NAD(P)H Oxidase–Dependent Self-Propagation of Hydrogen Peroxide and Vascular Disease

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Abstract—Excessive production of reactive oxygen species in the vasculature contributes to cardiovascular pathogenesis. Among biologically relevant and abundant reactive oxygen species, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) appear most important in redox signaling. Whereas $O_2^{\cdot-}$ predominantly induces endothelial dysfunction by rapidly inactivating nitric oxide ($NO^{\cdot}$), H_2O_2 influences different aspects of endothelial cell function via complex mechanisms. This review discusses recent advances establishing a critical role of H_2O_2 in the development of vascular disease, in particular, atherosclerosis, and mechanisms whereby vascular NAD(P)H oxidase–derived H_2O_2 amplifies its own production. Recent studies have shown that H_2O_2 stimulates reactive oxygen species production via enhanced intracellular iron uptake, mitochondrial damage, and sources of vascular NAD(P)H oxidases, xanthine oxidase, and uncoupled endothelial nitric oxide synthase (eNOS). This self-propagating phenomenon likely prolongs H_2O_2-dependent pathological signaling in vascular cells, thus contributing to vascular disease development. The latest progress on Nox functions in vascular cells is also discussed [Nox for *N*AD(P)H *ox*idases, representing a family of novel NAD(P)H oxidases]. **(*Circ Res*. 2005;96:818-822.)**

Key Words: reactive oxygen species ■ hydrogen peroxide (H_2O_2) ■ endothelial function ■ vascular NAD(P)H oxidases ■ Nox ■ uncoupled endothelial nitric oxide synthase (eNOS) ■ atherosclerosis

Vascular NAD(P)H oxidase–dependent overproduction of reactive oxygen species contributes to pathogenesis of cardiovascular diseases.[1–5] Among biologically relevant and abundant reactive oxygen species, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) appear most important in redox signaling. Whereas $O_2^{\cdot-}$ primarily modulates vascular function by rapidly inactivating $NO^{\cdot}$ (reviewed by Cai and Harrison),[6] H_2O_2 impacts on vascular function via complex mechanisms. Ambient production of H_2O_2 at low levels, likely maintained by pre-assembled NAD(P)H oxidases,[3] is necessary for endothelial cell growth and proliferation (reviewed by Griendling and Harrison; Eyries and colleagues).[7,8] Under pathological conditions, however, agonists-provoked activation of vascular NAD(P)H oxidases produces H_2O_2 in large quantities, which in turn amplifies its own production, resulting in compensatory or detrimental consequences. For instance, H_2O_2 is either compensatorily responsible for endothelium-dependent vasodilatation in hypertension where $NO^{\cdot}$ is substantially reduced,[9] or over the long term detrimentally involved in vascular smooth muscle cell proliferation and hypertrophy.[10–12] At biochemical levels, H_2O_2 signals by oxidizing low pK_a cysteine residues in protein phosphatases (reviewed by Rhee et al).[13,14] The current brief review complements previous reviews to discuss for the first time recent advances establishing the critical role of H_2O_2 in vascular disease development and mechanisms whereby vascular NAD(P)H oxidase-derived H_2O_2 amplifies its own production.

Hydrogen Peroxide and Vascular Disease

Though reactive oxygen species are clearly involved in vascular pathogenesis, the specific, individual reactive oxygen species that is most important in pathological signaling remains to be identified. Nevertheless, selectively overproducing or removing H_2O_2 in rodents was found highly influential of atherosclerotic development. Mice overexpressing NAD(P)H oxidase subunit $p22^{phox}$ (first developed by Dr David Harrison's group at Emory University) had markedly increased atheroma formation in a carotid ligation model.[15] This response was associated with enhanced H_2O_2 production in the vessel wall, and was abolished by scavenging H_2O_2 with ebselen, implicating a critical role of H_2O_2 in atherogenesis.[15] Parallel studies using different animal models and catalase scavenging of H_2O_2 from another group confirmed the same notion,[16] offsetting the concern that ebselen also removes peroxynitrite. Yang et al cross bred transgenic mice overexpressing Cu/Zn-SOD or catalase with mice deficient in apolipoprotein E ($apoE^{-/-}$), to examine a specific role of

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H_2O_2 versus $O_2^{\cdot-}$ in atherogenesis.[16] They found that whereas overexpressing Cu/Zn-SOD had no effect on atherosclerotic lesion formation in apoE$^{-/-}$ mice, overexpression of catalase or cooverexpression of catalase and Cu/Zn-SOD markedly retarded atherosclerosis in many aspects including lesion severity, lesion size, and area of affection throughout the aortic tree.[16] These observations were consistent with the findings by Tribble et al that overexpression of Cu/Zn-SOD failed to prevent atherosclerosis in high-fat diet–fed apoE$^{-/-}$ mice.[17] Taken together, these data indicate that H_2O_2 is more atherogenic than $O_2^{\cdot-}$. Of interest, the protective effects of catalase overexpression were found independent of plasma lipids.[16] One may argue that Cu/Zn-SOD is intracellular, and that the scavenging of $O_2^{\cdot-}$ by extracellular SOD (ecSOD) to prevent $NO^{\cdot}$ degradation during its trafficking to vascular smooth muscle is more relevant to atheroprotection. Indeed, evidence gained from ecSOD-null mice and adenovirus-mediated overexpression of ecSOD supports that ecSOD is the main determinant of $NO^{\cdot}$ bioavailability in the vessel wall and is thus involved in blood pressure regulation.[18,19] However, the impact of ecSOD overexpression on atherosclerosis is not yet reported. On the other hand, Sentman and colleagues found that mice deficient in ecSOD developed similar atherosclerotic lesions compared with wild-type mice.[20] Therefore, whereas $O_2^{\cdot-}$ is important in directly modulating $NO^{\cdot}$ bioavailability and serving as the precursor for H_2O_2,[6] relatively lasting H_2O_2 seems more important in mediating atherogenic signaling.

Of note, different from $O_2^{\cdot-}$ that is charged, hardly permeable, and extremely short-lived, H_2O_2 produced either intracellularly, within mitochondria, or at extracellular space is uncharged, relatively longer-lived, and freely diffusible. As for $NO^{\cdot}$, this property makes H_2O_2 an ideal signaling molecule. On the other hand, intracellular scavenging of H_2O_2 with ebselen or catalase could have removed H_2O_2 from all these sources. It thus remains unclear whether localized production of H_2O_2 at certain cellular compartment or vascular space is required in atherogenic signaling.

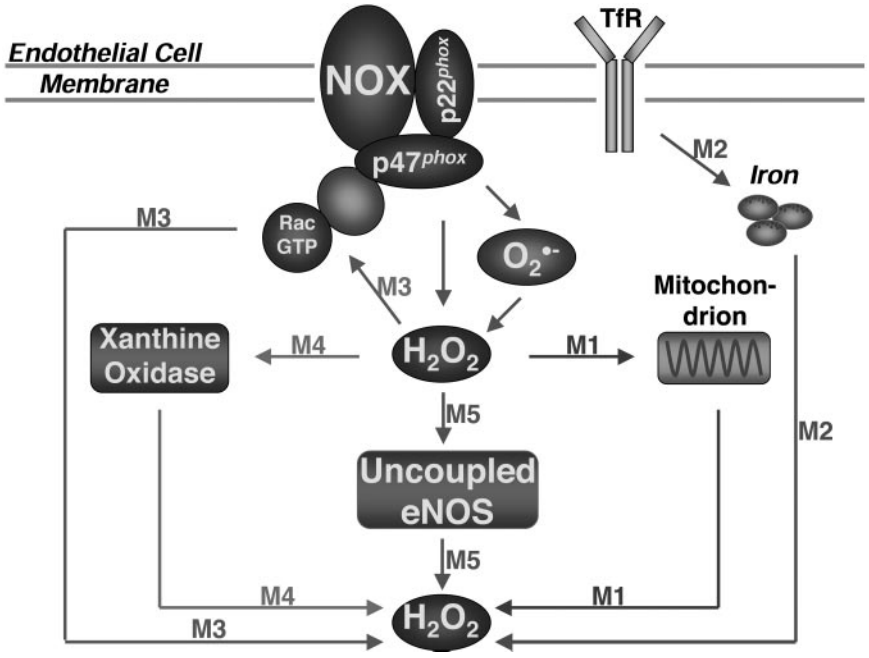
Interestingly, besides intracellular autocrine signaling, the capacity of diffusing among adjacent cells enables H_2O_2 for paracrine signaling. Of note, H_2O_2 produced by vascular smooth muscle can diffuse to endothelium to regulate endothelial cell function. For example, Laude et al recently showed that H_2O_2, produced in vivo in mice overexpressing $p22^{phox}$ in vascular smooth muscle, upregulates eNOS gene expression,[21] confirming our previous in vitro observations that H_2O_2 potently upregulates eNOS expression.[22,23] These data also indicate that H_2O_2, derived from adjacent vascular cells, is able to modulate endothelial function, further supporting a unique signaling role of H_2O_2 in the vasculature.

Hydrogen Peroxide Signaling and Vascular Function

Numerous signaling cascades are activated by H_2O_2 to mediate changes in vascular function including endothelial overgrowth,[7,8] angiogenesis,[24] smooth muscle proliferation and hypertrophy,[25] endothelial barrier dysfunction and cytoskeleton reorganization,[26,27] endothelial apoptosis,[28] induction of inflammatory proteins,[29] endothelium–leukocyte interaction, and vascular remodeling.[30,31] H_2O_2 potently activates MAPK members ERK1/2, p38MAPK, JNK, and ERK5 in both vascular endothelial and smooth muscle cells.[32–36] Our recent studies indicate that H_2O_2 activation of ERK1/2 and p38MAPK in endothelial cells requires CaMKII.[37] Receptor tyrosine kinases such as those for EGF, PDGF, FGF, VEGF,[38] and non-receptor tyrosine kinases such as JAK2,[22,39] Src,[33] Cas,[35] FAK, and Pyk2,[40,41] are responsive to H_2O_2 in vascular cells and often lie upstream of MAPK. Axl is a novel receptor tyrosine kinase identified in vascular smooth muscle, and its activation by H_2O_2 mediates neointima formation after vascular injury.[42,43] In addition, mitochondrial function was recently found necessary for H_2O_2-induced growth factor transactivation.[44] Redox-sensitive transcriptional factors including NFκB, AP-1, and HIF-1α are often activated via MAPK to modulate changes in gene expression and cellular function.[3,28] Phosphorylation-dependent posttranslational regulation of proteins also occurs in response to H_2O_2. For example, we and others have shown that H_2O_2 induces PI3-Kinase/Akt-dependent phosphorylation of eNOS, leading to a compensatory, transient increase in $NO^{\cdot}$ production,[36,45] which may serve as an intermediate step for long-term detrimental consequences.[46] Of note, many protein kinases are indirectly activated, subsequent to H_2O_2 inactivation of protein phosphatases.[13,14]

Mechanisms Underlying Hydrogen Peroxide Self-Propagation

Emerging evidence has demonstrated that uniquely, H_2O_2 is able to amplify its own production in vascular cells, and this phenomenon likely contributes to its long-lasting pathological effects. To date, at least 5 different mechanisms potentially underlie self-propagation of H_2O_2 (Figure). Earlier studies demonstrated that extra-mitochondrial H_2O_2 can induce mitochondrial DNA damage, destroying respiratory enzymes to produce reactive oxygen species.[47] Secondly, transferrin receptor (TfR)-dependent endothelial iron uptake is augmentable by H_2O_2, amplifying intracellular H_2O_2 formation to induce apoptosis.[48] Mitochondrial iron uptake can also be upregulated by H_2O_2.[49] Thirdly, in vascular smooth muscle and fibroblasts, NAD(P)H oxidase–derived H_2O_2 is capable of feed-forwardly activating NAD(P)H oxidase itself.[50] In endothelial cells, H_2O_2 was recently found capable of upregulating $p22^{phox}$ expression.[51] Likewise, McNally et al recently showed that in endothelial cells, oscillatory shear stress activation of NAD(P)H oxidases lies upstream of xanthine oxidase–dependent production of H_2O_2.[52] Last but not least; endothelial NAD(P)H oxidase–derived H_2O_2 mediates agonists-provoked tetrahydrobiopterin deficiency to induce eNOS uncoupling.[52a] This seems consistent with earlier findings that uncoupled eNOS lies downstream of vascular NAD(P)H oxidases in hypertension, and likely also, in diabetes.[9,53] Thus H_2O_2, originated by vascular NAD(P)H oxidases, propagates its own production via enhanced intracellular iron uptake, and sources of mitochondria, NAD(P)H oxidases, xanthine oxidase, and uncoupled eNOS. These feed-forward mechanisms form a vicious circle to amplify and sustain H_2O_2 production in large quantities, contributing to pathological signaling.



Mechanisms underlying NAD(P)H oxidase–dependent self-propagation of H_2O_2 in vascular cells. M1, H_2O_2 causes mitochondrial damage to produce reactive oxygen species; M2, H_2O_2 promotes transferrin receptor-dependent intracellular iron uptake to potentiate its own production; M3, H_2O_2 feed-forwardly stimulates reactive oxygen species generation from vascular NAD(P)H oxidases; M4, endothelial NAD(P)H oxidase is required for xanthine oxidase oxidation and activation, and subsequently H_2O_2 amplification in response to oscillatory shear stress; M5, uncoupled eNOS lies downstream of vascular NAD(P)H oxidases to propagate H_2O_2 production.

Vascular NAD(P)H Oxidases Origination of Hydrogen Peroxide

As discussed above, activation of vascular NAD(P)H oxidases is rate-limiting in H_2O_2 amplification of its own production.[1–5,54] Molecular activation of NAD(P)H oxidases in vascular smooth muscle has been elegantly reviewed.[1–5,54] In endothelial cells, though much to be learned, $p47^{phox}$ is confirmed to be critical in modulating enzymatic activity by interacting with catalytic unit $gp91^{phox}$ (Nox2, Nox for NAD(P)H oxidases, representing a family of novel NAD(P)H oxidases).[55–58] Studies using deficient mice or inhibitory peptide (gp91ds-tat) targeting Nox2 have established an essential role of Nox2 in producing reactive oxygen species in endothelial cells.[56,57,59,60] Functions of other newly identified $gp91^{phox}$ homologues (Nox1, Nox4 and Nox5), however, remain obscure but are under intensive investigation. A recent study reported that Nox4 is more abundantly expressed in endothelial cells compared with other Nox proteins, representing the major catalytic unit of the endothelial NAD(P)H oxidase that is activated by growth halting.[61,62] Nox1, on the other hand, was upregulated by oscillatory shear stress, mediating reactive oxygen species–dependent leukocyte adhesion to endothelium.[63] In addition, VEGF receptor–dependent activation of Nox1 was angiogenic, responsible for tube formation of endothelial cells.[64] The observations that Nox1 mediates growth signaling whereas Nox4 is growth suppressive in endothelial cells seems similar to what has been observed in vascular smooth muscle.[65,66] It is puzzling why the same reactive oxygen species–producing Nox proteins mediate different cellular responses. One possibility is that each Nox protein functions specifically based on their unique subcellular localization and tight regulation by different agonists.[1] For example, in vascular smooth muscle cells, Nox1 localizes to caveolae whereas Nox4 is found in focal adhesions.[67] In endothelial cells, however, Nox4 was found at endoplasmic reticulum[62] whereas Nox2 is localized to perinuclear cytoskeletal structure.[56]

Novel homologues of Nox-regulating proteins $p47^{phox}$ and $gp67^{phox}$ have been identified in epithelial cells ($p41^{phox}$ and $p51^{phox}$, respectively), serving as potent positive regulators for Nox1.[68–71] Duox1 and Duox2 are longer Nox proteins with peroxidase tails,[72,73] which have been shown to produce H_2O_2 in epithelial cells.[74,75] These proteins are studied for their presence and function in endothelium and vascular smooth muscle. Besides Nox, $p22^{phox}$ presents the only other membrane component of the vascular NAD(P)H oxidases. Overexpression of $p22^{phox}$ led to upregulation of Nox1 and Nox4 in vivo, likely via stabilization of proteins.[21] Recent studies have elegantly characterized physical interactions between Nox (Nox1 and Nox4) and $p22^{phox}$, and the functional, physiological consequences of these interactions regarding $O_2^{\cdot-}$ production in vascular smooth muscle.[76,77] Whether similar interactions occur in endothelial cells remains to be elucidated. Nonetheless, it was recently found that $p22^{phox}$ expression correlates well with expression of Nox4 in human arteries and that of Nox2 in veins.[78]

In summary, recent studies have established a critical role of H_2O_2 in the development of vascular disease, in particular atherogenesis. Uniquely, vascular NAD(P)H oxidase–derived H_2O_2 self-propagates via enhanced intracellular iron uptake, mitochondrion, vascular NAD(P)H oxidases, xanthine oxidase, and uncoupled eNOS. This phenomenon likely prolongs H_2O_2-mediated pathological signaling, thus contributing to vascular disease development. Initial activation of vascular NAD(P)H oxidases serves as the rate-limiting step for H_2O_2 amplification of redox signals. It is of significant importance to further investigate molecular mechanisms underlying vascular activation of Nox family proteins of the novel vascular NAD(P)H oxidases. This knowledge could lead to novel strategies effective in disrupting cascade production of reactive oxygen species, and of therapeutic potential for vascular disease.

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