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Oxidases and Peroxidases in Cardiovascular and Lung Disease: New Concepts in Reactive Oxygen Species Signaling

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Abstract

Reactive oxygen species (ROS) are involved in numerous physiological and pathophysiological responses. Increasing evidence implicates ROS as signaling molecules involved in the propagation of cellular pathways. The NADPH oxidase (Nox) family of enzymes is a major source of ROS in the cell and has been related to the progression of many diseases and even in environmental toxicity. The complexity of this family's effects on cellular processes stems from the fact that there are 7 members, each with unique tissue distribution, cellular localization and expression. Nox proteins also differ in activation mechanisms and the major ROS detected as their product. To add to this complexity, mounting evidence suggests that other cellular oxidases or their products may be involved in Nox regulation. The overall redox and metabolic status of the cell, specifically the mitochondria, also has implications on ROS signaling. Signaling of such molecules as electrophillic fatty acids has impact on many redox sensitive pathologies, and thus, as anti-inflammatory molecules, contributes to the complexity of ROS regulation. The following review is based on the proceedings of a recent international Oxidase Signaling Symposium at the

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University of Pittsburgh's Vascular Medicine Institute and Department of Pharmacology and Chemical Biology, and encompasses further interaction and discussion among the presenters.

Keywords

ROS; reactive oxygen species; hydrogen peroxide; superoxide; NADPH oxidase; Nox; mitochondria; lipooxygenase; myleoperoxidase; xanthine oxidase; heme oxygenase 1; arsenic; hypertension; fibrosis; electrophilic fatty acid

1. Introduction

In 1933, a "burst of extra respiration" was described in phagocytosing leukocytes [1]. This discovery identified what came to be known as the phagocytic oxidative or respiratory burst and triggered the beginning of reactive oxygen species (ROS) research. In 1961 hydrogen peroxide (H_2O_2) was identified as the product of this burst [2] and in 1973 it was demonstrated that the H_2O_2 is derived from the monovalent reduction of oxygen (O_2) to form superoxide ($O_2^{\bullet -}$) [3].

Since those discoveries, H_2O_2 and $O_2^{\bullet -}$ have been implicated in many physiological and pathophysiological processes including signal transduction, cell proliferation, gene expression, angiogenesis, sepsis, diabetes, heart disease, neurodegenerative diseases, pulmonary diseases, and cancer [4, 5] [6–10]. H_2O_2 and $O_2^{\bullet -}$ were also described as causative factors in aging as early as 1956 [11], initially as agents of oxidative damage. Over the last decade, H_2O_2 and $O_2^{\bullet -}$, often encompassed by the more generic term ROS, have emerged as mediators of intracellular signaling [12–14]. The concomitant identifications of reactive nitrogen species (RNS) and the interaction between ROS and RNS, specifically nitric oxide ($^{\bullet}NO$) and $O_2^{\bullet -}$, uncovered a new level of complexity in how these biological processes and pathological pathways are modulated. Furthermore, evidence is mounting on cross-talk and feed-forward interactions among the different cellular oxidases. The current short review derives from a recent international Oxidase Meeting at the University of Pittsburgh's Vascular Medicine Institute and Department of Pharmacology and Chemical Biology, focused on understanding the enzymology, structure-function, and signalling properties of oxidases in health and disease. This review highlights the mechanisms by which NADPH oxidase (Nox)-derived ROS interact with products of other oxidases and peroxidases, and covers new cutting-edge research tools and approaches in the study of ROS.

2. Reactive Oxygen Species

ROS are oxidants derived from the metabolism of oxygen which encompass a broad range of biochemical interactions and chemical properties. They include $O_2^{\bullet -}$, H_2O_2, hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), ozone (O_3) and singlet oxygen ($^1O_2$). $O_2^{\bullet -}$ is the precursor of most ROS in the cell and is dismuted to H_2O_2, either through the action of a family of enzymes, the superoxide dismutases (SOD), or spontaneously. Major cellular sources of ROS are mitochondria, cytochrome P450 family proteins, xanthine oxidoreductase, uncoupled nitric oxide synthase (NOS), peroxisomes, cyclooxygenases, lipoxygenases and the Nox family of enzymes [4, 15–22]. Of these, only the Nox enzymes possess pathways for the regulated generation of H_2O_2 and $O_2^{\bullet -}$. Recently, it has been suggested that mitochondria may be able to generate regulated levels of $O_2^{\bullet -}$ at different sites in the respiratory chain but this remains controversial [23].

ROS can induce oxidative damage through lipid peroxidation of cellular membranes, DNA strand breaks, and protein oxidation [10, 24–26]. However, under normal physiological conditions, an overall reducing environment exists in cells and a number of protective mechanisms including the amino acid cysteine, glutathione (GSH), thioredoxin (Trx), SOD and catalase, among others, scavenge ROS and maintain oxidative damage at a minimum [8, 24]. The current paradigm describes a more elaborate role of regulated levels of ROS, specifically from the NADPH oxidases and mitochondria, as modulators of intracellular signaling pathways including those of angiotensin II (Ang II) [27, 28], platelet-derived growth factor (PDGF) [29–31], tumor necrosis factor α (TNF-α) [32–34], thrombin [35, 36], interferon-γ [37], insulin [38], [39], and bacterial lipopolysaccharide (LPS) [40, 41], to name a few. Indeed, there is mounting evidence of redox sensitivity of a number of the major cell signaling molecules, especially phosphatidylinositol-3 kinase (PI3K), c-Jun N-terminal kinase (JNK), mitogen activated protein kinases (MAPKs), their upstream modulators such as apoptosis signal-regulating kinase-1 (Ask-1), phosphatases such as protein tyrosine phosphatase 1B (PTP1B) and Src homology 2-containing inositol phosphatase 2 (SHP-2), transcription factors such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1) [10, 24, 31, 42] [8, 9, 43–49], and more recently the cellular hemoglobin neuroglobin [50]. The specificity of ROS action on different signaling pathways is expected to depend on (a) the nature of the ROS produced, its amount and localization; (b) the redox status in the immediate proximity of the pathway; (c) the pKa of reactive thiols and/or nucleophilic moieties in signaling molecules; and (d) the overall redox status of the cell as determined by the balance of redox molecules including glutathione/glutathione disulfide (GSH/GSSG), NADPH/NADP, and NADH/NAD [10].

ROS are best known for their ability to effect cellular signaling via modifications of thiols on cysteine residues, or thioethers of methionines, on signaling proteins. 21,000 – 42,000 of all the unique cysteine residues in the human genome can be readily oxidized [42]. Under conditions of normal cellular pH, cysteine residues surrounded in the tertiary structure by basic amino acids have their thiol (-SH) group de-protonated to thiolate ion (-S) [10]. Thiolate is highly susceptible to oxidation resulting in formation of mixed disulfide bonds (-SS-), on the same or neighboring molecules, sulfenic acid or sulfenate ion (-SOH or $-SO^-$), sulfinic acid or sulfinate ion ($-SO_2H$ or SO_2^-), or sulfonic acid or sulfonate ion ($-SO_3H$ or SO_3^-). These modifications lead to structural changes in the target molecule, which can result in either activation or inactivation [31, 42]. For example, PTP1B is inactivated by oxidation at cysteine 215 and such inactivation is true of other phosphatases [51] [52, 53], whereas Ask-1 appears to be activated by oxidation at cysteine 250 among others, and this ROS mediated activation is also true for other kinases of the MAPK kinase pathways [54–56]. The degree to which these cysteine residues are oxidized determines the reversibility or irreversibility of the modification, with the sulfonic acid being least likely to be reversibly reduced. Moreover, $^{\bullet}NO$ and peroxynitrite ($ONOO^-$) can result in modified cysteine-nitrosothiol and nitrothiol. Sulfenic acid and nitroso-thiol modifications of proteins undergo further oxidation leading to other oxidation products and contributing to mixed disulfide formation and thus, protein adducts. The stability or instability of these modifications depends greatly on thioredoxin reductase, glutaredoxin and protein disulfide reductase activities, proximity, and the disulfide accessibility within the target molecule, all of which determine the steady state level of the modified thiol, and thereby, its signaling potential.

3. Oxidases and Peroxidases

Of the cellular sources of ROS mentioned above, Nox enzymes are unique in that either H_2O_2 or $O_2^{\bullet-}$ are detected as their primary and regulated products. In combination with their diverse localization in the cell and juxtapositions to specific signaling pathways, tight regulation of the activity of Nox enzymes appears to provide precise modulation of ROS

levels to control ROS-dependent signaling events. In addition, cells have developed elaborate mechanisms by which ROS and metabolites of different oxidases and peroxidases can interact with and regulate each other, thus introducing another level of complexity to the signaling of ROS in health and disease. Following is an overview of Nox enzymes and some of the cellular oxidases and peroxidases involved in these processes.

a. NADPH oxidases

NADPH oxidase activity was first described in liver microsomal isolates and bacteria in 1963 [57, 58]. Around the same time, human chronic granulomatous disease (CGD), a rare condition first described in 1957 characterized by susceptibility to recurrent infection due to failure of phagocytic microbicidal activity, was linked to defective respiratory burst of what was then referred to as an “NADH” oxidase [59–62]. The substrate of the oxidase was later revealed to be NADPH rather than NADH. In the 1980s and early 1990s, subunits of the phagocyte NADPH oxidase were characterized and a model for phagocytic NADPH oxidase was developed [16, 63–67]. The membrane component of the phagocyte NADPH oxidase that serves as the electron transferase is flavocytochrome b558, a heterodimer composed of gp91^{phox}, currently referred to as Nox2, and p22^{phox}, another membrane-spanning subunit. Based on sequence homology with gp91^{phox}, other members of the NADPH oxidase (Nox) family were identified and their expression characterized in many tissues, including vascular smooth muscle and endothelial cells, and fibroblasts. Moreover, their role in numerous pathological and physiological conditions including heart disease, atherosclerosis, rheumatoid arthritis, sepsis, asthma, cystic fibrosis, diabetes, immune diseases, neurodegenerative diseases and cancer, was demonstrated [68–71] [4, 15, 16, 20, 72–77].

Structure and Function—The Nox family is defined by seven distinct membrane-integrated catalytic subunits that form the basis of the enzyme, Nox1 to 5 and Duox1 and Duox2, which give the individual oxidases their names. Nox1 – 4 are associated with p22^{phox}, which appears to be involved in stabilization of these Nox proteins and is essential for their proper membrane targeting and activity. Nox5, Duox1 and Duox2 do not require p22^{phox} for their activity. The cytosolic regulatory subunits that are responsible for activation of the Nox1- through 3-based enzymes, but not Nox4 or 5, are p47^{phox} (also called Nox organizer 2, or NoxO2) or its homolog NoxO1, p67^{phox} (also called Nox activator 2, or NoxA2) or its homolog NoxA1, and p40^{phox}. p47^{phox} and p67^{phox} are the canonical regulatory subunits involved in Nox2 activation, whereas NoxO1 and NoxA1 are implicated as the major cytosolic subunits involved in the activity of Nox1. Nox3 seems to be activated by association with NoxO1 alone (human Nox3), NoxO1 and NoxA1 (murine Nox3), p47^{phox} and p67^{phox}, or p67^{phox} alone, but this remains controversial. The small Rho family GTP-binding proteins Rac2 and Rac1 are essential for the catalytic activity of Nox2 and Nox1, respectively, and evidence suggests a need for Rac1 in the Nox3 system as well. Rac1 has also been shown to be important for Nox2 activation in some cells, including endothelial cells. In addition, p40^{phox} and the GTPase Rap1A contribute to activation of the complex. Upon initiation of Nox2 oxidase activation, for example, p47^{phox}, p67^{phox} and p40^{phox} translocate *en bloc* to the membrane, with transfer regulated by conformational changes in p47^{phox} secondary to its phosphorylation. Concomitant with this translocation, Rac2 redistributes from cytoplasm to the membrane and completes the assembly process. In a similar process, Nox1 (and Nox3) likewise rely on NoxO1 and NoxA1 in complex activation. The activities of Duox1 and 2 depend on their association with the two membrane-bound proteins DuoxA1 and DuoxA2, which are important for trafficking of Duoxes from the ER to the Golgi and their final localization to vesicular structures or to the plasma membrane (figure 1) [4, 70, 78–103].

All Nox family members contain NADPH- and flavin adenine dinucleotide (FAD)-binding domains in their C-terminal cytosolic tails as well as predicted six conserved transmembrane domains containing two highly conserved heme-binding histidines on transmembrane domains III and V bound to two asymmetrical heme groups [4, 104]. FAD is constitutively bound to the enzyme while NADPH serves as its substrate (figure 2). Nox enzymes catalyze the transport of electrons across biological membranes from NADPH to O_2 generating $O_2^{\bullet -}$ [105]. In addition, Nox5, which has 5 isoforms (α, β, γ, δ and ε), displays an intracellular N-terminus that contains a calmodulin-like domain with four Ca^{2+}-binding EF-hand motifs in all its isoforms with the exception of NOX5ε[85, 96, 106]. Also, Duox1 and 2 possess a seventh transmembrane domain, an extracellular N-terminus with a peroxidase-like domain and two Ca^{2+}-binding EF-hand domains similar to Nox5, rendering these isozymes, as well as Nox5, highly regulated by changes in intracellular Ca^{2+} levels (figure 3). As with other Nox proteins, Nox5 is also regulated by PKC as well as c-Abl [107].

In the active enzyme, two electrons are transferred from NADPH to FAD reducing it to $FADH_2$ catalyzed by unique binding sites on the inner membrane C-terminal tail [108]. An electron is then transferred to the inner heme, which transfers it to the outer heme and then to a bound O_2 molecule on the external membrane, generating $O_2^{\bullet -}$ [92]. The second electron is transferred in the same way. Different members of the Nox family, however, vary in the major ROS detected from their activity. For Nox1 – 3 and Nox5 $O_2^{\bullet -}$ is readily detected, whereas mainly H_2O_2 is detected from Duox1, Duox2 or Nox4 [109–112]. This difference might be connected to differences in Nox4/Duox folding, leading to distinct formation and membrane insertion of the heterodimer with $p22^{phox}$ or DuoxA1/2, or to interaction with yet unknown proteins. Thus, rapid dismutation of $O_2^{\bullet -}$ to H_2O_2 as a result of these differences may preclude from detection of significant amounts of $O_2^{\bullet -}$ by currently available methods.

Expression—Cellular distribution and subcellular localization of different Nox isoforms play an important role in how individual Nox proteins regulate signaling. Nox2, the first catalytic subunit to be identified, is, in human cells, a heavily glycosylated protein that migrates as a broad band of 60 – 91 kDa on Western blots. It is noteworthy that in murine cells, Nox2 is less glycosylated and migrates as a 55 – 60 kDa protein on Western blots. Nox2 is expressed on membranes of neutrophils, macrophages, endothelial cells, some vascular smooth muscle cells, fibroblasts, skeletal muscle cells, cardiomyocytes and hepatocytes [4, 72, 80, 104, 113–117]. In myeloid cells, localization to the plasma membrane and/or phagosomal membrane is clear. While some evidence suggests that the same may be true for other tissue cells with respect to plasma membrane localization of this isoform, this remains controversial and other evidence suggests intracellular, perinuclear distribution in some cells [118]. Nox1, initially identified in colonic epithelial cells, is a major isoform in large artery SMC [119, 120], and is also expressed in endothelial cells, osteoclasts and reproductive organs [70, 121, 122], where it is localized intracellularly in the proximity of the endoplasmic reticulum (ER) but also on the plasma membrane associated with caveolae [123–125]. Nox3, discovered in 2000 [126], appears to be more limited in distribution, being highly expressed in the inner ear and in low levels in skull bone, brain and some fetal tissues [90]. Nox4, originally described in the kidney as Renox, has a more ubiquitous expression profile, including smooth muscle cells, fibroblasts, hematopoietic stem cells, osteoclasts, neurons, and endothelial cells [113, 123, 127–133]. Within the cell Nox4 has been detected near focal adhesions [123], at the endoplasmic reticulum [129], in the nucleus [134], and in the mitochondria [135, 136], depending on the cell type. Nox5, discovered in 2001, is found in bone marrow, lymph nodes, spleen, reproductive tissues, stomach and pancreas, in addition to some fetal tissues and exists in a truncated form (Nox5-S) in vascular smooth muscle and endothelial cells [85, 96, 137–139]. Duox1 and 2 are highly expressed in the thyroid and are also found in airway epithelia and the prostate.

Duox2 is also found in the digestive system. Within thyroid and epithelial cells, these two enzymes appear to localize to the apical membrane, but there is also evidence for an intracellular localization in these cells [93, 94, 140, 141]. It should be noted that species differences do exist in the expression profiles of Nox proteins. Specifically, Nox5 is not expressed in rodents and Duox are not expressed in mouse airway epithelial cells. Table 1 summarizes the cellular distribution and subcellular localization of Nox family members.

b. Peroxidases

Ubiquitously expressed throughout both plant and animal kingdoms, peroxidases consume H_2O_2 and catalyze the one- or two-electron oxidation of a remarkably diverse array of substrates. Recently, the heme-containing oxidoreductases have been classified based on their evolutionary relationships into the peroxidase-cyclooxygenase superfamily [142], which includes those enzymes previously designated as the “animal peroxidases”, namely myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), and thyroid peroxidase (TPO). Post-translational modifications of biological substrates by the action of peroxidases in general, and by mammalian peroxidases in particular, support a wide variety of physiologic functions, ranging from TPO-dependent thyroid hormone synthesis to the generation of hypobromous acid by EPO from stimulated eosinophils. With the exception of TPO, the members of this protein superfamily figure prominently in innate immunity, with each mediating functions specially tailored to their tissue location.

c. Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) catalyzes the terminal two steps of purine degradation (hypoxanthine → xanthine → uric acid) in humans. XOR is transcribed as a single gene product, xanthine dehydrogenase (XDH) where substrate-derived electrons at the molybdenum cofactor (Mo-cofactor) are transferred via two Fe/S centers to a FAD-cofactor where NAD^+ is reduced to NADH. During inflammatory conditions, post-translational modification by oxidation of critical cysteine residues or limited proteolysis converts XDH to xanthine oxidase (XO) [143, 144]. The key difference distinguishing XO from XDH is the structural conformation and electrostatic microenvironment surrounding the FAD-cofactor resulting in lower affinity for NAD^+ and enhancement of affinity for O_2 [145]. Substrate-derived electrons at the Mo-cofactor of XO reduce O_2 at the FAD-cofactor both divalently, forming H_2O_2 and univalently, generating $O_2^{\bullet -}$. However, conversion to XO is not requisite for $O_2^{\bullet -}$ and H_2O_2 production, as XDH displays partial oxidase activity under conditions in which NAD^+ levels are diminished, such as the ischemic/hypoxic microenvironment encountered in vascular inflammation [146]. This same inflammatory milieu leads to enhanced XO levels and thus increased XO-derived ROS formation resulting in activation of redox-dependent cell signaling reactions and alterations in vascular function. Evidence of this role for XO is exemplified by numerous studies in which XO inhibition attenuates symptoms of vascular disease including congestive heart failure, sickle cell anemia and diabetes [147–150].

d. Heme Oxygenase

Heme oxygenase (HO) catalyzes the degradation of Heme b (Fe-protoporphyrin-IX) to form tetrapyrrole biliverdin-IXα. Biliverdin-IXα is subsequently converted to bilirubin-IXα by a NADPH-dependent reductase [151]. HO enzymatic activity releases the heme core iron in the ferrous form Fe(II) and carbon monoxide (CO), a small gaseous molecule that is relatively stable in biological systems [151, 152]. Expression of HO-1, an inducible HO isoform, is usually undetectable under basal conditions, with the exception of spleen [153], the site of erythrocyte hemoglobin turnover, but responds to rapid transcriptional activation in response to biological stress, like LPS, NO, PDGF, Ang II, hypoxia and ROS. Besides its role in heme degradation, HO-1 also functions as an inducible stress protein with tissue and

cell protective effects in numerous models of cellular stress and organ pathology [154]. These protective effects have been largely attributed to the production of CO, biliverdin and bilirubin.

e. Mitochondria

The mitochondrion is the site of oxidative phosphorylation, a process by which electrons are transferred through the mitochondrial respiratory chain, leading to the reduction of each protein complex, until the electrons are ultimately accepted by oxygen, yielding water (figure 4). This process is coupled to the production of ATP at complex V to generate 50–95% of cellular energy (depending on cell type). The majority of electrons entering the electron transport chain ultimately are accepted by oxygen to form water in a process that does not yield free radicals. However, it is estimated that under basal conditions approximately 1–3% of electrons at respiratory complexes distal to cytochrome c oxidase (complex IV) do not make it through the chain and instead can catalyze the univalent reduction of O_2 to $O_2^{\bullet -}$. Initially, this was considered to be a “leak” of electrons from the respiratory chain since it was induced by pathological conditions such as hyperoxia. Recently, it has been suggested that under physiological conditions this can be a regulated process involved in cell signaling and cellular responses to stress. Indeed, hypoxia, nitric oxide, and the PI3-kinase pathway all contribute to regulation of mitochondrial ROS [155–158]. The major points of $O_2^{\bullet -}$ production from the respiratory chain are complex I (NADH dehydrogenase), and complex III (ubiquinone-cytochrome c reductase). $O_2^{\bullet -}$ formation occurs at multiple flavin and heme sites of single electron reactions that exist within these multi-subunit complexes [17, 159, 160]. Notably, the majority of $O_2^{\bullet -}$ generated by the electron chain is rapidly dismuted to H_2O_2 by manganese SOD (MnSOD) localized within the mitochondrial matrix. $O_2^{\bullet -}$ generation at complexes I and III is regulated by the rate of respiration, with slower respiration leading to higher rates of ROS generation due to increased reduction of the chain. Interestingly, generation of ROS at different segments of the respiratory chain results in differential oxidative modification of the mitochondrial proteome [161]. Additionally, physiological regulators of respiration, such as $^{\bullet}$NO or CO, also modulate mitochondrial ROS generation [158]. This type of modulation has been implicated in the adaptive signaling observed in response to hypoxia [162]. In contrast, dysregulation of mitochondrial ROS generation has also been shown to contribute to the progression of pathologies such as ischemia/reperfusion injury and sepsis [163].

4. Functional Distinctions Among Nox Isoforms: Heterodimerization and the “Nox” Domain

As mentioned above, the catalytic subunits Nox1 – 4 associate with $p22^{phox}$ and exist as integral membrane proteins. Heterodimer association is essential for Nox maturation, protein stabilization and function of these isoforms. Similarly, while $p22^{phox}$ does not associate with Duox proteins, heterodimerization with DuoxA1 and DuoxA2 is required for processing, subcellular localization and catalytic activity of Duox1 and Duox2, respectively [141, 164]. Not only do Nox isoforms differ in the presence of- and partners for- heterodimer formation, their dependence for activity on the different cytosolic subunits varies widely among members of this family [103]. The activities of Nox1 – 3/$p22^{phox}$ dimers require stimulus-mediated association with $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, NoxO1 and/or NoxA1, Rac activity and multiple phosphorylation events to form an active enzyme. In contrast to this spatial modulation, the Nox4/$p22^{phox}$ complex appears to be constitutively active and transcriptionally, rather than spatially, regulated. Some evidence, however, suggests also a possible regulation by polymerase [DNA-directed] delta-interacting protein 2 (poldip2) [165], but poldip2 associates with $p22^{phox}$ rather than with Nox4. Along the same lines, Laurindo and coworkers provide evidence for a possible regulatory association of Nox1,

Nox2, Nox4 and p22*phox* with protein disulfide isomerase, a redox chaperone and member of the thioredoxin family [166–168]. Nox5 and Duox enzymes are activated by agonist-induced increases in intracellular calcium in concert with various phosphorylations [169]. Finally, H_2O_2 is detected as the primary product from Duox1, Duox2 and Nox4 rather than $O_2^{\bullet -}$ as is the case with the other isoforms. All these factors will have important implications with respect to cellular signaling responses initiated by different Nox isoforms.

None of the Nox protein family members have been crystallized, but the putative structure of the "Nox" domain contains a cytosolic N-terminus (for Nox1 – 5 but not Duox 1 – 2), followed by six transmembrane α-helices (seven in the case of Duox 1 – 2) that are separated by three extracellular (A, C and E loops) and two intracellular loops (B and D loops). The cytosolic C-terminal region harbors the essential binding sites for FAD and NADPH, and may facilitate electron transfer via the heme coordination center embedded in the 3rd and 5th transmembrane domains by directly interacting with the B loop (figure 2) [170]. In the absence of a three-dimensional "Nox" structure, progress in understanding structural determinants of Nox function has relied on homology modeling, chimeric and mutagenesis studies, and analysis of naturally occurring Nox2 mutants identified in chronic granulomatous disease [171, 172]. These studies revealed that polybasic regions of the B loop are essential and unique for the catalytic activity of Nox2 and Nox4 [112, 170, 173]. Although replaceable among Nox family members, the D loop may control the electron transfer from FAD to O_2 in Nox2 [112, 174]. Detailed understanding of the mechanism by which the active conformation of "Nox" is created is incomplete. The constitutive activity of the Nox4-p22*phox* complex in absence of any regulatory subunits, however, may provide important insights. For example, in depth mutagenesis studies have identified structural elements that are important either for Nox4 catalytic activity or for permitting assembly of the Nox4-p22*phox* complex. Cell-free assays and transfected cell models reveal that the dehydrogenase domain located in the Nox4 C terminus, as well as the D loop, are linked to an intrinsically activated state and increased ROS production [175, 176]. Furthermore, despite not confirming higher ROS output upon expression of Nox4-Nox2 chimeras, two independent studies in which the complete C terminal region of Nox4 was replaced with the analogous Nox2 domain succeeded in transforming the constitutively active Nox4 into an inducible, phorbol ester-stimulated Nox that requires the presence of regulatory subunits [112, 175]. Studying the differences in these regions between different Nox isoforms may contribute to understanding the conformational changes that occur in the "Nox" domain leading to activation and how this activation differs from one isoform to the other.

As mentioned above, to date, Nox4-p22*phox* heterodimerization is the only known prerequisite for Nox4 catalytic activity [109]. The determinants for the formation of the Nox4-p22*phox* complex differ from those for the association of Nox1 – 3 with p22*phox*. Truncation of the p22*phox* C terminus or a mutation in the last of the presumed four p22*phox* transmembrane domains (p22*phox* Y121H) does not affect Nox4-p22*phox* heterodimerization, Nox4 maturation or ROS generation, whereas Nox1 – 3 function is reduced under these conditions [82, 177–179]. In addition, the Nox4 D loop is critically involved in creating a functional complex with p22*phox* [112]. Although at this point speculative, formation of a unique quaternary structure might be key to constitutive Nox4 activity.

Similarly, as only H_2O_2 is detected in the extracellular milieu when Duox enzymes are stimulated, distinctive features in their heterodimer assembly with DuoxA subunits may drive this phenomenon. Indeed, DuoxA isoforms, and potentially also p22*phox* and other proteins associating to these subunits, are not only crucial for Duox (or Nox) maturation and formation of a catalytically functional complex, but also seem to play a role in intracellular targeting of the assembled oxidase [141]. Thus, a combination of structural and sequential motifs, together with the geometry of the heterodimer and the utilization of regulatory

proteins, provide for individual functional dynamics and localization of Nox/Duox family members.

5. How Nox Isoforms Signal in Cells/Tissues: Compartmentalization

Most cells express multiple Nox isoforms, and in many cells, each Nox is linked to distinct cellular functions. This immediately raises the question of how enzymes that make identical products ($O_2^{\bullet -}$ and H_2O_2) can mediate different responses. The answer is most likely multifactorial, related to how their expression and activity is regulated, their subcellular localization, and the precise ratio of ROS species produced. For example, in vascular cells, as discussed above, Nox1 is found in caveolae [123] and signalosomes [125], is activated by growth-promoting stimuli such as Ang II, thrombin, prostaglandin $F_{2\alpha}$ and PDGF [76, 180–182], and produces more detectable intracellular $O_2^{\bullet -}$ than H_2O_2 [183]. In endothelial cells, Nox2 is similarly localized and growth factor-sensitive [184, 185] but is also found in the perinuclear region [118]. Of interest, in these cells, Nox-derived $O_2^{\bullet -}$ is produced both extracellularly and intracellularly [186, 187]. In contrast, Nox4 is regulated by many factors including shear stress, ER stress, mitochondrial dysfunction, hypoxia, growth factors, Ang II and transforming growth factor (TGF)-β [132, 188–191] [192–194], and H_2O_2 is mainly detected as its product [109].

As a consequence of these differences in subcellular localization, each Nox is expected to activate specific, context-dependent signaling pathways in individual cell types via localized production of ROS. Interestingly, however, neither $O_2^{\bullet -}$ nor H_2O_2 are particularly chemically reactive. This allows for the possibility of their signal being effectively transduced by other regulatory proteins in the immediate vicinity of the compartment in which they are produced. For example, these proteins could be iron-containing proteins which activate H_2O_2 or a particularly reactive protein thiol. In vascular smooth muscle cells stimulated to grow, Nox1 activates tyrosine kinase pathways downstream of Ras that include Src, the EGF receptor kinase, Akt and variably, the MAPKs [27, 195–198]. In endothelial cells, similar growth-related, anti-apoptotic pathways are regulated by Nox2 rather than by Nox1 [199]. Although not all the direct targets of Nox-derived ROS are known, some of this stimulatory effect is due to inactivation of lipid or tyrosine phosphatases [200]. Another example of compartmentalized signaling occurs when cells are exposed to pro-inflammatory cytokines, such as TNF-α or IL-1β, or microbial agonists such as LPS. In such situations, endosomal Nox1 in smooth muscle cells or Nox2 in endothelial and cardiac cells stimulates NF-κB, leading to transcription of genes encoding pro-inflammatory proteins [125, 201, 202]. Moreover, the role of Nox proteins in signaling can vary according to the phenotype of the cells. Nox4 is involved in maintaining smooth muscle differentiation [203], but is also involved in the proliferation response to urokinase plasminogen activator [204]. In endothelial cells, endoplasmic reticular Nox4-mediated activation of protein tyrosine phosphatase-1B terminates epidermal growth factor-induced signaling [205], but Nox4 regulates Erk1/2 to promote proliferation in response to serum [199]. Nox4 has also been linked to multiple signaling pathways such as AP-1, Jak/Stat, E2F, pigment epithelium-derived factor (PEDF), PKCα and p38 MAPK [192–194, 206]. Finally, Nox proteins can cooperate to modulate a given physiological response. Redox signaling in migrating smooth muscle cells is comprised of Nox1-mediated actin polymerization in lamellipodia by virtue of its activation of the cofilin phosphatase slingshot-1L phosphatase [207] and of Nox4-regulated focal adhesion turnover [165]. Thus, recognition of the cellular context is essential to an appreciation of Nox-mediated signal transduction; one must consider the cell type, the functional endpoint, the complement of Nox proteins expressed in the cell, and the tissue environment.

6. Hypertension and Nox1, Nox4 and Nox5

a. Oxidative Stress and Human Hypertension

Compelling findings from experimental and animal studies suggest a causative role for oxidative stress in the pathogenesis of hypertension and a critical role for ROS in cardiovascular remodeling and hypertension-associated target organ damage [208, 209]. Almost all experimental models of hypertension display some form of oxidative excess. Sources of ROS in experimental hypertension include Nox1, Nox2 and Nox4, xanthine oxidase, uncoupled NOS and mitochondria [5, 210]. Mice deficient in ROS-generating enzymes have lower blood pressure (BP) and Ang II dependent hypertension compared with wild-type counterparts [211, 212]. Moreover in experimental hypertension, antioxidants and Nox inhibitors attenuate vascular remodeling and the development of hypertension [213, 214].

Despite extensive data supporting a role for oxidative stress in experimental hypertension, it is unclear whether oxidative stress causes hypertension in humans, and only a few small clinical studies demonstrate a blood pressure-lowering effect of anti-oxidants, with most large anti-oxidant clinical trials failing to demonstrate any cardiovascular benefit or reduction in blood pressure [215–218]. On the other hand, NADPH oxidase polymorphisms and decreased antioxidant capacity are associated with hypertension and cardiovascular disease [219, 220]. Normotensive subjects with a family history of hypertension have greater plasma H_2O_2 levels than blood pressure – matched normotensive subjects without a family history of hypertension [216]. While most of these studies show correlations between high BP and markers of oxidative stress, a causal relationship is yet to be established. Nevertheless, evidence does suggest that ROS do play a critical role in the molecular mechanisms associated with cardiovascular and renal injury in hypertension, and that hypertension itself can trigger to oxidative stress [221].

A number of reasons for the inconsistency of clinical findings and inconclusivity of large trial data have been proposed [222, 223]. Importantly, traditional antioxidant therapies may have failed due to slow reactivity and/or bioavailability. Theoretically, agents that reduce ROS formation in a Nox-specific manner should be more efficacious than non-specific, inefficient antioxidant vitamin scavengers, as used in most clinical trials [224]. Indeed, specific Nox inhibitors as therapeutic agents are currently under development for potential clinical use. Inhibiting Nox enzymes by targeting their substrate, NADPH [225], may be another theoretically interesting strategy that would involve the use inhibitors of glucose-6-phosphate dehydrogenase, a major source of NADPH. However, since affecting the hexose monophosphate shunt may have widespread off-target deleterious effects, this approach would pose significant challenges.

b. Nox4 and Nox5 in the Vascular System

Although there has been enormous progress in the biochemical, chemical and structural characterization of Nox isoforms, the physiological role of individual Nox proteins still remains largely unknown and the exact function in pathological processes awaits clarification. One of the key areas where the biology of the Nox enzymes has been developed is the cardiovascular system where Nox1, 2, 4 and 5 are the isoforms expressed. Whereas Nox1 and 2 have been well studied in the context of vascular biology, less is known about vascular Nox4 and Nox5.

Nox4 shares only 39% homology with Nox2 and 35% homology with Nox1 [109, 192], and as mentioned above, it is constitutively active. Its localization to the endoplasmic reticulum (ER) suggests it may be involved in ER stress [167, 205] and its association with stress fibers and focal adhesions signifies its role in cell differentiation and migration [165, 203].

The biological roles of vascular Nox4 in health and disease are unclear and data are conflicting. On the one hand, Nox4 may be pro-angiogenic, anti-apoptotic and important in VSMC differentiation; and, on the other hand, it is involved in cell senescence, apoptosis, VSMC migration, and VSMC hypertrophy [226–228]. The conflicting results from these studies have not been resolved by in vivo evidence. To date, very few studies reported on Nox4−/− mice. In one study, Nox4 was shown to be important in stroke, since Nox4-deficient mice were protected against oxidative stress and neurodegeneration post acute ischemic stroke [229]. In contrast, another study on Nox4−/− mice and transgenic mice with cardiomyocyte-targeted overexpression of Nox4 revealed a protective role of Nox4 in animals subjected to chronic pressure overload [230].

Nox5, the newest member of the Nox family [96], has close homology to Nox1 and Nox2. Since it is not expressed in rodents, the study of Nox5 is challenging as mouse or rat models cannot be easily used to examine its role in vascular disease. Thus, the functional significance of vascular Nox5 is unknown, although it has been implicated in endothelial cell proliferation and angiogenesis, in PDGF-induced proliferation of VSMCs and in oxidative damage in atherosclerosis [231, 232]. Vascular Nox5 is activated by thrombin, PDGF, Ang II, ET-1 and ionomycin through PKC and cAMP response element-binding protein (CREB) [233, 234]. Thus, although our knowledge of many of the features of Nox4 and Nox5 has increased recently, there is still a paucity of information regarding the role of these oxidases in vascular biology.

7. Lung Fibrosis and NADPH Oxidase

Fibrosis of the lung is typically the result of an ongoing reparative response to injury of the alveolar epithelium and/or capillary endothelium due to injurious agents that are airborne or bloodborne, respectively. Many acute injuries, such as seen in adult respiratory distress syndrome (ARDS), resolve without significant fibrosis, whereas, chronic inorganic/organic dust exposures can result in fibrosis, as seen in asbestosis and hypersensitivity pneumonitis [235]. A particularly enigmatic form of lung fibrosis is idiopathic pulmonary fibrosis (IPF), a disease of the elderly that has not been linked to any single etiological agent. IPF carries a high mortality rate with median survival rates less than 3 years [236], and there are currently no effective therapies that have been shown to influence survival.

Oxidative stress has been implicated in the pathogenesis of IPF [237]. Although precise sources of ROS/RNS and mechanisms remain unclear, enzymatic sources of ROS that have been implicated in lung fibrosis are Nox2 and Nox4. The evidence for Nox2 in lung fibrosis comes primarily from animal models that show protection from lung injury induced by bleomycin [238] or carbon nanotubes [239] in mice with a genetic deficiency of Nox2. Studies in human subjects with IPF are limited; however, some studies have demonstrated enhanced generation of ROS from alveolar inflammatory cells, primarily macrophages, in promoting injury to alveolar epithelial cells [240, 241].

A role for Nox4 in lung fibrosis has been defined more recently. The mRNA expression of Nox4 was found to be induced by the pro-fibrotic cytokine, transforming growth factor-β1 (TGF-β1), while other Nox/Duox isoforms were unaffected [242]. Nox4 mediates myofibroblast differentiation, contractility, and extracellular matrix production in lung fibroblasts [242], and is associated with fibroblastic foci within alveolar-interstitial and vascular remodeling in human IPF lung tissues [242]. RNAi-mediated knockdown of Nox4 protects against development of lung fibrosis in the two different murine models of lung injury [242]. Another more recent study by Amara *et al*. [243] demonstrates that lung fibroblasts express Nox4 *in situ* in the fibrotic IPF lung and that Nox4 is upregulated in IPF lung tissue-derived fibroblasts. There is also emerging evidence that Nox4 may be involved

in the vascular remodeling associated with IPF [244–246]. These studies suggest that targeting of Nox4 may be an effective therapeutic strategy for IPF and potentially for hypoxia-induced vascular remodeling that leads to pulmonary hypertension [247].

8. Endothelial NADPH Oxidase in Arsenic Signaling: An Example of Environmental Vascular Pathogenesis

Among other environmental and nutritional insults, arsenic in the drinking water is a prototypical environmental hazard. It is a major worldwide public health concern, since exposure increases risks of cardiovascular and metabolic-related diseases, as well as a number of cancers. Arsenic directly affects the vasculature by enhancing angiogenesis and promoting vessel occlusion and atherogenesis [248]. However, the mechanisms that initiate arsenic signaling for pathogenic changes in the vasculature remain unclear. There is consensus from a number of reports that arsenic stimulates ROS production in vascular cells and that arsenic stimulation of Nox enzymes promotes both signaling events and toxicities in vitro [248–250]. Toxicity or vascular dysfunction may arise from generation of both H_2O_2 and peroxynitrite as arsenic stimulates both oxidant and reactive nitrogen formation [250–252]. Whereas cell culture-based studies provided a wealth of information regarding potential mechanisms for arsenic action through Nox, proof of functional consequence of this activation in arsenic-induced pathogenesis in vivo and identification of the mechanism through which arsenic activates the oxidase are lacking.

To address functional consequence in vivo, the role of Nox-derived arsenic stimulation of liver sinusoidal endothelial cell (LSEC) capillarization was investigated in genetic mouse models [253]. LSEC are critical for bulk removal of circulating lipids, lipoproteins, and modified proteins by the liver. Loss of these functions in capillarization, in LSEC differentiation, in aging and in oxidative injury may promote metabolic imbalance and age-related atherogenesis. Mice exposed to low levels (10–250 ppb) of arsenic (as arsenite) in drinking water have reduced LSEC fenestrations, as well as decreased protein and lipids scavenging by the liver. Capillarization and increased sinusoidal protein S-nitrosation are absent in arsenic-exposed $p47^{phox}$ null mice, indicating that Nox2-based oxidase activity is required for both events [253]. Ex vivo studies with primary LSEC isolates confirm the role of Nox2 in arsenic-stimulated $O_2^{\bullet -}$ generation, as this stimulation is inhibited by the Nox2 inhibitor, Nox2ds-tat peptide [253]. Furthermore, ex vivo studies with LSEC isolated from the $p47^{phox}$ null mice demonstrate that H_2O_2 is the primary oxidant signaling for defenestration [253]. Although these studies were the first in vivo confirmation that Nox2 mediates arsenic pathogenesis in the vasculature and that Nox2-derived oxidants promote capillarization, they did not identify the mechanism for arsenic stimulation of the enzyme complex. In vitro and in vivo studies indicate that arsenic causes Rac1 membrane mobilization [249, 254], and inhibition of Rac1 GTPase activity prevents arsenic-stimulated ex vivo LSEC capillarization [253]. In human microvascular lung endothelial cells (HMVEC) or LSEC treated with *Pertussis* toxin, arsenic fails to activate Rac1 GTPase activity or promote capillarization, respectively [255]. These results implicate a Gαi-protein coupled receptor in the activation pathway. A specific antagonist to the sphingosine-1-phosphate type 1 receptor (S1PR1) prevents arsenic-stimulated capillarization and $O_2^{\bullet -}$ generation in LSEC, as well as arsenic-stimulated increases in Rac1-GTPase activity, angiogenic gene expression, and in vitro tube formation in HMVEC. The primary role of S1PR1 in the actions of arsenite was confirmed with siRNA knockdown of the receptor in HMVECs [255]. These data support a mechanism in which arsenic co-opts signaling through S1P1 receptors to stimulate Rac1 and Nox2 activities required for the pathogenic ROS generation that underlies enhanced angiogenesis, vascular remodeling and endothelial dysfunction (e.g. loss of LSEC scavenging and increased peroxynitrite formation). These

pathogenic changes may contribute to the etiology of both hepatic and systemic vascular diseases that result from environmental exposure to arsenic (figure 5).

9. Examples of Oxidase/Peroxidase Interaction

a. Airway Epithelia: NADPH Oxidase and the Action of Peroxidases in Host Defense

Polymorphonuclear neutrophils (PMN) constitute the dominant circulating cell in humans and, as phagocytic cells, contribute to the immediate innate immune response to infection. Once ingested by PMN, an invading microbe is compartmentalized in a phagosome, where it is exposed simultaneously to oxidants generated *de novo* by the NADPH oxidase [103] and to proteins stored in granules that fuse with the nascent phagosome [256]. The combined actions of the granule proteins and the varied oxidants synergize to create an environment toxic to many microbes and culminate, when successful, in the death and degradation of ingested prey (reviewed in [257]). PMN contain relatively large amounts of MPO, ~ 5% of the cell by weight [258] and predicted to reach millimolar concentrations in the restricted space of the phagosome, where the volume is estimated to be ~ 1.2 femtoliters [259]. MPO is structurally and functionally unique in the peroxidase-cyclooxygenase superfamily in having three, rather than two, covalent bonds between the heme group and the protein backbone and, consequently, in catalyzing the two-electron oxidation of Cl^- at physiologic pH to generate $HOCl^-$ (reviewed in [260]). The chloride concentration in human PMN phagosomes is 73.3 ± 12.2 mM [261], with chloride delivered, in part, from the cytoplasm via the cystic fibrosis transmembrane conductance regulator (CFTR) [262]. Interestingly, CFTR is present in the secretory vesicles but not the plasma membrane of unstimulated human neutrophils [263]; during phagocytosis, secretory vesicles fuse with the nascent phagosome and thereby deliver CFTR to the membrane of the nascent phagosome. Patients with cystic fibrosis lack CFTR and their neutrophils exhibit depressed phagosomal chloride concentrations, defective chlorination of ingested targets, and depressed bacterial killing [263]. The HOCl produced by stimulated normal PMN represents as much as 72–90% of the oxygen consumed by the phagocyte NADPH oxidase [259, 264], demonstrating the extent to which phagocyte-generated oxidants supply HOCl to the antimicrobial machinery in the phagosomes. Given that HOCl targets vulnerable substrates regardless of their origin, there is extensive chlorination of host as well as microbial proteins present in the phagosome [265]. Overall, chlorination of bacteria directly correlates with the loss of microbial viability [265, 266], although identification of the critical targets that are modified remains the subject of study.

The principles of the peroxidase paradigm, exemplified by events in PMN phagosomes, apply to features of host defense in the airway. Airway epithelia constitutively produce H_2O_2 through the activity of Duox [267–269]. Goblet cells along the airway secrete LPO, which constitutes ~1% of the soluble protein in the airway lumen, thereby providing the peroxidase necessary to reduce the Duox-derived oxidants [270–272]. Airway epithelial cells also transport thiocyanate into the airway lumen, where $OSCN^-$ is generated and mediates bacteriostatic and bactericidal activities [268, 273]. Just as the combined activities of the phagocyte NADPH oxidase and MPO generate HOCl in phagosomes, Duox-derived H_2O_2 and LPO produce HOSCN in the human airway. The LPO-H_2O_2-OSCN system, earlier identified as the basis for the antimicrobial activity of saliva [274], oxidizes exposed and susceptible sulfhydryls to sulfenic acid and sulfenyl thiocyanite products, thereby compromising bacterial metabolism and viability [275].

Although the MPO- and LPO-mediated systems parallel each other in principle, significant differences exist. HOCl is a potent oxidant; for example, the k_2 for HOCl reaction with free cysteines is 3.0×10^7 M^{-1} s^{-1} vs. 2.9 M^{-1} s^{-1} for H_2O_2 at pH 7.4 [276–278]. In contrast, HOSCN is a relatively weak oxidant, reacts primarily with sulfhydryls, and promotes

modifications that are reversible in the absence of continuous SCN^- or in the presence of reductants [275]. As a consequence of its nonselective reactivity, HOCl can mediate irreversible damage to targets, including both those on host and microbial proteins. HOSCN, on the other hand, because of its lower reactivity and more limited spectrum of susceptible substrates, can mediate more selective and reversible changes. In some cellular systems, the ED_{50} of HOSCN for host tissue is ~ 10 times higher than that for microbes, although it should not be assumed that the oxidant is uniformly nontoxic to tissues. HOSCN can oxidize intracellular thiols, thereby inhibiting thiol-dependent enzymes and compromising key metabolic activities and cell viability [[279, 280] and reviewed in [281]]. With that caveat in mind, there are data suggesting that SCN may blunt MPO-dependent cytotoxicity towards host cells and thereby serve as a protective agent against the oxidant damage in the airway of patients with excess inflammation, such as seen in patients with cystic fibrosis [282]. Several mechanisms may provide relative protection to host tissue in an inflamed airway where both the LPO- and MPO-dependent systems coexist. As LPO consumes H_2O_2, there will be less available to MPO for HOCl production. Low concentrations of SCN^- compete effectively with Cl^- for reaction with MPO, and concentrations above 100 μM decrease HOCl production and MPO-mediated cytotoxicity [282]. Lastly, SCN^- directly and rapidly reduces OCl^- in solution [283], thereby consuming the potentially damaging oxidant. Taken together, these reactions may provide a means by which innocent bystander damage in the airway due to HOCl produced by activated PMN recruited into the airway can be attenuated.

b. Vascular Xanthine Oxidoreductase: A Molecule in Need of Restraint or Nitrite?

Xanthine oxidase (XO) is a critical source of reactive oxygen species contributing to vascular inflammation. However, reports of vascular production of ROS frequently assume XO as the $O_2^{\bullet-}$-producing form of xanthine dehydrogenase (XDH) and, if mentioned, H_2O_2 is merely a secondary byproduct of spontaneous or enzymatic dismutation of $O_2^{\bullet-}$. A crucial concept often overlooked is that XO-mediated $O_2^{\bullet-}$ production is directly proportional to O_2 concentration and pH. 100% $O_2^{\bullet-}$ production is achieved only at conditions of 100% O_2 saturation and pH 10 [284]. Under physiological conditions (21% O_2 and pH 7.4), however, XO-catalyzed O_2 reduction results in 72% of the product being H_2O_2 and only 28% being $O_2^{\bullet-}$. Moreover, under hypoxic conditions (1% O_2 and pH 7.4), XO-generated $O_2^{\bullet-}$ is decreased to 10% and H_2O_2 formation is increased to 90% [284, 285]. This O_2-dependent effect on the relative proportions of H_2O_2 and $O_2^{\bullet-}$ generated by XO is enhanced ~30% when XO is immobilized on endothelial glycosaminoglycans (GAGs) resulting in further diminution of XO-derived $O_2^{\bullet-}$ formation [285]. Therefore, under inflammatory conditions where both vascular O_2 and pH are significantly decreased while XO is released into the circulation and bound to endothelial GAGs, XO production of $O_2^{\bullet-}$ is greatly diminished, resulting in almost exclusive production of H_2O_2. The impact of these findings is exemplified in myograph studies where perfusion with 95% O_2 is standard procedure. In this severely hyperoxic setting, $O_2^{\bullet-}$ formation is favored producing results and conclusions that overshadow the role of XO-derived H_2O_2. This is critical as H_2O_2 differs greatly from $O_2^{\bullet-}$ with regard to mobility and reactivity. For example, the negative charge on $O_2^{\bullet-}$ limits its ability to traverse lipid bilayers as well as react with DNA whereas can H_2O_2 freely diffuse through membranes where it can participate in a myriad of both autocrine and paracrine cellular signaling events [286]. *In toto*, this new perspective of XOR biochemistry demands a more critical evaluation of XO-derived ROS with attention focused on H_2O_2 under clinically-relevant O_2 conditions.

Contrary to its deleterious effects as a source of ROS, emerging evidence has revealed a nitrite reductase activity of XO and, as such, identified XO as a source of $^{\bullet}NO$ under hypoxic/inflammatory conditions. For example, under strictly anoxic conditions, using xanthine or NADH as reducing substrate, XO catalyzes the single electron reduction of

NO_2^- to •NO at the molybdenum cofactor [287–289]. This evidence suggests a potentially beneficial role of XO as an •NO donor. However, the following major issues must be critically evaluated before an XOR-dependent mechanism for •NO generation from NO_2^- can be determined to be significant *in vivo*:

1) *substrate concentration*-reports of XOR-mediated •NO formation utilize excessive concentrations (1–50 mM) of NO_2^-, while tissue levels of NO_2^- rarely rise above 300 nM [290],

2) *O_2 concentration*-all reports to date demonstrate XOR-driven •NO formation under anoxic conditions, while these conditions rarely exist for any appreciable time in arteries and arterioles,

3) *allopurinol*-identification of XOR-catalyzed •NO formation in tissues has been accomplished solely by inhibition with allopurinol without verification with alternative XOR-specific inhibitors, such as febuxostat. If XOR-mediated •NO production is functionally significant and beneficial, then inhibition of this process should produce undesirable outcomes. This is in contrast to numerous studies reporting positive effects of XOR inhibition with allopurinol under similar conditions. Thus, while it appears that XOR can mediate the reduction of NO_2^- to •NO in vitro, the in vivo significance of this finding is uncertain at present. Therefore, studies to delineate the relative impact of XOR-dependent generation of ROS vs. the potential beneficial effects of XOR-catalyzed •NO formation are crucial, and the interplay between these potential products and Nox activity would present a very interesting area for investigation (figure 6).

c. Regulation of Vascular Nox by Heme Oxygenase and Carbon Monoxide

A major product of heme oxygenase is CO, which acts as a heme iron ligand and forms complexes with hemoproteins and metalloenzymes. At low concentrations, CO has profound effects on intracellular signaling processes, including anti-inflammatory, antiproliferative, antiapoptotic and anticoagulative effects [154]. Indeed, studies have shown that CO may inhibit NADPH oxidase activity in lung smooth muscle cells [291], neutrophils [292] and macrophages [293]. Recently it was demonstrated that induction of HO-1 expression in rat aortic smooth muscle cells inhibits PDGF-induced migration [294], a process integral to the development of atherosclerosis and restenosis [295]. CO, but neither biliverdin nor bilirubin, inhibits Nox1-dependent PDGF-stimulated migration in RASMC by directly interacting with the enzyme. Inhibition of Nox1 activity by CO correlates with decreased phosphorylation of Akt, and the MAPKs p38, Erk1/2, and JNK1. Taken together, these studies reveal a novel mechanism by which increased HO-1 expression and activity, and HO-1-derived CO may mediate their beneficial effects in arterial inflammation and injury [294].

10. New Concepts in Studying ROS in Cardiovascular and Lung Disease: Mitochondrial reserve capacity, bioenergetics and therapeutic implications

Mitochondria are not only a source of ROS, but also represent a major target for ROS within the cell. For example, mitochondrial DNA is known to be damaged prior to nuclear DNA by cellular oxidative stress [296]. Additionally, multiple studies associate oxidative stress with changes in bioenergetics, and this mechanism is thought to underlie the pathogenesis of a number of diseases, including ischemia/reperfusion injury and cardiac failure [297, 298]. The importance of understanding the relationship between oxidative stress and mitochondrial bioenergetics is further highlighted by studies suggesting that specific isoforms of Nox may be localized within or in close proximity to the mitochondrion [135,

136]. Traditional studies of the effects of oxidative stress on mitochondrial function have focused on examining modifications of individual proteins within the mitochondria. However, with the advent of new technology, it is now possible to study bioenergetics as a whole in intact cells. Emerging from this new line of study is the concept that mitochondrial bioenergetic reserve capacity is important in regulating cellular response to oxidative stress [299]. In basal physiological conditions, cells respire at a rate that is approximately 20–50% of their maximal capacity. Reserve capacity of mitochondria is defined as the difference between the maximal and basal rates of respiration. Emerging data demonstrate that the amount of mitochondrial reserve capacity determines the response of a given cell to oxidative insults, such as the electrophilic lipid 4-hydroxynonenol or hydrogen peroxide [300]. Interestingly, oxidative and nitrosative stresses deplete the mitochondrial reserve capacity of the cell, and thereby decrease its ability to respond to stress [299]. The mitochondrion is now emerging as a center in which ROS-signaling can be integrated with cellular metabolism and the program leading to cell differentiation or death through apoptotic mechanisms. A recent concept that has emerged is that specific oxidation of the phospholipid cardiolipin by cytochrome c exposed to hydrogen peroxide can modulate the apoptotic process [301, 302]. Clearly, the basal bioenergetic status and extent of the mitochondrial reserve capacity are likely determinants of whether cells initiate a ROS-dependent proliferation or cell death signaling. Interestingly, aging and chronic inflammatory conditions generate ROS/RNS that deplete the reserve capacity which has been proposed as an underlying contributory factor to the bioenergetic defects associated with cardiovascular and neurodegenerative diseases.

11. Nitrated Fatty Acids: New Anti-Inflammatory Signaling Mediators

Electrophilic fatty acids mediate post-translational protein modifications (PTPMs) and serve as potent anti-inflammatory cell signaling mediators. Two classes of electrophilic fatty acids, nitro- and keto-derived fatty acids, have recently been identified. Electrophilic fatty acid oxidation products (EFOX) are α, β-unsaturated keto derivatives derived from omega-3 fatty acids and generated by a cyclooxygenase-2 (COX-2)-catalyzed mechanism in activated macrophages [303]. Electrophilic nitro-fatty acid (NO_2-FA) derivatives are abundant bioactive oxides of nitrogen in blood and tissues. These electrophilic NO_2-FAs are formed by oxidative inflammatory reactions that involve redox reactions of $^{\bullet}NO$ and nitrite [304]. More recent data reveal that these electrophilic fatty acids can also be generated under mild acidic conditions in the presence of nitrite and unsaturated fatty acids (*in preparation*). Both classes of electrophilic fatty acids react with nucleophilic targets and thereby alter protein structure, function and cellular distribution through PTPMs.

The PTPMs induced by electrophilic fatty acids modulate the expression of mRNA and proteins under the regulation of Nrf2/Keap1 [305], NF-κB [306] and PPARγ [307]. Both EFOX and NO_2-FA derivatives activate phase II gene transcription through the Nrf2/Keap1 pathway. These electrophilic derivatives target highly reactive cysteines on Keap1, thereby liberating Nrf2 and resulting in nuclear translocation, binding to antioxidant response elements (ARE) and activation of phase II genes such as HO-1 [308–311]. Additionally, EFOX and NO_2-FAs inhibit LPS-induced NF-κB activation through similar electrophilic interactions. The dimeric interaction between p50 and p65 subunits of NF-κB promotes the progression of inflammation. The electrophilic fatty acid derivatives inhibit NF-κB activity by alkylating the p65 subunit. This electrophilic alkylation prevents NF-κB binding to promoter regions of inflammatory genes and thus exerts potent anti-inflammatory effects [303, 306]. Both EFOX and NO2-FA derivatives bind to a highly conserved cysteine residue in the PPARγ ligand-binding domain and thereby transactivate PPAR-responsive genes [303, 312]. These electrophilic fatty acid signaling mediators regulate metabolism and inflammation through PPAR activation [304, 311]. The clinically-relevant manifestations of

the anti-inflammatory actions of these electrophilic nitro- and keto-derived fatty acid derivatives have recently been described using several murine models of disease. The functional outcomes of these electrophilic fatty acids have been shown to reduce atherosclerosis in an apoE−/− model [313], inhibit neointima formation in a model of restenosis [314], mediate protective effects in a murine model of focal cardiac ischemia and reperfusion injury [315] and normalize blood glucose levels in *ob/ob* mice in a model of type 2 diabetes [316]. These findings are summarized in figure 7.

12. Summary and Future Directions

An emerging principle in cellular responses to various stimuli is the cross-talk between what initially were thought to be distinct signaling pathways. Oxidases are no exception. The different sources, localization and temporal patterns of oxidases' actions determine the final outcome in both physiological and pathophysiological conditions. Indeed, oxidases, especially Nox proteins, are involved in a plethora of pathophysiological conditions. We have highlighted only a few of these conditions here, showing that different Nox isoforms signal differently, and that they play an important role in hypertension, lung fibrosis and arsenic poisoning. Importantly, the roles of Nox proteins in different tissues are influenced by the activity of other oxidases and peroxidases, such as myeloperoxidase, heme oxygenase and xanthine oxidase. New discoveries on mitochondrial bioenergetic profiles and reserve capacity help in enriching our understanding of the role of mitochondria in regulation of cellular redox status, possibly providing the opportunity for the study of more elaborate regulation mechanisms between mitochondria and Nox proteins. Furthermore, the emergence of electrophilic fatty acids as anti-inflammatory agents with therapeutic implications and the advent of new approaches to regulating oxidases will further our knowledge of their actions and interactions, and more importantly, will pave the way for the development of novel therapeutic strategies. Technological advances in measurement of oxidase activity have contributed and will continue to contribute to this goal.

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14. List of abbreviations

ROS	Reactive oxygen species
Nox	NADPH oxidase
$O_2^{\bullet-}$	Superoxide
H_2O_2	Hydrogen peroxide
RNS	Reactive nitrogen species
$^{\bullet}NO$	Nitric oxide
$^{\bullet}OH$	Hydroxyl radical
$ROO^{\bullet}$	Peroxyl radical
$RO^{\bullet}$	Alkoxyl radical
O_3	Ozone
$^1O_2$	Singlet oxygen

SOD	Superoxide dismutase
NOS	Nitric oxide synthase
GSH	Glutathione
Trx	Thioredoxin
AngII	Angiotensin II
PDGF	Platelet-derived growth factor
TNF-α	Tumor necrosis factor-α
LPS	Lipopolysaccharide
PI3K	Phosphatidylinositol-3 kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
Ask-1	Apoptosis signal-regulating kinase-1
PTP1B	Protein tyrosine phosphatase 1B
SHP-2	Src homology 2-containing inositol phosphatase 2
NF-κB	Nuclear factor κB
AP-1	Activator protein 1
CGD	Chronic granulomatous disease
FAD	Flavine adenine dinuculeotide
MPO	Myeloperoxidase
EPO	Eosinophil peroxidase
LPO	Lactoperoxidase
TPO	Thyroid peroxidase
XOR	Xanthine oxidoreductase
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
HO-1	Heme oxygenase 1
CO	Carbon monoxide
poldip2	Polymerase [DNA-directed] delta-interacting protein 2
TGF-β/1	Transforming growth factor-β/1
PEDF	Pigment epithelium-derived factor
CREB	cAMP response element-binding protein
ARDS	Adult respiratory distress syndrome
IPF	Idiopathic pulmonary fibrosis
S1PR1	Sphingosine-1-phosphate type 1 receptor
EFOX	Electrophilic fatty acid oxidation products
COX-2	Cyclooxygenase-2

NO_2-FA Nitro-fatty acid

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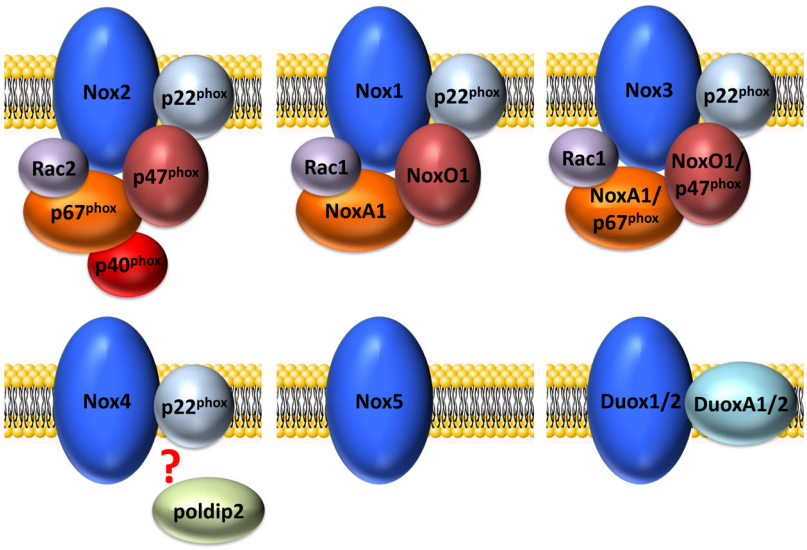

Figure 1. Current models of the active Nox complexes

Each of the 7 members of the Nox family requires a different set of conditions and protein associations. This figure shows the cytosolic subunits that are beleived to assemble onto each Nox isoform in the active oxidase state.

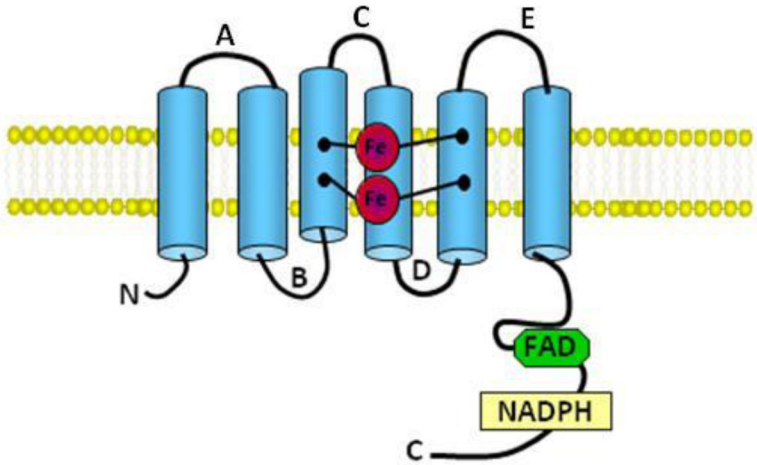

Figure 2. Schematic representation of common Nox features

The "Nox" portion of each of the Nox family members contains a similar "Nox" component. Shown here are the key features in common for all Nox family members. The letter designations A – E signify the cytosolic/extracellular loop domains. Shown also are the heme binding sites (Fe) and the FAD and NADPH binding domains.

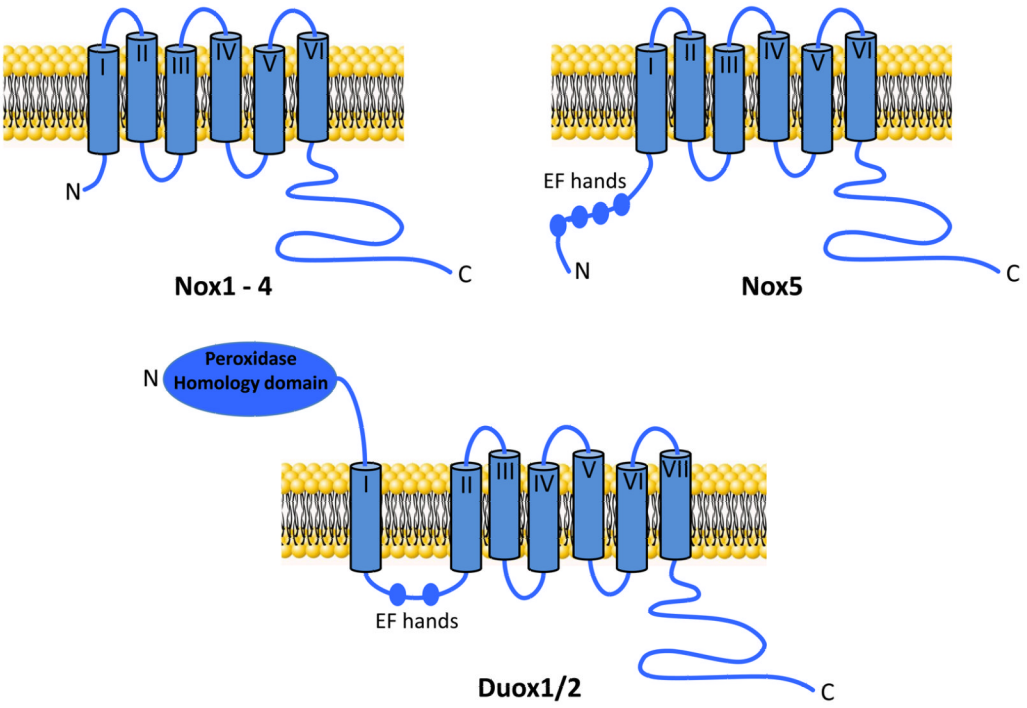

Figure 3. Outline of the structure of Nox family members

Shown are the structural elements of the different members of the Nox family. The transmembrane domains are numbered I to VII. Shown are the calcium binding motifs (EF hands) and the peroxidase homology domain of Duox1/2.

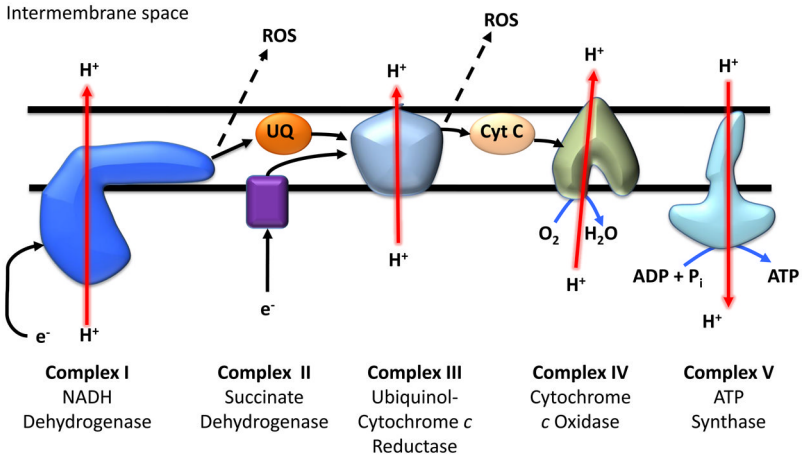

Figure 4. The mitochondrial electron transport chain

This simplified diagram of the electron transport chain on the inner mitochondrial membrane shows the direction of electron (e^-) flow along the chain (black arrows) and the direction of flow (red arrows) of hydrogen ion (H^+) across the mitochondrial membrane. Dotted arrows show ROS production as a result of the electron leak. UQ refers to ubiquinone; Cyt C refers to cytochrome *c*.

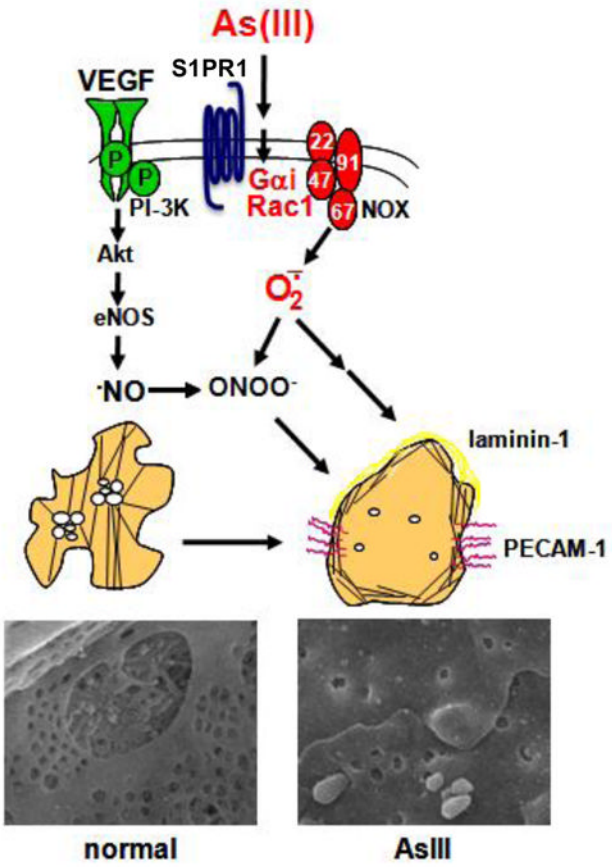

Figure 5. Arsenic-stimulated capillarization of fenestrated liver sinusoidal microvessels

A model for the proposed mechanism of arsenic (As) action via Nox2 to induce phenotypic changes. SEM images are of liver sinusoids from mice drinking normal water or water containing 100 ppb arsenite for two weeks (modified from [322]).

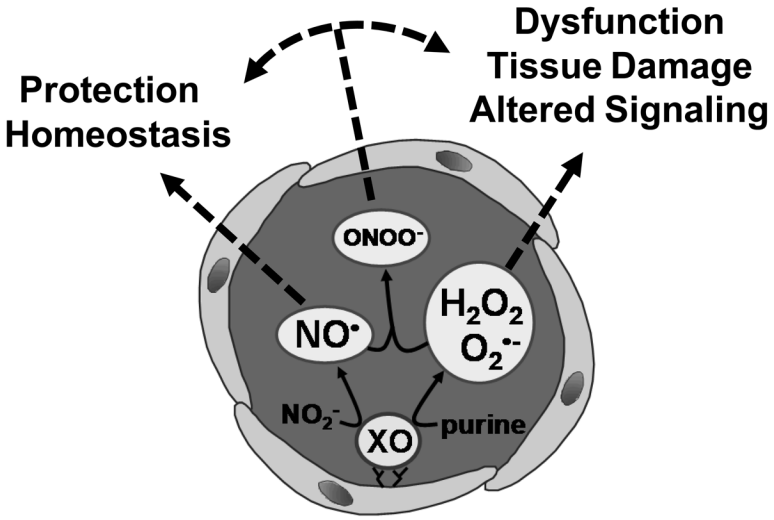

Figure 6. Scheme of xanthine oxidase-derived reactive intermediates

Xanthine oxidase (XO) may produce ROS or •NO thereby contributing to either tissue dysfunction or protection, or both.

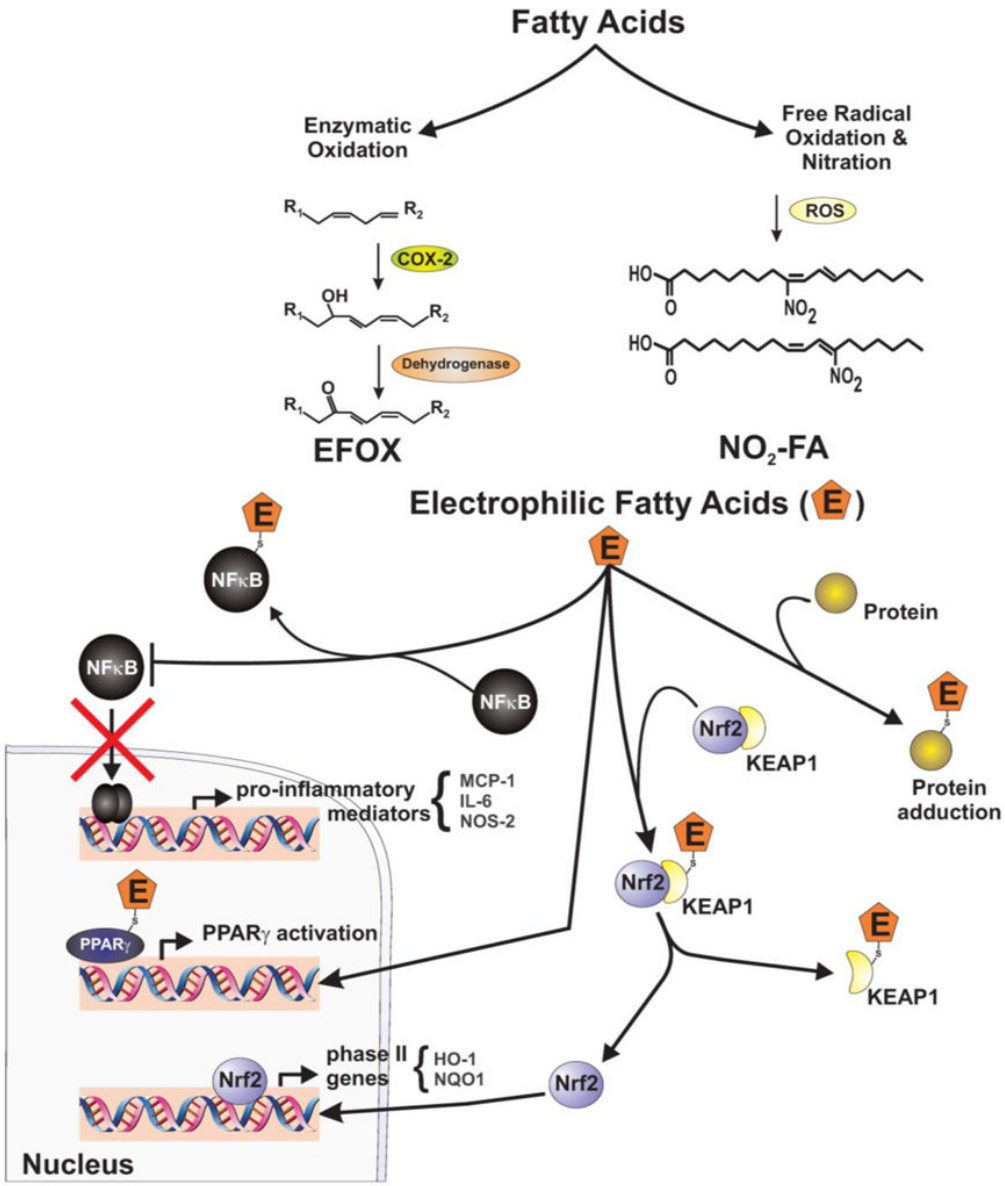

Figure 7. Electrophilic fatty acids are potent anti-inflammatory cell signaling mediators

They are generated from fatty acids through enzymatic and non-enzymatic pathways, and modulate transcription factors, regulating inflammation and metabolism. Electrophilic fatty acids 1) adduct to specific cysteine residues on NFκB, resulting in inhibition of pro-inflammatory cytokine expression; 2) bind to PPARγ and transactivate downstream responsive genes; and 3) adduct to highly reactive cysteines on KEAP1 releasing Nrf2, causing its nuclear translocation, binding to ARE and activation of phase II genes.

Table 1

Tissues Distribution and Subcellular Localization of Nox Proteins

Nox Isoform	Cellular Distribution	Subcellular Localization	References
Nox1	Colon epithelium, vascular smooth muscle cells, endothelial cells, osteoclasts, reproductive organs	Intracellular membranes close to ER, endosomes, signalosome, caveolae	[119, 120], [70, 121, 122], [123, 124], [125]
Nox2	neutrophils, macrophages, endothelial cells, vascular smooth muscle cells, fibroblasts, skeletal muscle cells, cardiomyocytes and hepatocyte	Cell membrane, phagosome, perinuclear	[4, 72, 80, 104,113 – 118]
Nox3	Inner ear (vestibular system, cochlea), skull, brain, fetal tissues	Plasma membrane	[82, 90, 317, 318]
Nox4	Kidney, vascular smooth muscle cells, fibroblasts, hematopoietic stem cells, osteoclasts, neurons, endothelial cells	Focal adhesions, ER, nucleus, mitochondria	[113,123,127 – 133], [134]
Nox5	vascular smooth muscle, endothelial cells, bone marrow, lymph nodes, spleen, reproductive tissues, stomach, pancreas, fetal tissues	Plasma membrane, ER	[85, 96, 107, 137 – 139, 319 – 321]
Duox1/2	Thyroid, airway epithelia, prostate, digestive system (Duox2)	Apical membrane	[93, 94,140]