**NADPH Oxidases: Functions and Pathologies in the Vasculature**

**Bernard Lassègue, Kathy K. Griendling**

**Abstract**—Reactive oxygen species are ubiquitous signaling molecules in biological systems. Four members of the NADPH oxidase (Nox) enzyme family are important sources of reactive oxygen species in the vasculature: Nox1, Nox2, Nox4, and Nox5. Signaling cascades triggered by stresses, hormones, vasoactive agents, and cytokines control the expression and activity of these enzymes and of their regulatory subunits, among which p22phox, p47phox, Noxa1, and p67phox are present in blood vessels. Vascular Nox enzymes are also regulated by Rac, ClC-3, Poldip2, and protein disulfide isomerase. Multiple Nox subtypes, simultaneously present in different subcellular compartments, produce specific amounts of superoxide, some of which is rapidly converted to hydrogen peroxide. The identity and location of these reactive oxygen species, and of the enzymes that degrade them, determine their downstream signaling pathways. Nox enzymes participate in a broad array of cellular functions, including differentiation, fibrosis, growth, proliferation, apoptosis, cytoskeletal regulation, migration, and contraction. They are involved in vascular pathologies such as hypertension, restenosis, inflammation, atherosclerosis, and diabetes. As our understanding of the regulation of these oxidases progresses, so will our ability to alter their functions and associated pathologies. *(Arterioscler Thromb Vasc Biol. 2010;30:653-661.)*

**Key Words:** atherosclerosis ■ blood vessels ■ hypertension ■ NADPH oxidase ■ reactive oxygen species
oxidosomal-induced cytosol acidification and accumulation of endosomal negative charges (Figure 1).\(^9\) Targeting anion channels could be used to inhibit oxidases, but their tissue and Nox subtype specificity remain to be investigated.

Nox1 is most highly expressed in intestinal epithelium, because of transcriptional upregulation of its promoter by cytokines involved in host defense.\(^{10,12}\) In VSMC, Nox1 is induced by growth factors and vasoactive agents (Table). This results from preferential activation of another promoter, located further upstream in the gene, in VSMC stimulated to grow by injury or angiotensin II (AngII).\(^{13}\) The resulting longer transcript produces a protein with a slightly longer N-terminus, whose activity is identical to epithelial Nox1. This promoter is responsible for Nox1 upregulation by platelet-derived growth factor (PDGF) or prostaglandin F2\(_\alpha\) via a signaling cascade including protein kinase C-\(\delta\) (PKC\(\delta\)), epidermal growth factor (EGFR) transactivation, and either PI3K, ATF-1, and MEF2B or ERK1/2 and JunB. Binding sites for transcription factors MEF2B and JunB have been identified.\(^{10,14,15}\)

Agonists also acutely activate Nox1 and its regulatory subunits by stimulating upstream signaling cascades, including phospholipases C and D, PKC, Src, and PI3K. These pathways are required for activation of p47phox and Rac.\(^1\) ROS produced by Nox1 then regulate downstream signaling pathways and essential cellular functions (Table), as discussed in greater detail elsewhere.\(^7\)

The role of Nox1 in cardiovascular disease has been studied mainly in hypertension. Vascular or kidney Nox1 is upregulated in experimental models, such as renin transgenics,\(^24\) implying that other mechanisms control chronic hypertension. Because growth factors stimulate ROS production in VSMC, the role of Nox1 in proliferative vascular disease was investigated. Nox1 is activated or upregulated in VSMC treated with AngII, low-density lipoprotein, or advanced glycation end products.\(^1,25,26\) Nox1 upregulation increases serum-induced proliferation and PDGF-induced migration in VSMC.\(^27\) Conversely, Nox1 deletion inhibits VSMC proliferation induced by serum,\(^1\) thyroid hormone,\(^28\) or PDGF,\(^27\) as well as PDGF or basic fibroblast growth factor-induced migration,\(^27,29\) which is rescued by Nox1 transfection.\(^29\) Moreover, Nox1 is activated or upregulated in vessels from diabetic animals.\(^30,31\) Remarkably, not only is injury-induced intimal hyperplasia accompanied by Nox1 upregulation\(^1\) but also it is inhibited by Nox1 deletion.\(^27\) Thus, Nox1 can clearly mediate abnormal vascular growth and possibly atherosclerosis.

In this context, a possible link between Nox1 and vascular inflammation was also investigated. Thrombin increases VSMC Nox1 expression, IL-6 secretion, and NF\(\kappa\)B translocation. These latter effects may be mediated by Nox1, as they are inhibited by atorvastatin,\(^32\) which downregulates Nox1.\(^1\) Furthermore, tumor necrosis factor-\(\alpha\) or IL-1\(\beta\) induces Nox1-dependent VSMC NF\(\kappa\)B activation.\(^5\) Likewise, increased Nox1 expression and activity by hyperhomocysteinemia in rat coronary arteries appear to be mediated by tumor necrosis factor-\(\alpha\).\(^33\) No less important, Nox1 in macrophages is required for foam cell formation, thereby contributing to vascular lesion formation.\(^34\) Therefore, Nox1 appears to mediate vascular inflammation via multiple mechanisms.

Although Nox1 is clearly implicated in vascular pathology, it also has beneficial physiological roles. For example, it is likely responsible for shear-induced outward vessel remodeling, because this response is blocked by deletion of p47phox, but not Nox2.\(^35\)
**Nox2**

Nox2 is found in all vascular wall cells, except VSMC from large arteries, and produces intracellular or extracellular superoxide (Figure 1).\(^1,36,37\) Even in large arteries, EC and adventitial fibroblasts are important sources of Nox2-derived superoxide.\(^2\) Nox2 is activated by pathways similar to vascular Nox1 or phagocytic Nox2, such as p47phox phosphorylation by PKC and Src.\(^38–40\) Similar downstream signals are also affected by Nox1 and Nox2 as described at length in other reviews.\(^7\) For example, Nox2 promotes EC proliferation by p38MAPK and Akt stimulation.\(^37,41\) On a larger scale, Nox2 also affects vascular function and pathology.

Because \(\text{O}_2^{-}\) rapidly neutralizes vasodilator NO, Nox2 is expected to induce contraction. Nox2 expression is inversely correlated with endothelium-dependent relaxation in isolated aorta.\(^42,43\) Furthermore, Nox2 deletion inhibits PKC-mediated aortic contraction,\(^40\) restores acetylcholine-induced cerebral artery vasodilation,\(^44\) and increases contraction in males.\(^45\) These results suggest that Nox2 may affect blood pressure.

A correlation between Nox2 expression and hypertension has been observed repeatedly. Aortic Nox2 is elevated in stroke-prone spontaneously hypertensive rats, in rats exposed to aldosterone plus salt and in AngII-infused mice.\(^1,46\) Tempol improves both small intrapulmonary artery Nox2 expression and pulmonary hypertension in renin transgenic rats.\(^47\) As expected, hypertension is worsened by Nox2 overexpression in endothelial-targeted transgens after acute or chronic AngII treatment.\(^48\) In converse experiments, hypertension can be improved by Nox2 deletion, but results depend on the experimental model. Nox2 ablation in mice reduces systemic hypertension in 2-kidney 1-clip or deoxycorticosterone acetate salt animals,\(^49,50\) and prevents hypoxia-induced pulmonary hypertension.\(^51\) In contrast, Nox2 deletion does not prevent hypertension induced by AngII infusion but decreases medial hypertrophy,\(^1\) possibly affecting blood pressure later. Likewise, hypertension is unaffected in renin transgenics.\(^52\) These results suggest that treatments targeting Nox2 could improve some forms of hypertension.

Accumulating evidence suggests that Nox2 in resident EC and adventitial fibroblasts as well as recruited macrophages promotes inflammation and atherosclerosis.\(^2,53–55\) Nox2 mRNA, \(\text{O}_2^{-}\) production, and monocyte binding are increased by oscillatory shear stress in EC.\(^56\) Furthermore, in animal models of surgical vascular injury or high-cholesterol diet, Nox2 is significantly upregulated, at least during the chronic phase of the disease, even in the absence of inflammation.\(^1,57\) Similarly, in human vessels Nox2 expression is correlated

---

**Table. Examples of Nox1 Signaling in Vascular Smooth Muscle**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Upstream Signals</th>
<th>Nox1 Regulation</th>
<th>Downstream Signals</th>
<th>Downstream Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Basal activity</td>
<td>Caveolin phosphorylation, AT1R at plasma membrane</td>
<td></td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>Upregulation</td>
<td></td>
<td></td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>AngII</td>
<td>(\text{Ca}^{2+}) Upregulation</td>
<td>P-Akt</td>
<td>Hypertrophy</td>
<td>1, 112</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>Activation</td>
<td></td>
<td></td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>Rac1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Src</td>
<td>EGFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K</td>
<td></td>
<td>PI3K</td>
<td>JNK</td>
<td>Migration</td>
<td>29</td>
</tr>
<tr>
<td>Cyclic stretch</td>
<td>Upregulation</td>
<td></td>
<td></td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Activation</td>
<td></td>
<td>NF(\kappa)B</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>LDL</td>
<td>Upregulation</td>
<td></td>
<td></td>
<td></td>
<td>1, 25</td>
</tr>
<tr>
<td>oxidized LDL</td>
<td>PKC(\delta)</td>
<td></td>
<td>NFKB</td>
<td>Hypertrophy</td>
<td>1, 27, 114, 115</td>
</tr>
<tr>
<td>PDGF or PGF2(\alpha)</td>
<td>EGFR and either PI3K, ATF-1, MEF2B or ERK, JunB</td>
<td>Upregulation</td>
<td>MCP-1</td>
<td>Migration</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>Upregulation</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Serum</td>
<td>Upregulation</td>
<td></td>
<td>Proliferation</td>
<td>1, 27</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>Downregulation</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>T3</td>
<td>Upregulation</td>
<td></td>
<td></td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Upregulation</td>
<td></td>
<td>Proliferation</td>
<td>1, 32</td>
<td></td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>Activation</td>
<td></td>
<td>NF(\kappa)B</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

EGFR indicates epidermal growth factor receptor; LDL, low-density lipoprotein; TNF, tumor necrosis factor.
with lesion severity, a although part of the effect derives from macrophage infiltration in advanced lesions. Moreover, Nox2 deletion greatly reduces descending aortic lesion burden in hyperlipidemic mice. b Overall, these results favor a causal role of Nox2 in atherosclerosis.

**Nox 4**

Nox4 mRNA is present in all vascular wall cells and is significantly more abundant than other Nox enzymes. The protein is located in perinuclear space or endoplasmic reticulum in EC, nucleus in EC and VSMC, and in focal adhesions and stress fibers in VSMC. Because the only regulatory subunit Nox4 shares with Nox1 and Nox2 is p22phox, Nox4 was thought to be constitutively active and responsible for basal ROS production; however, a recent report shows that Poldip2 enhances its activity (Figure 1). Nox4 is consistently upregulated and acutely activated by transforming growth factor-β in all cell types tested. In fact, transforming growth factor-β is responsible for Nox4 activation in response to other stimuli, such as hypoxia. Investigations of downstream signaling pathways suggest that p38MAPK is a target of Nox4. Nox4 also activates the Ras/ERK pathway, JNK and Akt. In addition to tyrosine kinase signaling pathways, Nox4 has been implicated in the activation of Rho in VSMC, consistent with its effects on the cytoskeleton.

Because there is no report to date of genetic models with altered Nox4 expression, current knowledge derives mostly from interventions in cell culture. Nox4 is linked to decreased vascular cell growth in many studies. For example, whereas serum withdrawal upregulates Nox4 mRNA in VSMC and EC, growth-promoting agents such as AngII, PDGF, IL-1β, thrombin, and phorbol esters downregulate Nox4 mRNA in VSMC and adventitial fibroblasts. However, these results may need to be re-evaluated because of a recent study showing that Nox4 mRNA and protein expressions are not always correlated. Nevertheless, Nox4 is required to maintain the quiescent VSMC phenotype and tumor necrosis factor-α–induced apoptosis in EC. In contrast, Nox4 appears to stimulate growth in other instances. Thus, proliferation induced by urotenin or hypoxia (via transforming growth factor-β) in pulmonary VSMC requires Nox4. Similarly, in EC, Nox4 promotes growth and inhibits apoptosis, and Nox4 knockdown impairs epidermal growth factor– and human immunodeficiency virus–tat-induced proliferation. Further studies are required to reconcile these conflicting results.

A similar conundrum holds for the role of Nox4 in migration. Whereas Nox4 siRNA decreases PDGF-induced VSMC migration and wounding-activated EC migration, Nox4 overexpression also decreases migration in PDGF-stimulated VSMC and adventitial fibroblasts exposed to AngII. It is likely that too little Nox4 prevents focal adhesion formation, whereas too much Nox4 prevents focal adhesion dissolution, both of which are required for cell motility.

In some cases Nox4 appears linked to pathways protecting vessels against disease. In EC, Nox4 is upregulated by physiological shear stress and downregulated by pathological stress. Similarly, Nox4 is increased during redifferentiation of smooth muscle after vascular injury. However, Nox4 expression is also correlated with deleterious responses. The proinflammatory mediators tumor necrosis factor-α and oxidized phospholipids increase Nox4 expression or activity, respectively. Basal and urotenin-induced profibrotic plasminogen activator inhibitor-1 gene expression in pulmonary artery VSMC is dependent on Nox4, and Nox4 downregulation blocks IL-8, monocyte chemoattractant protein-1, low-density lipoprotein receptor, and ROS production in EC stimulated with oxidized phospholipids. Furthermore, Nox4 is elevated in cerebral aneurysms and in vessels from diabetic mice (except ). Of interest, treatment with the peroxisome proliferator-activated receptor-γ agonist rosiglitazone mitigates Nox4 upregulation.

Overall, our knowledge of Nox4 in the vascular system is incomplete. Current evidence suggests that it is involved in numerous essential cellular pathways mediating differentiation, growth, and migration. Its role is still controversial, suggesting that a central aspect of its function remains to be discovered.

**Nox5**

Nox5 differs from other Nox enzymes by its additional N-terminal regulatory domain. Nox5, which requires no additional subunit, is activated by calcium binding to N-terminal EF-hands (Figure 1). Calcium sensitivity is increased by calmodulin and phosphorylation by PKC. In transfected leukemia cells, Nox5 is activated by H2O2 in a positive feedback loop involving c-Abl. Five splice variants with different tissue distributions are known: Nox5α, Nox5β, Nox5γ, Nox5δ, and Nox5S, a short form without calcium-binding domain. Multiple isoforms are expressed in the vasculature. Within cells, Nox5 can be found in the cytoskeletal fraction, endoplasmic reticulum, or plasma membrane.

Because Nox5 is not present in rodent, experimental models are limited to cultured cells and isolated tissue. In VSMC, Nox5 participates in PDGF-induced proliferation via the JAK/STAT pathway. In EC, Nox5 overexpression slightly increases proliferation and formation of capillary-like tubes, whereas Nox5 depletion reduces thrombin-stimulated growth and tube formation. In intact vessels, adenoviral-mediated Nox5 overexpression paradoxically increases endothelial NO synthase activity, but as expected, reduces bioavailable NO via inactivation by . In arteries, Nox5 is detected in the endothelium of control arteries, in the neointima of diseased arteries, and in smooth muscle underlying complex lesions in arteries with advanced atherosclerosis.

These studies are provocative, suggesting an important role for Nox5 in vascular function. Clearly, additional work is needed to further explore its physiological and pathological roles.
Interactions Between Oxidases

Because most cells simultaneously express multiple Nox enzymes, some of their functions may be redundant. For example, both Nox2 and Nox4 in EC are required for the angiogenic response and participate in serum-induced proliferation. Both enzymes are similarly regulated by shear stress and colocalize with endoplasmic reticulum markers, and knockdown of one subtype upregulates the other, suggesting that their functions are overlapping, although not entirely identical.

Most often, however, Nox enzymes in the same cell mediate distinct functions. For example, in VSMC from large arteries, Nox1 is required for hypertrophy and proliferation, whereas Nox4 mediates differentiation. These enzymes...
respond to different agonists, participate in migration via different mechanisms, and have distinct localizations.

Similarly, in transfected HEK293 cells, Nox2 and Nox4 are expressed in different compartments, and each responds to different agonists and activates distinct signaling pathways. Therefore, subcellular localization seems to be an essential factor in determining Nox function.

With regard to vascular disease, multiple NADPH oxidases appear to contribute to the pathophysiological response. Both Nox1 and Nox2 have been implicated in hypertension because they contribute directly to blood pressure elevation and to end-organ damage or remodeling. The expression of Nox1, Nox2, and Nox4 is altered after vascular injury, with the former two mediating the proliferative response of VSMC and EC, and the latter likely contributing to redifferentiation of VSMC. In diabetic vascular disease, all 3 are upregulated, however, their specific contributions to diabetes-related endothelial dysfunction and inflammation remain to be elucidated.

It is also important to consider that Nox enzymes in multiple tissues contribute to the pathological response in a coordinated fashion. In hypertension, evidence exists for a role of brain, vascular, cardiac, and kidney Nox enzymes. Furthermore, a large part of the hypertensive response to AngII infusion or deoxycorticosterone acetate salt administration is dependent on T lymphocytes. Harrison and Gongora have proposed that stimulation of Nox enzymes in circumventricular organs by AngII increases efferent sympathetic activity, leading to activation of T cells in lymph nodes, which home to vessels and kidneys. Importantly, T cells release cytokines that stimulate NADPH oxidases in these tissues, causing vasoconstriction and sodium retention (Figure 2). A similar coordinated response is likely to occur in the vessel wall in atherosclerosis, where infiltrated macrophages release cytokines in a Nox2-dependent manner, which then activate Nox1 and Nox2 in VSMC, EC, and adventitial fibroblasts.

Once ROS production is initiated by Nox activation, other oxidase systems often amplify this initial response. For example, EC exposed to pathological shear stress exhibit a Nox-dependent activation of xanthine oxidase. Similarly, AngII stimulation of these cells enhances mitochondrial ROS production in a Nox-dependent manner. In hypertension, Nox activation leads to the formation of peroxynitrite, which uncouples endothelial NO synthase, leading to a dramatic enhancement of O$_2^-$ production. Moreover, ROS can also activate Nox enzymes and increase their own production in a positive feedback loop (Figure 2). Such self-reinforcing loops may set new homeostatic points of ROS production and lock the organism in a sustained pathological state.

Conclusion

The evidence summarized in this review clearly shows that Nox enzymes are essential to normal vascular function and participate in the development of vascular disease. They modulate intracellular signaling pathways to regulate cell survival, vessel contraction, and relaxation. In pathological conditions, they contribute to VSMC proliferation and migration, angiogenesis, inflammation, hypertension, and medial hypertrophy. Because of their varied and essential functions, it is not surprising that antioxidants designed to scavenge ROS produced from these enzymes provide ineffective protection against vascular disease. The work described here strongly argues for a change in therapeutic strategies to target only deleterious sources of ROS while leaving intact those that are required for normal vascular function.

That said, more research is necessary to fully understand the roles of different Nox enzymes in the vasculature. Additional studies should focus on identifying the specific molecular pathways that are targets of each Nox. The creation of tissue-specific, genetically modified animals will permit a better analysis of their roles in physiology and disease. Moreover, subtype-specific inhibitors are needed to allow the field to advance therapeutically once pathological targets have been identified. Studies such as these will allow us to truly test the oxidative hypothesis of vascular disease.

Sources of Funding

This work was supported by National Institutes of Health grants HL38206, HL058863, and HL095070.

Disclosures

Emory University is the owner of a patent on Nox1 with both authors cited in the list of inventors.

References


Castier Y, Brandes RP, Lesegue G, Tedgui A, Lehoux S. p47phox


Gupte SA, Kaminski PM, George S, Kozunzestova L, Olson SC, Mathew R, Hintze TH, Wolin MS. Peroxide generation by p47phox-Src acti


Takenouchi Y, Kobayashi T, Matsumoto T, Kamata K. Gender dif


