Published in final edited form as:

Free Radic Biol Med. 2007 August 1; 43(3): 332–347.

Nox Enzymes, ROS, and Chronic Disease: An Example of Antagonistic Pleiotropy

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Summary

Reactive oxygen species (ROS) are considered to be chemically reactive with and damaging to biomolecules including DNA, protein and lipid, and excessive exposure to ROS induces oxidative stress and causes genetic mutations. However, the recently described family of Nox and Duox enzymes generates ROS in a variety of tissues as part of normal physiological functions, which include innate immunity, signal transduction and biochemical reactions, e.g. to produce thyroid hormone. Nature's "choice" of ROS to carry out these biological functions seems odd indeed, given its predisposition to cause molecular damage. This review describes normal biological roles of Nox enzymes as well as pathological conditions that are associated with ROS production by Nox enzymes. By far the most common conditions associated with Nox-derived ROS are chronic diseases that tend to appear late in life, including atherosclerosis, hypertension, diabetic nephropathy, lung fibrosis, cancer, Alzheimer's disease and others. In almost all cases, with the exception of a few rare inherited conditions (e.g., related to innate immunity, gravity perception, and hypothyroidism), diseases are associated with *overproduction* of ROS by Nox enzymes; this results in oxidative stress that damages tissues over time. I propose that these pathological roles of Nox enzymes can be understood in terms of antagonistic pleiotropy: genes that confer a reproductive advantage early in life can have harmful effects late in life. Such genes are retained during evolution despite their harmful effects, because the force of natural selection declines with advanced age. This review discusses some of the proposed physiologic roles of Nox enzymes, and emphasizes the role of Nox enzymes in disease and the likely beneficial effects of drugs that target Nox enzymes, particularly in chronic diseases associated with an aging population.

Introduction

The enzymology and regulation of Nox enzymes, and their function in the generation of ROS are described in the preceding article. It is apparent from that review that ROS are generated by Nox/Duox enzymes in a variety of tissues, and that ROS production by these enzymes is normally tightly regulated. Herein, I consider ---- insofar as is known or suspected ---- the normal biological roles of Nox and Duox enzymes, as well as the clinical conditions that are associated with either underactivity (rare) or overactivity/overexpression (common) of these enzymes. This review is organized more or less according to specific tissues or organ systems, and in each case, considers Nox/Duox expression in a given organ, normal biological functions and finally pathological conditions that are suspected or proven to be associated with particular Nox enzymes. As with the enzymology of Noxes, the phagocyte Nox system provides the clearest and most thoroughly studied precedent for understanding the biology and pathobiology

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of Nox enzymes, and this review therefore first consider this classical ROS-generating system as a prelude to discussing the more recently discovered novel Nox and Duox enzymes that are expressed in non-phagocytic cell types.

In reviewing conditions that are proven or suspected to be associated with Nox/Duox overactivity or overexpression, it became apparent to the author that for the most part, these diseases represent chronic conditions, and that these conditions are often associated with tissue damage, fibrosis and in some cases probable genetic damage. Such changes are consistent with the well-established involvement of ROS in causing damage to biomolecules including protein, DNA and lipid membranes. This raises the important question: why has evolution retained enzymes that are capable of causing such destruction? The short answer is that in addition to causing molecular and tissue damage, the ROS generated from Nox and Duox enzymes must provide an evolutionary advantage that outweighs their harmful effects. The normal biological functions of ROS generated by Nox and Duox enzymes is still being worked out but already has been shown (*vide infra*) to include such diverse processes as calcium signaling regulating smooth muscle contraction, regulation of protein tyrosine phosphatase activity, generation of thyroid hormone, cell differentiation, mitogenic regulation and other functions. Obviously, these normal functions must provide an evolutionary advantage, allowing Nox and Duox enzymes to be retained during evolution. A second observation about Nox/Duox-associated diseases is that for the most part, they tend to be expressed late in life, generally after reproduction has already occurred. Genes that confer an advantage during the time during which the organism is reproductively active, but that have harmful effects late in life are subject to positive rather than negative selection during evolution, resulting in retention of such genes. This phenomenon, well-known in genetics, is referred to as "antagonistic pleiotropy". This review covers a growing list of both normal biological functions of Nox/Duox enzymes, and Nox/Duox disease associations, which together provide an outstanding example of this genetic phenomenon. These examples provide a reminder that evolution often occurs through a series of trade-offs that do not take into account a comfortable, healthy old age. In particular, the evolutionary "choice" to use ROS to carry out biological functions represents a double edged sword. Nevertheless, I believe that an understanding of the Nox and Duox enzymes --- and the development of drugs targeting these enzymes ---- provides a tool with which the harmful effects of this biological choice can be diminished or eliminated.

Nox enzymes in Innate Immunity

a. General considerations

Nox-generated ROS can participate in immune function in a variety of ways, which are not mutually exclusive. First, the reactive oxygen itself or its byproducts such as HOCl and peroxynitrite can directly oxidize biomolecules in invading microbes in a fairly non-specific manner, resulting ultimately in molecular damage and microbial cell death. High abundance Nox enzymes and those that are co-expressed with cooperating enzymes such as peroxidases and Nitric Oxide (NO) synthase are the most likely candidates for this sort of mechanism. Second, the reactive oxygen can participate in signal transduction mechanisms linked to immunity and inflammation. This occurs through the selective oxidation of specific signaling enzymes/proteins that are linked to processes such as the secretion of cytokines or the activation of other killing mechanisms. Such signaling targets include transcription factors such as NF-kappaB, signaling proteins such as protein kinases and phosphatases and ion and/or proton channels. The second group of mechanisms is a specialized case of the more general function of Nox enzymes in signal transduction and are covered in detail elsewhere in this review. In this section, the focus is primarily on the direct mechanisms of killing by ROS.

b. Nox2 and professional phagocytes

The first role to be definitively established for Nox-derived ROS was in innate immunity mediated by professional phagocytes such as neutrophils and macrophages. These cells express very large amounts of gp91^{phox}, now also called Nox2, along with its regulatory subunits p47^{phox}, p67^{phox}, p40^{phox} and Rac2, reviewed in [1,2]. It can be calculated that the concentration of ROS produced in the phagosome is extremely high, probably in the molar range [3]. In addition, myeloperoxidase (MPO) is secreted into the phagosome where it converts H₂O₂ (produced by Nox2) plus chloride into HOCl; the latter has a direct microbicidal effect [4,5] (although surprisingly, MPO-deficient individuals do not suffer from markedly increased rates or apparent severity of infections [6]). In addition, macrophages (but possibly not neutrophils) produce large production of NO during phagocytosis; when NO reacts with superoxide, it generates the highly cytotoxic chemical species peroxynitrite (HONO) [7]. The activity of the phagocyte NADPH-oxidase also triggers opening of proton [8–11] and possibly potassium channels [3], that are proposed to change the ionic environment of the phagosome thereby activating microbicidal proteases and contributing to microbial killing [3]. Regardless of the precise mechanisms, it is clear from the inherited condition chronic granulomatous disease (CGD) that mutations resulting in defects in ROS generation by the respiratory burst oxidase are associated with an inability of phagocytes to kill bacteria and other microbes [12], convincingly demonstrating a role for the Nox2 system in innate immunity mediated by professional phagocytes.

c. Nox2 and tissue inflammation

Neutrophil-derived ROS, including superoxide and H₂O₂ generated by Nox2, HONO generated from superoxide and nitric oxide, and HOCl generated by MPO, have been implicated in the tissue damage seen in acute and chronic inflammatory conditions in which there is at some stage in the disease a neutrophil or macrophage infiltrate. Such conditions include acute and chronic infections, autoimmune conditions such as inflammatory bowel disease, adult respiratory distress syndrome (also called “shock lung”), arthritis, and any number of other inflammatory conditions. This area has been reviewed extensively, and the reader is referred to several excellent articles and books for a comprehensive discussion of this area [13–19].

d. Nox enzymes and mucosal immunity

Lactoperoxidase (LPO) catalyzes the H₂O₂-dependent oxidation of the anion thiocyanate to form the antimicrobial compound HOSCN that prevents growth of bacteria, fungi, and viruses [20,21], but the origin of the H₂O₂ was until recently unclear. Epithelial cells in salivary ducts express Duox2, and those in trachea and bronchus express Duox1; these Duox enzymes are likely to play a role in humans as a source of H₂O₂ for LPO-dependent antimicrobial activity [22]. Induction of Nox1 and other mucosal Nox enzymes by cytokines [23] and bacterial products [24] provides circumstantial evidence for a role for Nox/Duox enzymes in mucosal innate immunity, although it should also be noted that Nox1 is induced by a variety of other agonists including growth factors, consistent with other roles such as mitogenic regulation.

Genetic studies thus far have not provided conclusive support to a role in mucosal immunity. Examination of 45 polymorphisms in ten Nox/ROS-related genes including p47^{phox}, p67^{phox}, p40^{phox}, p22^{phox}, gp91^{phox}, Duox1, and Duox2 in a cohort of 95 lung disease individuals and 95 control individuals did not show an association of these polymorphisms with increased susceptibility to infectious or inflammatory lung diseases including tuberculosis, asthma, and sarcoidosis [25]. A role for Duox in insect innate immunity has been proposed based upon the finding that when Duox expression is reduced in *Drosophila* intestine using RNAi, there is increased lethality upon exposure to food-borne bacteria [26]. On the other hand, suppression of Duox expression is also associated with a molting defect that results

in the maturation of a small number of adults that are particularly susceptible to death by stresses, for example ether anaesthesia (Ritsick, D. and Lambeth, D., unpublished), making it unclear in my opinion whether increased lethality upon exposure to bacteria represents a specific role for Duox or an example of increased lethality in response to a stress in a generally compromised animal. Additional studies are needed to clarify the universality of a role for Nox and/or Duox enzymes in mucosal immunity, and/or to document their other roles in the lung and GI tract.

Nox enzymes in the Lung

a. Immune mechanisms/Microbial defense

In addition to the role of Duox1/2 in LPO-mediated immunity described above, ROS produced by Nox(es) plays a role in the response of human lung fibroblasts to rhinovirus infection. NADPH-oxidase components p47^{phox}, p67^{phox} and Nox4 (but not Nox2) were expressed in lung fibroblasts, and p67^{phox} was induced by rhinovirus, accompanied by increased ROS and elaboration of IL-8 [27].

b. Hypoxia

Hypoxia sensing and related signaling events including activation of hypoxia-inducible factor 1 (HIF-1) represent important features of lung cell physiology and lung function. Up-regulation of Nox1 mRNA and protein occurred during hypoxia, accompanied by enhanced reactive oxygen species (ROS) generation; the latter was accompanied by activation of HIF-1-dependent gene expression, which was blocked by catalase. Thus, hypoxic upregulation of Nox1 and subsequently augmented ROS generation may activate HIF-1-dependent pathways and participate in adaptation to high altitude [40].

c. Emphysema and fibrotic diseases of the lung

Increasing evidence points to a role for Nox-dependent ROS in both fibrotic diseases and the alveolar cell death that leads to emphysema. Airway epithelial cells are both exposed to and produce cytokines and ROS in inflammatory settings and upon exposure to cigarette smoke. Some lung fibrotic diseases are associated with increased TGF- β 1 in the airway. This cytokine induces H₂O₂ production in lung fibroblasts, which in the presence of heme-type peroxidases such as LPO and MPO, can mediate oxidative cross-linking of tyrosine residues in extracellular matrix proteins, resulting in lung fibrosis [28]. Fibroblasts isolated from the lungs of patients with idiopathic pulmonary fibrosis generated H₂O₂ in response to TGF- β 1, and induced death in co-cultured small airway epithelial cells [29]. ROS produced in lung epithelial cells activated JNK and caused cell death via TNF-RI and the TRAF2-ASK1 signaling axis [30]. Cigarette smoke may also contribute to obstructive pulmonary disease via Nox-generated ROS. Cigarette smoke and the bacterial product LPS both up-regulate NOXO1, the activator of Nox1 [31]. TLR4 deficiency, which causes emphysema in mice, up-regulated Nox3 in lung and endothelial cells resulting in increased oxidant generation and elastolytic activity. Treatment of Tlr4^(-/-) mice or endothelial cells with chemical Nox inhibitors or Nox3 siRNA prevented the disease development [32].

d. Nox enzymes and asthma

Oxidant/antioxidant imbalance is recognized as an important contributor to asthma [33,34]. In addition to the Nox2 system, which is highly expressed in inflammatory cells including the eosinophils that are recruited to the asthmatic lung, airway smooth muscle cells express Nox enzymes, particularly Nox4, which have been proposed to contribute to tissue destruction in asthma [35]. In addition, pollen itself contains an endogenous NADPH-oxidase activity, which functions to generate local signals in airway epithelium. These signals in turn trigger the early

recruitment of granulocytes, contributing to allergic inflammation in the lung [36,37] and eye [38].

e. Nox and pulmonary hypertension

Human urotensin II, which is implicated in pulmonary hypertension, potentially induced p22^{phox} and Nox4 in lung smooth muscle, markedly increasing ROS levels that activate ERK1,2, p38 MAPK, Jun Kinase and Akt. This perturbed redox-dependent signaling is proposed to contribute to smooth muscle hypertrophy and proliferation that is associated with pulmonary hypertension [39].

Nox enzymes in the Cardiovascular System

a. Nox enzymes in cardiovascular physiology and pathology

Nox enzymes are expressed in vascular smooth muscle, adventitia and endothelium, and are the major physiologic source of ROS in these tissues in the absence of infiltration by inflammatory cells. Nox2 and Nox4 are both expressed in endothelium [41,42] where under some conditions they participate in cell proliferation [43] while Nox1, Nox4 [42,44] and Nox2 [45] are expressed in vascular smooth muscle cells. Nox1 in vascular smooth muscle participates in cell proliferation [46], while Nox4 in these cells participates in maintenance of the differentiated phenotype [47,48]. Nox2 and associated regulatory proteins are also expressed in adventitia, where they contribute to constitutive ROS generation [49] and to Angiotensin II-induced vascular tone in part through inactivation of NO by superoxide [50]. In addition, Nox5 is expressed in vascular smooth muscle [51] and [Petumnetcha and Lambeth, unpublished]. Probable physiological roles (*e.g.*, regulation of vascular tone, differentiation, growth, oxygen sensing) and pathophysiological roles of Nox enzymes in the cardiovascular system have been reviewed recently [52]. As elaborated below, pathological processes include endothelial dysfunction, inflammation, hypertrophy, apoptosis, migration, angiogenesis, and vascular and cardiac remodeling.

b. Nox enzymes in the myocardium

Both Nox2 and Nox4 are expressed in cardiomyocytes. Nox4 is necessary for the differentiation of mouse embryonic stem cells or fibroblasts into myocytes [53,54]. In addition, Nox4 may contribute to the pathological activation of cardiac fibroblasts in cardiac fibrosis associated with heart failure [54]. Increased ROS is also associated with left ventricular hypertrophy (LVH), and this correlated with overexpression of Nox2 in Angiotensin II-induced LVH, and, in pressure overload LVH, with both Nox4 [55] and Nox2 [56]. Aldosterone/Angiotensin II-mediated interstitial cardiac fibrosis is mediated by Nox2-dependent ROS generation [57]. Nox enzymes are likely to contribute to the occurrence of and tissue damage seen in myocardial infarction by several mechanisms. Nox2 overexpression in cardiomyocytes is seen following myocardial infarction [58], and may result in a sustained increase in ROS. Ischemia followed by reperfusion is implicated in increased myocardial injury following infarction, and is mediated by increased ROS; reperfusion injury is associated with increased circulating and myocardial levels of cytokines, which are associated with increased levels of Nox1, Nox2 and Nox4 [59]. Myocardial Nox2 also contributes to superoxide production in the fibrillating human atrial myocardium where it may play an important role in the cardiac oxidative injury and electrophysiological remodeling seen in patients with atrial fibrillation [60].

c. Nox enzymes and vascular hypertension

In normal physiology, Nox enzymes participate in vascular smooth muscle signaling: Nox-derived ROS in vascular smooth muscle cells regulate the activity of the signaling proteins p38 MAPK and Akt [42], and are essential for Angiotensin II-induced calcium fluxes (Petumnetcha

and Lambeth, unpublished). Nox-derived ROS can also affect the local bioavailability of the vaso-protective signal molecule NO [61].

Nox1 in the vasculature plays a central role in hypertension. Oxidative stress has been recognized as an important contributor to hypertensive disease since the mid 1990's [62], although the major source of ROS has only more recently been proven to be Nox enzymes [63]. Hypertension of vascular origin is associated with increased vascular contractility and with hypertrophy and proliferation of vascular smooth muscle and other cells. Nox enzymes (particularly Nox1) and ROS are induced in vascular cells by growth stimuli [Angiotensin II, PDGF, lyso-phosphatidylcholine, thrombin [42,44,64], urokinase plasminogen activator [65], by PGF2 α and ATF-1 [66]] and by inflammatory stimuli [e.g., TNF α and IL1 β [67]]. Nox1 was also markedly overexpressed in transgenic hypertensive rats overexpressing the Ren2 gene [64] and in stroke-prone spontaneously hypertensive rats [68]. In the latter animals, overexpression of Nox1 and to a lesser extent Nox4 was dependent upon Angiotensin II type 1 receptors. PGF2 α -induced hypertrophy of smooth muscle cells is associated with elevated Nox1, and reduction of Nox1 using ribozymes protected against hypertrophy [69]. Decreased expression of Nox1 in vascular smooth muscle cells using antisense RNA also resulted in decreased cell proliferation [46]. In mice, overexpression in vascular smooth muscle of Nox1 [70] or of p22^{phox} [71] (which indirectly increases Nox1 expression, [72]) resulted in a marked increase in systolic blood pressure and hypertrophy in response to Angiotensin II. In Nox1 knockout mice, there was a lowering of basal blood pressure [73] and a complete protection against Angiotensin II-induced increase in blood pressure and medial hypertrophy [73,74] which resulted in part from sparing of NO when superoxide production was eliminated. Angiotensin II-induced vasoconstriction and reduced blood flow in kidney also occurred by mechanisms that are independent of NO and involved superoxide rather than hydrogen peroxide as a mediator [75].

Nox2 has also been implicated in some models of hypertension. Nox2 accounts for significant ROS generation in vascular smooth muscle in resistance arteries [45] and in endothelium [42,76]. In a model of renovascular hypertension Nox2-derived superoxide decreased NO bioavailability, and there was marked protection from hypertension in the Nox2^(-/-) mice [61]. In low renin salt-sensitive hypertension, a tat-peptide inhibitor of Nox2 normalized ROS generation and endothelium-dependent vascular relaxation [77]. Thus, increasingly, evidence points to the critical role of Nox enzymes in the vascular remodeling associated with hypertension. Nox1 and Nox2 therefore provide promising targets for therapeutic intervention in hypertensive cardiovascular disease [41,78,79].

d. Nox enzymes and atherosclerosis

Accumulating evidence points to a key role for Nox enzymes in atherogenesis and peripheral artery disease. Human atherosclerotic plaques express large amounts of Nox2 [77], which was localized to the plaque shoulder, an area that is rich in macrophages [63]. Approximately 60% of the ROS in atherosclerotic plaques arises from Nox enzymes, particularly Nox2. In human coronary vessels, superoxide was markedly elevated in patients with coronary artery disease, even in vessels without overt atherosclerotic plaque, and was double at branch points [79]. Oscillatory shear stress occurs preferentially in branched or curved regions of arteries and is associated with atherogenesis. Oscillatory shear stress results in several-fold induction of Nox1, Nox2 and Nox4 in vascular endothelium (with opposite effects of the anti-atherogenic laminar flow that occurs in straight portions of vessels) [80,81]. Oscillatory shear stress is associated with induction of bone morphogenic protein 4 (BMP4) [82], which induces Nox1 and p47phox, resulting in an oxidative stress that leads to ICAM-1 expression and monocyte adhesion [81]. Nox1 expression increases ~3-fold following balloon injury and precedes restenosis and atherosclerosis [83]. This in turn led to monocyte infiltration and a vicious cycle

of increasing oxidant stress. Peripheral artery disease, like coronary artery disease, is also associated with evidence of oxidative stress and treatment with an antioxidant improved arterial flow parameters [84].

The mechanisms by which Nox-derived oxidative stress induces atherogenesis and arterial disease may include direct molecular damage by ROS [including “uncoupling” of nitric oxide synthase that results in a further increase in ROS [85]], increased expression of pro-atherosclerotic genes [42], induced differentiation of adventitial fibroblasts into myofibroblasts (a feature of the vascular remodeling seen in atherosclerosis) [86,87], and induction of VEGF [88,89] which contributes to the growth of new microvessels into atheromatous plaques. Chronic activity of Nox enzymes also inactivates telomerase and may promote senescence of endothelial progenitor cells [90]. Therefore, inhibition of Nox2 and/or Nox1 is likely to be useful for the prevention and treatment of atherosclerosis.

e. Diabetic vascular disease

Chronic hyperglycemia is directly linked to microvascular complications that cause blindness, atherosclerosis and neuropathy. This link involves biochemical abnormalities including increased polyol pathway flux, increased formation of advanced glycation end-products (AGEs), activation of protein kinase C and increased flux through the hexosamine pathway. Mechanistically, all of these pathways all seem to result from overproduction of ROS [91–93], and antioxidants are protective against deleterious effects of high glucose on vascular endothelial cells [94]. Nox enzymes, particularly the Rac-regulated enzymes Nox1 and Nox2, play a role in endothelial dysfunction in the setting of diabetes mellitus [95]. Consistent with a role for these Nox isoforms, dominant negative Rac1 protected against oxidative stress and endothelial dysfunction in a mouse model of diabetes [96]. Indeed, impaired activation of Rac1 and Nox-dependent oxidative stress has been proposed to underlie some of the vascular protective effects of statins [97]. Some of the cardiovascular pathologies seen in diabetes may be mediated by the glycosylated proteins that result from high glucose. For example, glycosylated BSA stimulated Nox2-dependent ROS production via a protein kinase C-dependent mechanism, resulting in NF-kappaB activation and induction of inflammatory genes [98].

Nox enzymes, ROS and Renal Disease

a. Renal Nox enzymes

Nox4 is expressed in high levels in kidney [99,100], while other Nox1, Nox2 and Nox regulatory subunits are expressed at lower but quantitatively significant levels [101,102], making Nox enzymes attractive candidates for the origin of renal ROS including the relatively high levels of H₂O₂ seen in urine. In kidney, Nox-dependent ROS is produced in response to agonists that bind to D1-like receptors [103], to Angiotensin II [104,105] and to H⁺ fluxes [106]. Although physiological roles are not well understood, Nox enzymes have been suggested to function in normal renal physiology in secretion of erythropoietin [100], in renal regulation of blood pressure [103], regulation of mesangial cell protein synthesis [107] and in innate immunity [23]. In addition to their normal roles in renal physiology, Nox-generated ROS are implicated in the pathogenesis of variety of kidney-related diseases:

b. Diabetic nephropathy

While elevated glucose in diabetes affects a variety of tissues, kidney is particularly susceptible, responding with renal hypertrophy, fibrosis, glomerular enlargement and hyperfiltration of protein. In proximal tubules, high glucose stimulates ROS production, resulting in increased expression of angiotensinogen [108] with consequent systemic effects *e.g.* on blood pressure. Apocyanin, an inhibitor of Nox2 and probably other Nox enzymes, was used in rat to test the hypothesis that ROS from a Nox underlies the development of diabetic nephropathy. Diabetes

mellitus increased excretion of H₂O₂, lipid peroxidation and protein [101,109]. Kidneys of rats with diabetes mellitus had increased expression of Nox2, p47^{phox} and Nox4 [101,109], increased membrane translocation of p47^{phox} (reflecting Nox2 activation) [101] and increased mesangial matrix [101]. Apocyanin prevented the increased H₂O₂, lipid peroxidation, and protein in diabetic rats, prevented the increased renal expression of Nox2 and membrane translocation of p47^{phox}, and blocked the mesangial matrix expansion [101]. Biochemical effects of elevated ROS included inhibition of Na⁺/glucose co-transport, increased secretion of TGF-beta1 and activation of NF-kappaB signaling [110]. PKC-beta^(-/-) diabetic mice were protected against induction of Nox2, Nox4 and glucose-induced renal dysfunction and fibrosis, indicating a role for PKC in Nox expression and renal pathology [109]. Nox4 is a major source of ROS in diabetic nephropathy, based on protection against high glucose-induced ROS generation and fibronectin expression in kidney cells transfected with Nox4 antisense oligonucleotides [111]. Thus, drugs targeting Nox4 and possibly Nox2 appear to be promising for the treatment and prevention of diabetic nephropathy.

c. Non-diabetic renal failure and glomerulonephropathies

ROS play an important role in the pathogenesis of glomerulopathies and renal failure, and antioxidants are useful in preventing or treating disease [112]. Active Heymann nephritis (AHN) is a model of human membranous nephropathy, and is associated with oxidant-antioxidant imbalance, which contributes to renal damage [113]. Likewise, glomerular mesangial injury in rats treated chronically with aldosterone and salt is associated with induction of Nox2, Nox4 and p22^{phox} [114], increased p47^{phox} and p67^{phox} in the membrane fraction (indicating activation of the Nox2 system), and increased renal ROS and [102]. In an Angiotensin II-induced mesangioproliferative model of glomerulonephritis, Nox2 and Nox4 induction were associated with disease progression, and treatment with the antioxidant probucol in combination with Angiotensin II receptor blockade fully arrested disease progression and proteinuria [115].

d. Acute Tubular Necrosis

Acute tubular necrosis secondary to ischemic renal failure is a common and serious clinical problem. Reactive oxygen and phagocyte-mediated inflammation play central roles in this process, and antioxidant therapy is beneficial [116].

e. Nox enzymes and renal hypertension

In general, renal oxidative stress can precede and contribute to hypertension from several origins, and if corrected, can lower blood pressure. Angiotensin II-infused rodents show increased renal and systemic expression of Nox1 [117], which contributes to the development and maintenance of hypertension. Nox enzymes may also contribute to a genetic predisposition to renal hypertension. Genetically salt-sensitive rats show a 3-fold higher expression of renal Nox1 compared with control rats, and overexpression was associated with increased activity of ERK1,2 and JNK kinases [118]. Mechanistically, the regulation of tubular transport by ROS is important to overall salt and water balance and therefore to blood pressure: superoxide stimulates NaCl absorption by the thick ascending limb by activating protein kinase C and by blunting the effects of NO [119]. Hyperleptinemia also induces hypertension, which may be mediated by its stimulation of both systemic and renal oxidative stress, which decreases the amount of bioactive NO and causes renal sodium retention by stimulating tubular sodium resorption [120].

f. Hemodialysis-associated renal disease

Inflammation and consequent oxidative stress is induced by hemodialysis and is linked to the acceleration of tissue damage in end-stage renal disease. Interleukins and anaphylatoxins

produced during hemodialysis are potent activators of Nox enzymes, providing a possible link between Nox activation and tissue damage. The resulting oxidative stress is implicated in long-term complications including anemia, amyloidosis, accelerated atherosclerosis, and malnutrition [121].

Nox Enzymes and Cancer

Nox enzymes have been implicated in cell proliferation, angiogenesis, inhibition of apoptosis, and integrin signaling, as discussed below. These enzymes are likely to function in normal physiology in these processes, but to date, most studies have been done in the context of cancer.

a. Association of ROS, Nox enzymes and cancer

In studies over the past 2 decades, cancer and rapidly proliferating cells were frequently noted to overproduce reactive oxygen [122,123], and antioxidants and inhibitors of NADPH-oxidases were associated with decreased cell proliferation [124–126]. In many of cases, the source of this ROS is Nox enzymes: this includes melanoma (Nox4, [127]), prostate cancer (Nox5 [128] and Nox1 [129]), glioblastoma (Nox4 and sometimes Nox5 [130]), *H. pylorus*-induced gastric inflammation leading to gastric cancer (Nox1 [131]) and Barrett's esophageal adenocarcinoma (Nox5 [132]). Some, but not all reports have observed an increase in Nox1 expression in colon cancer, see [133–136]. In recent studies, we found that Nox1 protein and mRNA are over-expressed beginning at the adenoma (precancerous stage), and did not further increase at later stages [Laurent et al. (unpublished data)], consistent with most other reports. Over-expression showed a strong correlation with oncogenic mutations in K-Ras, and markedly elevated Nox1 levels in the intestinal tract were also seen in mice that expressed V12 K-Ras in intestinal epithelium. These studies are consistent with earlier studies in a V12-K-Ras-expressing model epithelial cell line that showed a marked induction of Nox1 mRNA and ROS [137]. While Nox over-expression seems to be a feature of many cancer cells, altered expression of many genes is a frequent feature of cancer. Several studies have begun to address whether Nox enzymes play a causal role in the cancer phenotype.

b. Is Nox-derived ROS a causal factor in cancer?

A causal role for Nox overexpression or activation in cancer is supported by several lines of evidence. In early studies, non-phagocyte Nox enzymes were implicated in cell division and suggested to play a role in cell transformation and cancer [46]. Decreasing the expression of Nox1 (originally called Mox1) decreased cell division in vascular smooth muscle [46] and in V12-K-Ras transformed NRK cells [137]. Suppression of Nox5 expression in Barrett's esophageal adenocarcinoma cells likewise inhibited proliferation [132]. While *in vivo* studies are needed to definitively link Nox-derived ROS to the cancer phenotype, Nox-dependent effects on cell division, angiogenesis, cell survival and integrin signaling provide plausible mechanisms by which Nox enzymes may be causally linked to cancer development, and justify interest in Nox enzymes as drug targets for cancer prevention and treatment.

c. Nox enzymes and cell division

Nox overexpression may influence cancer is by increasing the rate of cell division. Proliferating keratinocytes showed higher ROS generation and Nox1 levels than quiescent cells [123]. Over-expression of Nox1 in several cell types is associated with increased cell division [46,138, 139]. In fibroblasts that over-expressed heterologous Nox1 and also harbored an oncogenic mutation in Ras, overexpression of catalase markedly decreased mitogenic growth, the transformed phenotype and tumorigenicity in athymic mice [140], implicating Nox-derived H₂O₂ in the tumor phenotype. The mechanism of mitogenic stimulation involves several redox-sensitive steps. In actively cycling cells, Nox1 stimulated proliferation by reducing the

requirement for growth factors to maintain expression of cyclin D1, whereas during cell cycle re-entry, Nox1 activity was required for transcriptional activation of Fos family genes [138].

d. Nox enzymes and angiogenesis

In studies in prostate tumor cells that over-expressed Nox1, Nox1-derived H₂O₂ had only a small effect on mitogenic rate in culture. However, in animals, Nox1 over-expression markedly increased angiogenesis by inducing the angiogenic factor VEGF [89] correlating with an aggressive tumor phenotype. A similar role for Nox1 in angiogenesis in atherosclerosis has been proposed [88].

e. Nox enzymes and cell survival

In contrast to the frequently reported pro-apoptotic effect of ROS, ROS from Nox4 in pancreatic cells [141] and from Nox1 in colon adenoma and carcinoma [136] inhibit apoptosis. In pancreatic cancer cells, depletion of Nox4 or ROS triggered apoptosis [142] predicting a therapeutic effect of Nox inhibition in treating this type of cancer. Cell survival involved activation of NF kappa-B- [136] and Akt- [142] dependent pro-survival pathways.

f. Nox enzymes and integrin signaling

Cancer cell phenotype including mitogenic rate and response to chemotherapy is profoundly affected by attachment to extracellular matrix (ECM) [143]. Nox enzymes both affect ECM synthesis and structure, and mediate the cellular effects of ECM [144]. In colon carcinoma cells, Nox1 controls the expression of specific integrins at the cell surface, and integrin-dependent attachment/signaling stimulates the G1/S transition [145]. In A431 carcinoma cells, the growth factor EGF activates Nox-dependent ROS generation, and this in turn regulates expression of integrins, cell attachment properties and cell survival [146]. In pancreatic cancer cells, ECM stimulated ROS production through Nox4 resulting in increased cell survival [147]. These studies emphasize the interplay between ROS and ECM in effecting the cancer phenotype.

Nox enzymes in Brain and Nerve

a. Roles for Nox-derived ROS in brain and nerve

ROS play a role in both normal neurological processes and in neurological disease states. NGF stimulates ROS generation in PC12 cells in a Rac1-dependent manner, and NGF-generated ROS participates in neuronal differentiation [148]. ROS in neurons also enhances voltage-gated K⁺ currents elicited by NGF, mediated by activation of NF-kappaB [149]. H₂O₂ inhibits synaptic transmission in hippocampus and other areas of the brain [150,151] by complex mechanisms that are not yet fully elucidated [152,153]. In guinea pig striatal slices, H₂O₂ production was Ca²⁺-dependent and modulated neurotransmitter release, revealing a signaling role for ROS in synaptic transmission [154]. A possible target is the fusion protein SNAP25, which may function as a presynaptic ROS sensor [155].

Specific roles for Nox enzymes in nerve and brain are beginning to come to light. Nox2 is expressed in relatively high levels in microglia [156], the principal immune effector cell in the brain, where it participates in host defense and inflammatory responses in this organ [157]. Nox4 is expressed in neurons and capillaries of the brain, and is up-regulated during ischemia [158]. Neuronal Nox1 is induced in response to NGF, and suppresses neurite outgrowth [159]. Nox5 shows significant expression in cerebrum and Duox1 is highly expressed in cerebellum (Cheng and Lambeth, unpublished data). To date, there have been no detailed reports cataloging the expression of specific Nox enzymes in subregions of the brain. Remarkably, Nox1 knockout mice show a marked change in their ability to perceive inflammatory pain, *i.e.*, decreased thermal hyperalgesia produced by inflammation, compared

with wild-type mice (Ibi and Yabe-Nishimura, unpublished personal communication). This suggests that drugs inhibiting Nox1 activity could find applications in inflammatory pain control.

b. Nox enzymes and Alzheimer's disease

Among the characteristic changes seen in Alzheimer's Disease (AD) are extracellular deposits of fibrillar β -amyloid protein (plaques) and neuronal loss resulting in progressive cognitive impairment. While the underlying early cause(s) of AD remains elusive, evidence points to inflammatory reactions as a key component in the progressive neuronal loss, reviewed in [160]. Microglia are thought to be a major cell type that mediates this inflammatory response, which involves secretion of inflammatory cytokines and release of ROS and reactive nitrogen species [161], although astrocytes may also play a role. Chronic production of inflammatory mediators results in neuronal death [162], either by direct oxidative damage or by over-activating death-promoting signaling systems including NF-kappaB [163].

Evidence for excessive oxidant production in AD comes from autopsy studies and animal models. Markers of oxidative stress in AD brains occurred early, and increased with severity of the disease [164,165]. Oxidative damage could be detected prior to observation of plaques in both human and in animal models, pointing to inflammation as an early event in AD [166, 167]. The major source of oxidants is generally thought to be the microglial and/or astrocyte Nox2-type system [166,168–170], although there is also increased expression of Nox1 and Nox3 in AD brain [164].

c. Nox enzymes and Parkinson's disease

Parkinson's Disease (PD) is a complex disorder that results in the progressive degeneration of dopaminergic neurons in the substantia nigra. Although the origin is unclear, oxidative stress has been thought to play a role in its pathogenesis [171–173], and the condition can be recapitulated experimentally by administration of MPTP (1,2,3,6-tetrahydropyridine), which results in increased ROS by inhibiting mitochondrial respiration and by activating Nox2 in microglia [171]. While defects in mitochondrial Complex I may underlie sporadic PD, activated or induced Nox enzymes in microglia may play a synergistic role [174]. In model systems, LPS administration acutely activates the microglial inflammatory response, releasing proinflammatory factors, activating glial Nox2 and producing neuronal loss [175,176]. In this model of Parkinson's Disease, Nox2 knockout mice were significantly protected against loss of nigral dopaminergic neurons [176]. In a more natural setting, substance P produced in substantia nigra can also activate glial Nox2, and could play a role in PD [177].

d. Nox enzymes and amyotrophic lateral sclerosis (ALS)

ALS is a progressive and ultimately fatal loss of spinal cord motor neurons. Although a role for oxidative stress and oxidative damage has been well documented [178], this has generally been assumed to be due to decreased oxidative defense mechanisms because SOD1 is mutated in familial forms of the disease [179]. However, markers of oxidative damage are also seen in sporadic cases of ALS, and an inflammatory interplay between neuronal and glial cells mediated by cytokines has been observed [180]. Recently, the Nox2 system was found to be activated in the spinal cord of patients with ALS and in genetic animal models of this disease [181]. Importantly, inactivation of Nox in ALS mice delayed neurodegeneration and extended survival. Thus, treatments aimed at decreasing Nox2-dependent inflammation are likely to be therapeutic in ALS.

Nox3 and Inner Ear Function

a. Nox3 and gravity perception

The highest expression of Nox3 is in the inner ear, specifically in vestibular and cochlear sensory epithelia and the spiral ganglions [182]. The vestibular system is responsible for the perception of motion and gravity, and this process involves tiny biomineralized particles called otoconia. In response to acceleration or gravity, these particles deflect stereocilia of hair cells, which transduce this physical signal into a neural signal. Mutant mice that show an absence of otoconia exhibit a “head slant” phenotype characterized by defective gravity sensing. Several of these mouse strains showed mutations in either Nox3 [183] or its regulatory subunit NOXO1 [184].

b. Nox3, ototoxicity and deafness

Deafness and the ototoxicity of certain drugs and toxins may result from Nox3-mediated ROS generation. In CD/1 mice, age-related hearing loss is associated with ROS formation leading to HIF-1 α induction in the cochlea [185]. Nox3 in the inner ear is induced by cisplatin, suggesting that it could mediate the ototoxic effects of this chemotherapeutic agent [182, 186].

Nox enzymes and the Endocrine System

a. Duox and the thyroid gland

The thyroid gland carries out the thyroid peroxidase-dependent iodination of thyroglobulin, a key step in the biosynthesis of thyroid hormone. This reaction requires H₂O₂, which derives from a previously unidentified thyroid NADPH oxidase. Duox was first shown to be this long-sought oxidase based on purification and partial cDNA cloning [187]; two forms of this oxidase were subsequently identified by molecular cloning [188–190], and both are expressed in thyroid [191]. The essential role for Duox2 in thyroid hormone biosynthesis is demonstrated conclusively by the occurrence of mutations in this gene in genetic variants of hypothyroidism [192,193].

b. Pancreatic islets, diabetes and Nox enzymes

Nox1, Nox2 and Nox4 were found in pancreatic islet cells, and glucose-stimulated insulin secretion was suppressed by a general Nox inhibitor, supporting a role for one or more Nox enzymes in normal pancreatic islet function [194]. In addition to this normal function, excessive ROS production may damage pancreatic islets leading to type 1 diabetes. In early type 1 diabetes, systemic markers of oxidative stress correlate with insulin requirements, suggesting that oxidative stress in the pancreatic islets damages insulin-secreting beta-cells [195]. In type II diabetes, increased expression of Nox1 occurs in islets, and may exacerbate disease over time by damaging insulin-producing cells [196]. In a model of pancreatitis, Nox1 and/or other Nox enzymes were implicated in the inflammatory response that resulted in tissue inflammation and destruction [197].

c. Signaling role of Nox enzymes in hormonal responses

Nox1 induction and activation is implicated in the hormonal response to Angiotensin II and growth factors such as PDGF and EGF (*vide infra*). In addition, recent studies implicate Nox4 in the tissue response to insulin [198,199]. In fat cells, insulin triggered H₂O₂ production; H₂O₂ was essential for transduction of the insulin signal, in part by ROS inhibition of the protein tyrosine phosphatase PTP1B.

Nox enzymes in Muscle, Cartilage and Bone

a. Bone resorption

ROS play a role in bone resorption, and are necessary for bone remodelling [200]. The mechanism by which superoxide participates in bone resorption is not clear, but may involve direct oxidation of calcium binding sites and/or acidification of the local environment near osteoclasts. Nox4 in osteoclasts may be the source of the ROS in bone [201].

b. Cartilage and osteoarthritis

In osteoarthritis, interleukin 1 β is implicated in cartilage destruction through a ROS-dependent mechanism. In chondrocyte cell lines, Nox4 is implicated as the source of the interleukin 1 β -dependent ROS generation [202].

c. Smooth muscle

In recent studies, we (D. Ritsick and D. Lambeth, unpublished) made the surprising observation that knocking down the expression of the *Drosophila* Nox5 with si-RNA resulted in female sterility. This was traced to an inability to lay eggs which resulted from a defect in contraction of the smooth muscle of the ovary. This, in turn, resulted from a co-signaling role of Nox-generated H₂O₂ in triggering an agonist-induced calcium flux in these cells. Additional studies (M. Petumnetcha and D. Lambeth, unpublished) show that the Angiotensin II-stimulated calcium flux human vascular smooth muscle is likewise dependent upon the generation of a Nox (probably Nox1)-generated H₂O₂ signal. Thus, we suggest a general function of Nox enzymes in smooth muscle calcium signaling.

Concluding remarks

Proposed roles of specific Nox and Duox enzymes in human diseases are summarized in Table I. In many cases, the role of a Nox enzyme has been established either by human or mouse mutations (chronic granulomatous disease, gravity perception, hypothyroidism) or by convincing cellular studies, while in other cases the evidence is still circumstantial. It seems clear, however, that Nox and Duox enzymes are emerging as critical contributors to a variety of disease states. Inspection of Table I reveals that only in a few cases do diseases result from *decreased* production of ROS. By far, the majority of diseases involving Nox and Duox enzymes are associated with *increased* ROS, and in many cases this has been demonstrated to result from increased expression of these enzymes and/or their regulatory subunits. A second broad conclusion is that the majority of diseases represented in Table I are chronic illnesses, very often appearing late in life. These include various degenerative diseases of the central nervous system, cardiovascular diseases, cancer, diabetes and others.

These results emphasize the conclusion that biology seems to have embarked on a risky enterprise when enzymes whose main function is to produce ROS appeared on the evolutionary scene. While there are clearly beneficial functions for ROS, including in innate immunity, bone remodeling, otolith production, signal transduction and biosynthesis of biologically important molecules such as thyroid hormone, the use of ROS for these functions appears to have had a down-side, as illustrated by the many diseases listed in Table I. Because most of these diseases appear late in life and do not affect the organism during its reproductive period of life, I believe that the occurrence of Nox enzymes and their expansion during evolution (*C. elegans* has 1 Nox, *Drosophila* has 2, and humans have 7) can be understood as an archetypal example of antagonistic pleiotropy. This term refers to the propagation and expansion of genes that confer a survival advantage early in life, but which have harmful effects later in life. This occurs because reproduction takes place before the negative effects of such genes can be expressed, resulting in a decline in the force of natural selection for these genes with increasing age. Thus,

the fact that Nox and Duox enzymes are associated with a variety of chronic diseases has had little impact on their propagation and expansion during evolution, and it is their positive biological functions rather than their later negative effects that have governed the evolutionary history of these genes.

It is also apparent from this review that drugs that target specific Nox and Duox enzymes or even broad groups these enzymes can be expected to be useful in arresting the progression of a variety of chronic diseases, particularly those that appear in the second half of life. The development of Nox inhibitors is in its infancy, with the pharmacy limited to the non-specific and non-clinically useful diphenylene iodonium and apocyanin. The development of Nox/Duox inhibitors represents an important new direction for biomedical investigation and eventual clinical treatment and prevention, particularly of chronic diseases and their complications.

Acknowledgements

Supported by NIH Grants CA105116 and CA084138. My thanks to Susan Smith for carefully reading and helping revise the manuscript.

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List of Abbreviations

Nox

	NADPH oxidase
Duox	Dual oxidase
ROS	reactive oxygen species
AD	Alzheimer's disease
PD	Parkinson's disease
phox	<u>phagocyte oxidase</u>
PDGF	platelet-derived growth factor
NOXO1	Nox organizer protein 1
NOXA1	Nox activator protein 1
FAD	flavin adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

Table 1

Diseases involving Nox or Duox enzymes

Shown is the disease and the tissue affected along with the particular Nox or Duox that is thought to be involved.

Disease	Tissue	Candidate Nox/Duox
<i>Diseases of Decreased Nox Activity</i>		
Chronic granulomatous disease	Phagocytes	Nox2
Defective gravity perception	Inner ear	Nox3
Hypothyroidism	Thyroid gland	Duox2
<i>Diseases of Increased Nox Activity</i>		
Arthritis	Phagocytes	Nox2
Inflammatory bowel disease	Phagocytes, Intestinal epithelium	Nox2
Shock lung (adult respiratory distress syndrome)		Nox1
Lung fibrosis	Lung	Nox2, others?
Emphysema	Lung	Nox4, Duox
Asthma	Lung	Nox1, Nox3
Pulmonary hypertension	Lung	Nox4, Nox2
Cardiac fibrosis in heart failure	Lung	Nox4
Cardiac hypertrophy	Heart	Nox2
Electrophysiologic remodeling in atrial fibrillation	Heart	Nox2, Nox4
Hypertension	Heart	Nox2
Atherosclerosis	Vasc. sm. muscle	Nox1, Nox2
Diabetic vascular disease (includes blindness, neuropathy, atherosclerosis)	Vascular	Nox1, Nox2
Diabetic nephropathy	Vasc. endothelium, other cell types	Nox1, Nox2
Renal hypertension	Kidney	Nox4, Nox2
Glomerulonephritis	Kidney	Nox2
Acute tubular necrosis	Kidney	Nox2, Nox4
Hemodialysis-induced renal failure	Kidney	Nox2
Cancers	Kidney	Nox2, others?
Melanoma	Skin	Nox4
Barrett's adenocarcinoma	Esophagus	Nox5
Prostate cancer	Prostate	Nox1, Nox5
Gastric cancer	Stomach	Nox1
Colon cancer	Colon	Nox1
Glioblastoma	Brain	Nox4, Nox5
Inflammatory pain	Peripheral nerves	Nox1
Alzheimer's disease	Brain	Nox2, Nox3, Nox1?
Parkinson's disease	Brain	Nox2
Amyotrophic lateral sclerosis	Spinal motor neurons	Nox2
Deafness	Inner ear	Nox3
Type I Diabetes	Pancreatic islets	Nox1, Nox2
Pancreatitis	Pancreas	Nox1