of kynurenine (240). The latter is thought to participate in regulating the immune response, and its absence may result in a hyperinflammatory state. Regardless of the explanation, it is obvious that phagocytes from CGD individuals are markedly abnormal, not only in their ability to kill certain microbes, but also in their significantly altered expression of inflammatory, apoptotic, and other genes, which is expected to result in altered function.

In addition to granuloma, there are a number of other clinical manifestations of an overly exuberant immune response in CGD. For example, fungal products result in a life-threatening “mulch pneumonitis” (271). In addition, CGD patients show an increased frequency of certain autoimmune diseases, including rheumatoid arthritis, a systemic lupus erythematosus (SLE)-like syndrome, and Guillain-Barré syndrome, an autoimmune demyelinating disease (86, 107, 108). Experiments in rodent models summarized next add further insights into mechanisms by which an absence of NOX2-dependent ROS generation in cells of the innate immune system may propagate effects through cell types of the adaptive immune system.

Because the majority of CGD patients have residual levels of ROS below 5% of normal, the extent to which drug targeting the NOX2 system may result in autoimmune side effects is not clear. In the study of 287 patients described earlier (148), the occurrence or severity of granulomatous complications did not correlate well with the extent of residual NOX activity, which might suggest that autoimmune

TABLE 3. GENETIC ORIGINS OF CGD

<i>Subunit</i>	<i>Function</i>	<i>Gene</i>	<i>% of cases</i>	<i>Inheritance</i>
NOX2	Catalytic	<i>CYBB</i>	65	X-linked
p22 <i>phox</i>	Regulatory	<i>CYBA</i>	<5	Autosomal recessive
p47 <i>phox</i>	Regulatory	<i>NCF1</i>	30	Autosomal recessive
p67 <i>phox</i>	Regulatory	<i>NCF2</i>	<5	Autosomal recessive
p40 <i>phox</i>	Regulatory	<i>NCF4</i>	Single case	Autosomal recessive

dysfunction may still occur when residual ROS levels are 5–15% of normal. Additional models of partial loss of NADPH-oxidase activity are needed to better assess the risk of side effects, and some are presented next.

Autoimmune disease in mothers of CGD patients

Mothers of X-linked CGD patients show an increased incidence of autoimmune disorders, including an SLE-like syndrome and rheumatoid arthritis (177, 275). In kindreds with X-linked CGD, 9% have one individual diagnosed with lupus-like symptoms. A study of 19 CGD carrier mothers revealed a high incidence of lupus-like symptoms limited to cutaneous lesions, including 58% reporting photosensitive skin rashes and 42% with mouth ulcers. In addition, 37% showed joint pains (36). However, it is important to note that random X inactivation (a.k.a. Lyonization) implies that female carriers will have two populations of neutrophils, one that is entirely normal in its NADPH-oxidase activity, and a second which is CGD-like, lacking NOX2 activity (177). Depending on the developmental stage at which Lyonization occurs, the effect on the cell lineage can be skewed and can even give rise to mild CGD symptoms in female carriers. Skewed cell lineage has been verified in CGD carriers using a nitroblue tetrazolium (NBT) dye reduction test which stains only neutrophils that actively produce ROS (124); while normal individuals showed 98% NBT positive cells, female carriers showed a wide range (16–88%) of positive cells. Thus, while the ROS generation in CGD carriers is, on average, about half of normal, the average arises from normal ROS generation in some cells and absent ROS generation in others. Since CGD cells have grossly abnormal gene expression and immune function compared with normal cells, it seems likely that the same subpopulation of phagocytes which lacks ROS generation in the carrier state will closely resemble a CGD phagocyte in terms of immune function, apoptosis, *etc.*, and that these severely compromised cells will predispose the carrier to autoimmune disorders. With regard to bacterial killing, neutrophils of CGD carriers, indeed, show a range of functional abilities, from near normal to inactivation almost as profound as that seen in CGD (235). While it also would be of great interest to investigate whether there is a correlation between the fraction of abnormal cells and the frequency of autoimmune disease, to our knowledge, this has not been done. Still, the presence of a severely compromised subpopulation of immune cells raises questions about the extent to which the CGD carrier state can be used as an appropriate model to predict the likelihood of autoimmune complications that might arise from partial NOX2 inhibition. Due to these considerations, in our opinion, the CGD carrier state is a poor model for predicting NOX2 drug side effects. In the next few sections, we consider inactivating (including partially inactivating) mutations in animals and humans as predictive models for possible complications of NOX2 inhibition.

Animal model association of autoimmune disease with compromised NOX2-dependent ROS generation

Studies in rats and mice have provided important insights into the physiological mechanisms by which a compromised NOX2 system may increase the risk or severity of autoimmune disorders. In genetic studies comparing inflammation-resistant E3 rats with Dark Agouti (DA) rats, which are

predisposed to developing pristane-induced arthritis and other autoimmune conditions, the Pia4 region of the chromosome correlated with susceptibility to or severity of inflammatory diseases (101, 253). Positional cloning identified *NCF1* (encoding p47*phox*) as the gene that was responsible (204, 313). Depending on the activating agonist used, neutrophils from affected animals showed between 25% and 50% of normal $O_2^{\bullet-}$ generation. Similarly, mice with a mutation in *NCF1* had no detectable oxidative burst and showed enhanced collagen-induced arthritis as well as increased severity of experimental autoimmune encephalomyelitis (106, 107). Some of the female *NCF1* mutated mice developed spontaneous severe arthritis without pristane. Other mouse strains mutated in other *NOX/phox* genes also show low or absent ROS generation, and are similarly susceptible to increased arthritis severity in various models (108).

Studies in rodents have provided possible insights into the mechanisms by which a compromised NOX2 system increases arthritis severity (108). In one study of arthritis-susceptible rats, a higher content of reduced thiols on the T-cell surface was seen and correlated with increased ability to induce arthritis in adoptive transfer experiments (85). T cells do not express the NOX2 system, so any oxidation of the T-cell surface most likely results from an interaction with phagocytes. It is difficult to understand, however, how the redox state of thiols on the cell surface would fail to equilibrate rapidly in a new host animal, and it seems unlikely that such changes alone would account for the observed results. Since it readily diffuses through membranes (197, 327), H_2O_2 can also, in theory, affect intracellular proteins in nearby cells, for example by oxidizing regulatory low pKa thiols in enzymes such as PTPs and in transcription factors (153), and this might lead to reprogramming of protein expression patterns and/or differentiation in target cells. In another study, cellular changes were tracked in *NOX2* knockout mice that displayed aging-dependent spontaneous development of arthritis (161). The NOX2 deficiency was associated with changes in immune cell populations, with marked alterations in subpopulations of myeloid cells as well as lymphomegaly, splenomegaly, and increased levels of inflammatory cytokines, including interferon- γ and IL-17. Additional studies are needed to fully elucidate the mechanism by which decreased NOX2 activity is linked to the development of arthritis in rodents.

While rodent models have proved useful in dissecting cell types and pathways involved in NOX2-ROS suppression of hyperinflammation, they do not provide an adequate model for predicting whether therapeutic NOX2 inhibition is likely to cause side effects related to autoimmune disorders. In addition to the issue of species difference, most of the models described earlier (including the aging-dependent spontaneous arthritis model) used animals in which NOX2 activity was undetectable, providing a model for immunologic changes in CGD but not for a partially or intermittently inhibited state as would be seen with drug treatment. In the DA rat models of arthritis, a polymorphism in the *NCF1* gene was associated with partial inhibition of NOX2-dependent $O_2^{\bullet-}$ generation, but the DA rat represents an animal that is already genetically predisposed to arthritis. In addition, strong arthritis-inducing stimuli such as pristane and collagen were used to cause disease in many of the studies, and it is not clear how relevant this will be to spontaneous arthritis in

humans. Therefore, it is important to evaluate associations in humans between decreased (but not absent) NOX2-dependent ROS generation and autoimmune diseases.

Human disease associations with polymorphisms in NOX2 and phox components

In rheumatoid arthritis patients, a subset of male patients had a single nucleotide polymorphism in *NCF4*, the gene encoding p40phox, at a higher frequency than that of the general population (205). The polymorphism occurred in a non-coding region in the beginning of intron 4, but the effect on p40phox expression or NOX2 enzyme activity was not reported. The results were interpreted as consistent with the view that both multiple genes and also sex differences contribute to disease progression in different subsets of rheumatoid arthritis patients. A separate study by the same group reported a decreased risk of rheumatoid arthritis associated with increased copy number of *NCF1* (p47phox) (206). *NCF4* has also been associated with Crohn's disease (239). SLE patients (120) had a higher incidence of the 389 Q/Q polymorphism in *NCF2* (encoding p67phox) than the control population, which has the more common 389 H/H allele. The Q mutation resulted in a weaker association of p67phox with the guanine nucleotide exchange factor Vav, resulting in 50% lower Fc receptor-activated $O_2^{\bullet-}$ generation. Similar to rheumatoid arthritis, multiple genes contribute to the development of SLE, and a change in a single gene is not sufficient to cause the disease (123). Since the disease-associated allele for both lupus and rheumatoid arthritis is rare in the general population, estimates regarding the increased risk associated with carrying the disease-associated allele are not reliable. However, it is safe to say that in the absence of additional predisposing genotypes, the likelihood of an individual with such an allele developing either disease is low. Indeed, in a study of the *NCF2* polymorphism in China, no increased risk of SLE was seen (336). To our knowledge, no polymorphisms in the genes for NOX2 or p22phox have been reported to be associated with autoimmune or inflammatory diseases.

On the other hand, polymorphisms in the genes encoding NOX2 (*CYBB*) or phox proteins are associated with beneficial effects in some conditions. Williams-Beuren Syndrome (WBS) is a developmental disorder that is marked by arterial defects leading to hypertension. Deletion of one of the two functional copies of *NCF1* protects a subset of WBS patients against hypertension, a finding that was recapitulated in a mouse model of the disease using the NOX inhibitor apocynin (37, 146). However, in another study of two patients with WBS and CGD who lacked any functional *NCF1* gene, there was no protection against hypertension, suggesting that factors other (or in addition to) than NOX in vascular tissue may be involved in hypertension (281).

These studies imply that while decreased ROS generation from the NOX2 system correlates with certain autoimmune diseases, this association seems to be less pronounced in humans than it is in rodent models, and does not correspond to the increased frequency of autoimmune diseases seen in mothers of X-linked CGD patients. Therefore, in our opinion, in the absence of additional predisposing genotypes, existing evidence does not strongly support the hypothesis that partial inhibition of NOX2 poses a significant increased risk of autoimmune disease.

Conclusions regarding the likelihood of side effects from inhibiting the NOX2 system with small molecule drugs

Based on available literature, it seems probable that side effects from inhibiting the NOX2 system are not likely to be common or severe, although, undoubtedly, this will vary with the indication, duration of therapy, and drug dosage. The most serious concern surrounding NOX2 inhibition has been immunosuppression, resulting in life-threatening infections. However, the studies summarized here indicate that the NOX2 system has a large excess of microbicidal capacity, and we suggest that only under continuous, long-term inhibition of NOX2 would serious impairment of innate immunity occur. Co-administration of antibiotics could provide a further safeguard against life-threatening complications. The possibility of triggering an autoimmune disorder is another important consideration. While polymorphisms in human phox genes have shown an association between decreased NOX2 activity and autoimmune diseases and arthritis, these diseases are associated with multiple susceptibility genes as well as unknown environmental factors. Thus, the individual risk of developing an autoimmune disease as a result of inhibiting any single susceptibility gene product such as NOX2 seems to be low. As with many drug therapies, some genetically predisposed subpopulations will very likely be more susceptible to side effects, and therapeutic benefits should always be balanced against possible harmful effects.

Small-Molecule NOX Inhibitors: Progress and Prospects

Early attempts to inhibit the phagocyte NOX are summarized in Cross (52), and subsequent progress is summarized in Kim *et al.* (137). To date, however, only a limited number of NOX2 small-molecule inhibitors have been identified and very few of these appear to be promising for drug development. This section summarizes those commonly used for *in vitro* and *in vivo* studies, and describes recent progress toward the development of inhibitors that may show eventual clinical promise. Inhibitor structures are shown in Figure 2, and their proposed sites of action where information is available are mapped onto a diagram of the NOX2 system in Figure 1.

NOX2 inhibitors commonly used as in vitro (and occasionally in vivo) tools

Diphenylene iodonium. DPI is a non-drug like molecule that was originally described as a general flavoprotein dehydrogenase inhibitor (179). Its mechanism of action involves abstraction of an electron from reduced flavin (FAD or flavin mononucleotide) to form a DPI radical, which then reacts to form a covalent adduct with the flavin, inactivating the coenzyme (201). Depending on its proximity to other groups, covalent adducts may also form with other cofactors such as the heme of NOX2 (68). This non-selective chemical mechanism causes DPI to inhibit a large number of flavin-dependent enzymes, including not only all of the NOX/DUOX enzymes, but also NOS, xanthine oxidase, and at higher concentrations mitochondrial respiration (8). NOX enzymes represent one of the more sensitive targets of DPI, which inhibits the neutrophil NOX2 system with an IC_{50} of 0.9 μM (92), while related analogs show IC_{50} values in the

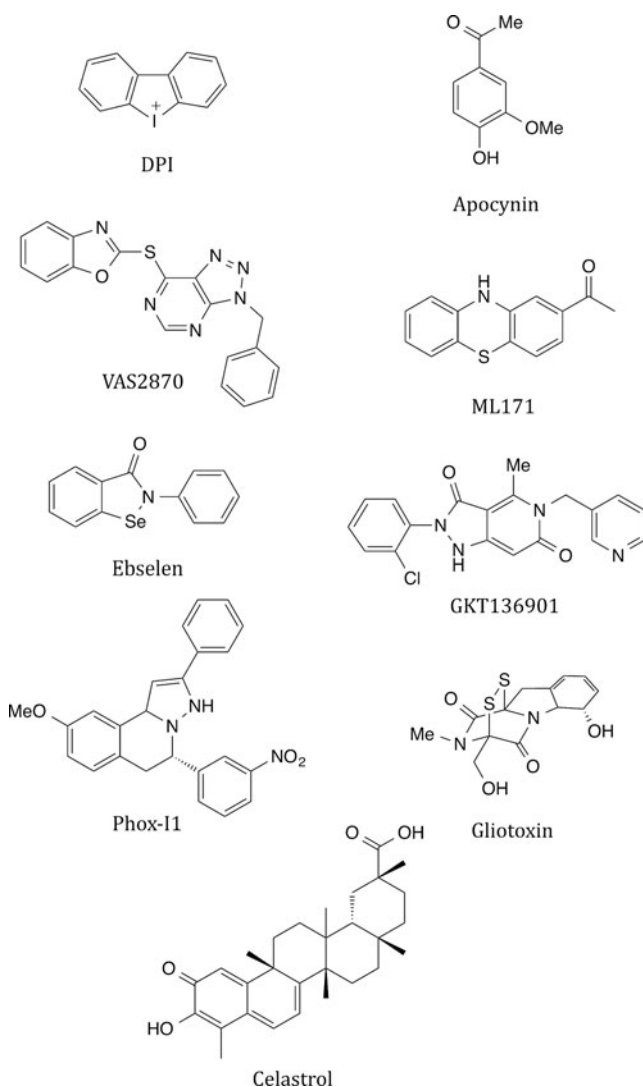


FIG. 2. Structures of some commonly used NOX inhibitors.

0.5–0.75 μM range. Its relative potency against NOX enzymes has led to wide and extensive use of DPI as a tool to study the NOX enzymes *in vitro* (68). Toxicity limits the use of DPI *in vivo*; the LD_{50} of DPI is < 10 mg/kg in rodents (83) and long-term administration at a lower dose (1.5 mg/kg/day, 4–5 weeks) led to cardiomyopathy (50). Low doses of DPI have been used, however, to support target validation of NOX-dependent pathologies *in vivo* (3). Although DPI can be considered a useful tool for *in vitro* NOX studies in situations where isoform selectivity is not an issue, its off-target effects, low solubility, and toxicity eliminate its development as a drug candidate.

Apocynin. Also known as acetovanillone, apocynin occurs naturally in certain plants (172) and was isolated from the root of *Canadian hemp* in 1883 and *Picrorhiza kurroa* in 1971 (73, 310). Plants containing the compound have been used as a traditional medicine, for example, for the treatment of jaundice, asthma, liver, and heart problems (219, 286). Since the early 1980s, apocynin has been extensively used as both an *in vitro* and *in vivo* NOX inhibitor (274). There is

considerable debate about both its mechanism of action and the active form of the molecule. Reportedly, the metabolism of apocynin by myeloperoxidase generates the active dimeric and trimeric species (282). A trimeric form inhibited a NOX2 cell-free system with an IC_{50} of 30 nM, whereas apocynin itself was ineffective (187). Thus, the effectiveness of apocynin as a NOX2 inhibitor may be limited *in vivo* to inflamed regions harboring neutrophils or other myeloperoxidase (MPO)-expressing inflammatory cells; other cells and tissues such as vascular cells lack MPO and cannot generate the active oligomers, suggesting that apocynin may not inhibit NOX2 in such regions. The apocynin oligomer inhibits the NOX2 system by covalently modifying Cys196 of p47 phox , thus preventing its assembly with p22 phox , as in Figure 1 (187). Apocynin treatment of monocytes prevents the translocation of p47 phox to the membrane, decreases NOX2-dependent ROS generation, and inhibits cyclooxygenase expression (16), which is proposed to account for protective effects of the compound in some experimental models of inflammation. While it blocked ROS-dependent signaling in vascular cells, apocynin failed directly to block O_2^- production by NOX1, NOX2, or NOX4 over-expressed in HEK293 cells. Rather, it interfered in assays detecting H_2O_2 or $\cdot\text{OH}$, indicating that in this setting apocynin functions as an antioxidant (96).

Since it decreases markers of oxidative stress *in vivo* and is non-toxic, apocynin has been extensively used in both chronic and acute animal models of disease (including many of the experiments described in previous sections), including collagen-induced arthritis (106–108), in which apocynin was found to have remarkable preventative properties when administered before (but not after) collagen. Similarly, apocynin has been shown to prevent inflammation in models of inflammatory bowel disease and asthma (138). In a model of stroke-prone spontaneously hypertensive rats, apocynin significantly decreased the occurrence of stroke (332). In addition, apocynin has been investigated in a Phase 2 human clinical trial for asthma (284), where it showed anti-inflammatory properties, including a decrease in H_2O_2 in exhaled breath condensates. Thus, while apocynin shows promise as an anti-inflammatory molecule, it remains unclear as to whether its primary mode of action *in vivo* involves the inhibition of NOX2, its antioxidant properties, or both.

gp91(NOX2)*ds-tat* and other peptide inhibitors

Peptide-based inhibitors, by their nature, have the potential advantage of being more specific and having fewer off-target effects than small-molecule organic compounds, but they have numerous problems as drugs having to do with bio-availability, stability, delivery, and—over time—induction of neutralizing antibodies. Since the 1990s, a variety of NOX-targeted inhibitory peptides representing many regions of the NOX subunit have been developed (47, 56, 57, 70, 71) and employed *in vitro*, although to our knowledge, only one of these has been tested *in vivo* (47). gp91(NOX2)*ds-tat* is an 18-amino-acid peptide that includes a part of the B-loop of NOX2 that was designed to block the interaction between NOX2 and its regulatory subunit p47 phox . However, effects on subunit assembly may be indirect, and we tentatively assign its effect to interrupting the interface between the dehydrogenase domain and the transmembrane domain (Fig. 1),

based on structural considerations that place the B-loop at this interface (117). NOX2ds-tat inhibited NOX2 with an IC_{50} of $0.74 \mu M$, but did not inhibit NOX1 or NOX4 in cell lines specifically expressing these isoforms (53). NOX2ds-tat also inhibited $O_2^{\bullet -}$ anion production from cultured endothelial cells (169), human resistance artery smooth muscle cells (304), and platelets (147). Moreover, the peptide showed protective effects in *ex vivo* and *in vivo* models. NOX2ds-tat improved acetylcholine-induced endothelium-dependent relaxation in aortic rings from mice with renovascular hypertension (126) and also suppressed angioplasty-induced neointimal proliferation in rat carotid artery (66).

Peptide-based inhibitors show a loss of bioactivity when administered orally, as most peptides are rapidly inactivated by gastrointestinal enzymes. The development of new technologies such as drug-encapsulating polymeric microparticles or nanoparticles may enhance the efficacy of peptides and other active pharmaceutical ingredients in the future (54). However, at this time, the development of non-peptide small-molecule NOX inhibitors seems more likely to result in clinically useful drugs.

Other reported NOX inhibitors

Other chemical compounds have been reported to inhibit NOX, but because of non-specific mechanisms of action or unresolved questions regarding effectiveness, these compounds are infrequently used as tools.

Phenylarsine oxide. This compound reacts covalently with vicinal cysteine residues, which is thought to be its mechanism of NOX2 inhibition. Specifically, phenylarsine oxide (PAO) at $50\text{--}100 \mu M$ reacted with NOX2 subunit and prevented its assembly with regulatory subunits; the compound failed to react with the NOX2 subunit after assembly had already been triggered (67). The administration of PAO (1 mg/kg intraperitoneal) provided an anti-inflammatory action on both hind paw edema induced by carrageenan and lung inflammation induced by lipopolysaccharide inhalation (242). However, given its mechanism of action, it is not clear whether beneficial effects were due to the inhibition of NOX2 or an unknown off-target effect.

Aminoethyl-benzenesulfonyl-fluoride. Aminoethyl-benzenesulfonyl-fluoride was originally characterized as an irreversible serine protease inhibitor, and it was subsequently discovered to inhibit NOX2 activity in a cell-free system when added before, but not after, enzyme activation. Specifically, the reagent inhibited the binding of p47phox to membranes containing NOX2/p22phox (60). The high concentration required for inhibition (nearly 1 mM) as well as off-target effects make this compound inappropriate for cell or *in vivo* applications.

Celastral. This triterpenoid natural product isolated from the Chinese *Thunder of God* vine or *Tripterygium wilfordii* has been used in traditional Chinese medicine for the treatment of fever, chills, edema, and carbuncle (132). Recently, Jaquet *et al.* (121) tested celastral on various NOX isoforms, and found that it acts as a general NOX inhibitor with some selectivity for NOX1 and NOX2 (IC_{50} values of 0.4 and $0.6 \mu M$, respectively) compared with NOX4 and NOX5 (both $\sim 3 \mu M$). For the NOX2 system, celastral binds to p47phox,

disrupting its interaction with p22phox, but its mode of action may differ between NOX1/2 and other NOX isoforms.

Fulvene-5. Long used as a dye, Fulvene-5 is an aromatic molecule with high water solubility. It was recently reported to inhibit NOX2 and NOX4 (218), and also blocked the growth of endothelial tumors in mice (22). However, Fulvene-5 inhibited these NOX isoforms by only $\sim 40\text{--}50\%$ at $5 \mu M$ and assay controls were not reported. Thus, additional studies are needed to determine whether *in vivo* effects of this compound are a result of NOX inhibition or other mechanisms.

Gliotoxin. Gliotoxin is a disulfide-containing mycotoxin extracted from *Aspergillus* species that blocks NOX2 activity in neutrophils ($IC_{50} = 8 \mu M$) (335). In a cell-free system, pretreatment of membranes containing NOX2 prevents subsequent activation, but the compound is ineffective post-activation, suggesting modification of a site on NOX2 that is blocked once the active complex is assembled (199). In addition, the compound inhibits phosphorylation of p47phox by blocking the translocation of protein kinase C to the membrane, suggesting multiple or complex modes of action (199). The proposed chemical mechanism of the compound involves dithiol exchange at cysteine residues of target proteins; simultaneous addition of the reducing agent dithiothreitol abolished inhibition.

Other compounds. In addition to the compounds described earlier, other plant-derived natural products—many that have been used as traditional medicines—have been reported to inhibit NOX enzymes (122). These include Honokio (269, 307), Norathyriol (104), Abruquinone A (103), Magnolol (113, 319), and Prodigiosin (193, 208). However, little is known about their chemical or biochemical mechanisms of action, and some (*e.g.*, Norathyriol, Abruquinone A, Magnolol) appear to target signaling pathways that lie upstream of NOX enzymes. Therefore, unless additional studies point to more specific effects, these compounds cannot currently be recommended as tools to study biological roles for NOX enzymes.

Systematic discovery of NOX inhibitors

While the NOX inhibitors described earlier were discovered fortuitously, the past decade has seen more systematic approaches in the search for NOX inhibitors. Such approaches involve cell-free or cell-based activity screens or screens involving disruption of protein–protein interactions between NOX regulatory subunits that are essential for activity. Practically speaking, cell-free activity assays are limited to the NOX2 system, due to the difficulty in obtaining sufficient amounts of material of other NOX isoforms as well as, in some cases, stability issues that make some isoforms insufficiently robust for robotic screening efforts. In some of these cases, binding assays can, in theory, be used as a surrogate for activity. Activity or binding assays are then applied to screening libraries of chemical compounds, which, depending on the size of the library can be considered medium-throughput or high-throughput screens, and that are carried out in an automated manner. Hits are then subjected to a series of counter-screens which are designed to establish the

hits as true inhibitors and not artifacts, for example, compounds that interfere with the screening assay itself. Depending on the potency of the initial hit, compounds may then be subjected to an iterative process of chemical modification and re-testing in order to develop compounds with enhanced potency, improved isoform selectivity, improved solubility, decreased toxicity, and increased bioavailability. The goal of this process is to develop compounds not only as research tools but also with properties that are appropriate for drug development.

Screens using inhibition of NOX activity

VAS2870. This compound was found using a screen of small molecules to identify inhibitors of NOX2, carried out by the pharmaceutical company Vasopharm. The compound inhibited $O_2^{\bullet-}$ anion production with an IC_{50} of $11 \mu M$ in neutrophil cell lysates and $2 \mu M$ in whole neutrophil assays (301). The compound was subsequently characterized as an inhibitor of all NOX/DUOX isoforms (326). However, in a contradictory study, VAS2870 decreased ROS levels by an unidentified mechanism unrelated to direct NOX2 catalytic activity or subunit (84). The compound inhibited ROS generation and PDGF-mediated migration of VSMCs from rat thoracic aorta, and also blocked ROS generation induced by oxidized low-density lipoprotein in human umbilical vein endothelial cells (285). In addition, VAS2870 decreased cell growth and promoted apoptosis in a rat liver tumor cell line that is dependent on NOX1 for growth (252). VAS2870 was effective in decreasing tissue damage in a stroke model, an effect that was attributed to the inhibition of NOX4 and not NOX2 (140). Despite its effectiveness, recent studies showed that VAS2870 given at the same doses that inhibit NOX enzymes have off-target effects based on alkylation of cysteines by the benzyltriazolopyrimidine moiety of VAS2870; one important off-target effect was shown to be alkylation of cysteine residues in the ryanodine receptor 1 (290). Therefore, at this time, while VAS2870 appears to be promising in the treatment of certain conditions, its *in vivo* mode of action is controversial, and additional studies are needed to clarify its mechanism. Caution is, therefore, advised in its use as a specific biochemical tool or *in vivo* probe.

ML171. ML171 and its phenothiazine analogues were identified in a medium-throughput (16,000 compounds) activity screen of NOX1-expressing HT-29 cells. ML171 and a related 2-(trifluoromethyl)-phenothiazine were identified as NOX1 inhibitors, and these and related compounds inhibited NOX1 with IC_{50} 's in the range of 0.2 – $1.0 \mu M$, but showed little inhibition of NOX2. However, in our hands (Smith *et al.*, Unpublished), ML171 also showed submicromolar inhibition of NOX2 and NOX4 in cell-free and whole cell L-012 assays, suggesting that the isoform selectivity of this compound requires additional evaluation. This inhibitor was used to elucidate the relevance of NOX1 in mechanisms of cancer invasion, where it blocked the formation of functional invadopodia in human colon cancer cells (88).

GKT136901 and related compounds. The pharmaceutical company Genkyotex used an NOX4-expressing cell line in a high-throughput screen (HTS) of 136,000 compounds to discover moderate potency pyrazolopyridine dione hits that

were then optimized using medicinal chemistry, resulting in GKT136901 as a lead compound (151). In cell-free systems, GKT136901 demonstrated high potency for both NOX4 ($K_i = 165 \text{ nM}$) and NOX1 ($K_i = 160 \text{ nM}$) and weaker inhibition of NOX2 ($K_i = 1.5 \mu M$) (262), making this compound a dual inhibitor of NOX1/4. Counterscreening against panels of biomedically important enzymes revealed no major off-target effects. In addition, the compound showed excellent pharmacokinetic properties and good oral bioavailability.

GKT136901 and related molecules were highly effective in *in vitro* assays of human fibroblast differentiation, epithelial cell apoptosis, and epithelial-mesenchymal transition (262). In addition, the compound was effective in preventive and curative murine models of bleomycin-induced pulmonary fibrosis, and in protection against diabetic nephropathy (263). A related compound, GKT137831, has completed Phase I clinical trials, where it has shown excellent safety and pharmacokinetic properties. To our knowledge, this compound is currently the most advanced NOX inhibitor in the drug development pipeline. While showing great promise for NOX4- and NOX1-related diseases, its lack of significant NOX2 activity makes it inappropriate for the treatment of most disease indications listed in Table 2.

Screens for disruption of subunit interactions

Ebselen and congeners. NOX2 activation *in vivo* depends on the binding of the C-terminal proline-rich domain (PRD) of its heterodimeric partner p22*phox* to the regulatory subunit p47*phox* (288). A HTS was designed to monitor this interaction *via* fluorescence polarization. In this screen (276), the binding of a synthetic, rhodamine-labeled peptide corresponding to the PRD of p22*phox* (rho-PRD) to recombinant glutathione-S-transferase-p47-*bis*-SH3 showed increased fluorescence polarization, while displacement of rho-PRD by the unlabeled PRD caused decreased signals. Using this principle, 230,000 compounds were screened; dose dependencies were carried out among initial hits; and 55 compounds were identified for confirmation in activity assays, resulting in the identification of 3–5 *bona fide* inhibitors. Among these was ebselen (272), a selenium-containing compound that had previously been identified as a glutathione peroxidase mimetic. Ebselen showed inhibition of cell-free NOX2 activity with an IC_{50} value of $0.6 \mu M$. Another hit compound, Thr101, an analog of ebselen with sulfur in place of selenium, was also inhibitory. Systematic modifications of the structure identified many other potent (sub-micromolar IC_{50} values) analogs containing either Se or S. These compounds, in general, showed excellent selectivity for NOX2 and NOX1 compared with NOX4 and NOX5, and some showed marked selectivity for NOX2 compared with NOX1. For the NOX2 system, the compounds also inhibited the translocation of p47*phox* to the membrane, consistent with its targeting of the *bis*-SH3 domain of p47*phox*. While the selenium-containing compounds have been shown to have a number of off-target effects (257), the sulfur-containing compounds show promise for selectively targeting NOX2 and possibly NOX1, and may prove useful as *in vitro* probes.

Phox-I1. The binding of Rac-GTP to p67*phox* is thought to result in a conformational change in p67*phox* that allows an interaction with and activation of NOX2 (185). A recent

study (25) used an *in silico* screening approach to identify potential inhibitors, which were then tested in an NOX2 activity assay. A crystal structure of the p67phox-Rac1 complex (158) was used to build a model of the Rac binding site on p67phox. Docking simulations (189) were then used to virtually screen 340,000 compounds for potential binding to the model binding site, and Phox11 was identified among the top hits. Although the compound bound tightly to recombinant p67phox with a K_d of $\sim 100 \mu M$, it inhibited NOX2-dependent ROS generation in intact neutrophils with an IC_{50} of $\sim 10 \mu M$, suggesting that Rac2 competition for the binding site inside the cell significantly decreases its *in vivo* potency. While this level of potency is unlikely to be sufficient for drug development, improved analogs may prove useful and are likely to show significant isoform selectivity.

Future prospects

NOX2 is a potentially important and novel target for the development of therapeutic agents for a range of serious diseases shown in Table 2, but additional approaches are needed to identify new isoform-selective small-molecule inhibitors. The high degree of structural and catalytic homology within the catalytic core of various NOX isoforms makes finding isoform-selective inhibitors a challenge. For example, the NADPH binding site is highly conserved among the NOX isoforms, suggesting that inhibitors targeting this site may be non-selective for NOX isoforms. Other regions of NOX subunits and their regulatory proteins, however, are structurally unique among the isoforms. Most screening approaches to date have not taken advantage of isoform-specific structural features, and, in some cases, have identified non-selective inhibitors. Fortunately, some general screens have already identified promising inhibitors, some of which show significant isoform selectivity and potential for drug development. GKT136901, for example, shows selectivity for NOX4 and NOX1, and has completed Phase 1 clinical trials. While probing new chemical libraries using existing screening methods will, undoubtedly, result in new hits, future breakthroughs are likely to result from a combination of techniques relying on ultra high-throughput and/or virtual screening, while simultaneously taking advantage of isoform-specific structural features. Figure 1 shows a summary of the locus of action, where known, of various inhibitors on the NOX2 system. An inspection of Figure 1 reveals that there are specific protein-protein interactions which could, in principle, be targeted with new high-throughput assays. For example, neither the binding of p67phox to p47phox nor the interaction of p40phox (not shown) with its targets has been exploited. Similarly, targeting the binding of p47phox or NOXO1 to its phospholipid in the membrane is another possible approach that could be used to block assembly of the active complex. Since some of these interactions require structural interactions that differ significantly among different NOX isoform systems; such an approach may be selective, compared for example with targeting the NADPH binding site.

In addition, improved ROS assay methods may help identify new inhibitors. For example, currently, many of the reagents used for screening ROS are non-selective and detect a variety of other reactive molecules (*e.g.*, NO, ONOO⁻, and HOCl); while others show high background signals. This

results in a high false-positive rate, and is likely to miss weaker but valid inhibitors that could provide the foundation for novel chemical series.

NOX2 inhibitors show particular promise for the treatment of inflammatory diseases, both acute and chronic. Theoretical side effects include pro-inflammatory and autoimmune complications, and while these should be considered in any therapeutic program, in our opinion they do not appear to be serious enough to eliminate NOX2 as a drug target, particularly when weighed against the seriousness of many of the indications listed in Table 2. As with any first-in-class drug, proof of concept for efficacy with minimal side effects is needed for the acceptance of NOX2 inhibitors as therapeutic agents.

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Author Disclosure Statement

Dr. Lambeth is co-founder of Genkyotex (Geneva, Switzerland), a pharmaceutical company that develops drugs targeting NADPH oxidases and serves on its scientific advisory board. None of the other authors have anything to disclose.

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Address correspondence to:

Prof. J. David Lambeth

Department of Pathology and Laboratory Medicine

Emory University School of Medicine

Whitehead Biomedical Research Building, Room 148

615 Michael Street

Atlanta, GA 30322

E-mail: noxdoc@mac.com

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Abbreviations Used

AD	= Alzheimer's disease
ALI	= acute lung inflammation
ALS	= amyotrophic lateral sclerosis
Ang II	= angiotensin II
ARDS	= acute respiratory distress syndrome
CF	= cystic fibrosis
CFTR	= CF transmembrane conductance regulator
CGD	= chronic granulomatous disease
CIH	= chronic intermittent hypoxia
COPD	= chronic obstructive pulmonary disease
CYBA	= gene name for p22phox
CYBB	= gene name for NOX2
DA	= Dark Agouti
DPI	= diphenylene iodonium
DUOX	= dual oxidase
FAD	= flavin adenine dinucleotide
GSH	= glutathione, reduced
GSSG	= glutathione, oxidized
GST	= glutathione-S-transferase
H ₂ O ₂	= hydrogen peroxide
HOCl	= hypochlorous acid

Abbreviations Used (Cont.)

HTS = high-throughput screen
IL = interleukin
KC = keratinocyte-derived chemokine
LIRI = lung ischemia-reperfusion injury
MCP-1 = monocyte chemoattractant protein-1
MPO = myeloperoxidase
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADPH = nicotinamide adenine dinucleotide phosphate, reduced form
NBT = nitroblue tetrazolium
NCF1 = gene name for p47phox
NCF2 = gene name for p67phox
NCF4 = gene name for p40phox
NET = neutrophil extracellular trap
NF-kappa B = nuclear factor-kappa B
NMDA = *N*-methyl-D-aspartate
NO = nitric oxide
NOS = Nitric oxide synthase

NOX = NADPH oxidase
 $O_2^{\bullet-}$ = superoxide
 $\bullet OH$ = hydroxyl radical
ONOO⁻ = peroxynitrite
PAO = phenylarsine oxide
PD = Parkinson's disease
PDGF = platelet-derived growth factor
PH = pulmonary hypertension
phox = phagocytic oxidase
PRD = proline-rich domain
PTP = protein tyrosine phosphatase
ROS = reactive oxygen species
SLE = systemic lupus erythematosus
SOD = superoxide dismutase
TBI = traumatic brain injury
TNF = tumor necrosis factor
TTRhRen = transgenic mice overexpressing human renin
VSMC = vascular smooth muscle cell
WBS = Williams-Beuren syndrome