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## Biochemistry, Physiology and Pathophysiology of NADPH Oxidases in the Cardiovascular System

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### Abstract

The NADPH oxidase (Nox) enzymes are critical mediators of cardiovascular physiology and pathophysiology. These proteins are expressed in virtually all cardiovascular cells, and regulate such diverse functions as differentiation, proliferation, apoptosis, senescence, inflammatory responses and oxygen sensing. They target a number of important signaling molecules, including kinases, phosphatases, transcription factors, ion channels and proteins that regulate the cytoskeleton. Nox enzymes have been implicated in many different cardiovascular pathologies: atherosclerosis, hypertension, cardiac hypertrophy and remodeling, angiogenesis and collateral formation, stroke and heart failure. In this review, we discuss in detail the biochemistry of Nox enzymes expressed in the cardiovascular system (Nox1, 2, 4 and 5), their roles in cardiovascular cell biology, and their contributions to disease development.

### Keywords

NADPH oxidases; vascular smooth muscle; endothelial cells; cardiomyocytes; atherosclerosis; hypertension; cardiac hypertrophy

## INTRODUCTION

In the dozen years since the discovery that the neutrophil respiratory burst NADPH oxidase was only one member of a family of homologous enzymes, tremendous progress has been made in understanding the role of these proteins in the cardiovascular system. The NADPH oxidase (Nox) family is comprised of 7 catalytic subunits termed Nox1-5 and Duox1 and Duox2 (for Dual Oxidase), regulatory subunits p22phox, p47phox or Noxo1, p67phox or Noxa1, p40phox and the major binding partner Rac. Of these, the Nox1, 2, 4 and 5 enzymes are expressed in cardiovascular tissues and not only participate in normal vascular and cardiac function, but also contribute to the development of cardiovascular disease.

Nox enzymes are heteroprotein complexes (except Nox5) with very specific regulatory mechanisms, tissue and subcellular patterns of expression, downstream targets and functions. They are found in endothelial cells (ECs), vascular smooth muscle cells (VSMCs), macrophages, adventitial fibroblasts, cardiac myocytes and fibroblasts, as well as adipocytes and stem cells. These proteins work independently or in concert to control not only cell survival, growth and death, but also cell-specific functions such as differentiation, angiogenesis and contraction. When acutely or chronically upregulated, they have been

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### DISCLOSURES

Emory University is the owner of a patent on Nox1 with BL and KKG cited in the list of inventors.

implicated in hypertension, atherosclerosis, heart failure, ischemia reperfusion injury and cardiac remodeling, but upregulation can be physiologically advantageous, as in angiogenesis and collateral formation. The molecular mechanisms that differentially control the expression, activation and cellular pathways linked to Nox enzymes, as well as the physiological and pathophysiological contexts in which they exert their effects, are the focus of this review.

## BIOCHEMISTRY

### Nox2

Historically the first discovered member of the NADPH oxidase family,<sup>1</sup> also known as gp91phox, Nox2 is the catalytic subunit of an enzymatic complex responsible for the phagocyte respiratory burst. Upon activation, Nox2 uses NADPH to reduce molecular oxygen to superoxide anion, which, in concert with its metabolites, is used by phagocytes to destroy invading microorganisms. Nox2 is also found throughout the cardiovascular system (Table 1), where its expression and activity are much lower than in phagocytes,<sup>2-4</sup> consistent with signaling functions described in a following section. However, in pathological circumstances, excess Nox2 can lead to oxidative stress and disease development (see Physiology and Pathophysiology in vivo). Here, we will first summarize salient features of Nox2, mostly derived from studies conducted in phagocytes, that apply to the cardiovascular enzyme. This enzyme will later serve as a reference against which to compare other NADPH oxidases.

The structure of Nox2 is not known in complete detail, but current models are based on homology to proteins of known architecture, mapped epitopes of monoclonal antibodies and mutations from X-linked chronic granulomatous disease (CGD) patients or site-directed mutagenesis.<sup>5</sup> Briefly, Nox2 is composed of two main domains of equal sizes with very different properties. The amino-terminal moiety includes six transmembrane  $\alpha$ -helices (numbered I-VI) connected by five loops (numbered A-E, Figure 1). Because both amino- and carboxy-termini are cytosolic, three loops are extracellular and include consensus asparagine glycosylation sites, while the other two are intracellular and accessible to cytosolic regulators. The degree of Nox2 glycosylation and apparent molecular mass differ between species. In western blots of human neutrophil proteins, Nox2 is detected as a smear from about 65 to 91 kDa. Transmembrane helices III and V each include two conserved histidine residues that coordinate iron atoms at the center of two heme molecules.<sup>6</sup> The cytosolic carboxy-terminal moiety of Nox2 constitutes a dehydrogenase domain that includes consensus binding sites for its NADPH substrate and FAD cofactor. Upon activation, two electrons are transferred from cytosolic NADPH to FAD and in succession across the membrane, via redox changes in heme irons. Finally, each electron reduces a molecule of oxygen to a superoxide radical, which is subsequently released outside the cell or in a topologically equivalent compartment, such as a vesicle lumen. Superoxide is the first reactive oxygen species (ROS) in a cascade of metabolites including hydrogen peroxide and peroxynitrite.<sup>7</sup> A charge compensation mechanism, required to balance electron transport by Nox2 and sustain its activity, is provided by a voltage-gated proton channel<sup>8</sup> and the chloride/proton antiporter CIC-3.<sup>9</sup>

Tight regulation, critical to avoid excessive production of deleterious superoxide, is evident from the large number of proteins involved in oxidase assembly (Figure 1). These include Nox2 itself, p22phox, p47phox, p67phox and p40phox; all essential subunits whose mutations can cause CGD.<sup>10, 11</sup> Also crucial is Rac GTPase, which binds to the dehydrogenase domain of Nox2.<sup>12</sup> In the resting state, Nox2 and p22phox form an inactive membrane complex known as cytochrome b558,<sup>1, 5</sup> while the other dormant subunits form a trimer in the cytosol with p67phox linking p47phox and p40phox.<sup>13</sup> Signaling cascades

activate Rac, p47phox and p40phox, allowing translocation of cytosolic subunits to the membrane and association with the cytochrome to form an active enzyme.<sup>1, 5</sup> Phosphorylation of Nox2 by protein kinase C (PKC) also promotes activity and recruitment of cytosolic subunits.<sup>14</sup> We will now summarize essential features of these regulatory subunits.

Numerous structural and functional studies revealed that p47phox is composed of an N-terminal PX domain, two central SH3 domains, an autoinhibitory region and a C-terminal proline-rich region that binds p67phox.<sup>5</sup> At rest, intramolecular folding masks the PX domain and the SH3 tandem, which binds to the autoinhibitory region.<sup>15</sup> In leukocytes and transfected cells, unfolding of p47phox is triggered by PKC phosphorylation of serine residues, including 303, 304 and 328 in the autoinhibitory region.<sup>16-20</sup> Other kinases that can phosphorylate p47phox on serine and tyrosine may contribute to oxidase activation.<sup>21-23</sup> Similar phosphorylation events occur in vascular cells<sup>24-26</sup> as well as tyrosine phosphorylation by Src.<sup>27, 28</sup> Once exposed, the PX domain allows translocation of p47phox to the membrane by binding to phosphoinositide 3-kinase (PI3K) lipid products<sup>20, 29</sup> and actin fibers,<sup>30-32</sup> while the SH3 domains interact with the cytosolic C-terminal proline-rich region of p22phox.<sup>33, 34</sup> Thus, PKC, PI3K, and Src can cause oxidase assembly by recruiting p47phox bound to p67phox and p40phox. Because p47phox is an essential component of the oxidase *in vivo*, many pathophysiological studies have taken advantage of a spontaneous null mutant,<sup>35</sup> as well as two independent knockout mouse lines.<sup>36,37</sup> These models are useful, but it should be remembered that p47phox can also be part of the Nox1-based oxidase (see below).

Functionally, p67phox is indispensable for Nox2 activation, whereas p47phox can be omitted from cell-free assays.<sup>38, 39</sup> Results from many studies indicate that p67phox is composed of an N-terminal region including four tetratricopeptide repeat (TPR) motifs, followed by an activation domain, an SH3 domain, a PB1 domain and finally a second SH3 domain at the C-terminus. The TPR motifs, important for Rac-GTP binding, are defective in many CGD mutants.<sup>10, 40-42</sup> Interestingly, in the cell-free system, a fragment of p67phox, including the N-terminus and the activation domain, fused with Rac1, is highly active presumably because it is targeted to the membrane.<sup>43</sup> Most importantly, the activation domain of p67phox triggers FAD reduction by Nox2.<sup>39</sup> The V204A mutant in this region is a competitive inhibitor of wild-type p67phox.<sup>44</sup> The first SH3 domain increases oxidase activity, but its target is not known.<sup>45</sup> The PB1 domain allows binding to p40phox, which is abolished by a K355A mutation.<sup>46, 47</sup> Finally, the C-terminal SH3 domain of p67phox is responsible for binding the proline-rich region of p47phox and therefore allows p67phox translocation to the membrane after activation.<sup>48-50</sup> An additional degree of regulation is provided by phosphorylation, which is both constitutive and stimulated by agonists.<sup>51-53</sup> Phosphorylation of p67phox, mediated by PKC $\delta$ , ERK2 and p38MAPK, increases superoxide production by Nox2.<sup>54-56</sup> Physiological studies still await the creation of a p67phox knockout model. Although a line of mice with a mutation in the PB1 domain of p67phox and increased susceptibility to infections has been reported, additional work is required to verify that this mutation is the only cause of the phenotype.<sup>57</sup>

Structural and functional studies indicate that p40phox is composed of an N-terminal PX domain,<sup>40</sup> a central SH3 domain and a C-terminal PB1 domain. While masked by interaction with the PB1 domain at rest,<sup>58</sup> the PX domain sustains oxidase activation after stimulation by interaction with lipid products of PI3K.<sup>59-61</sup> Because p40phox binds inositol headgroups with lower phosphate content than p47phox,<sup>59</sup> it preferentially interacts with endosomes. Thus, p40phox appears to increase oxidase activity in cooperation with p47phox<sup>47, 62, 63</sup> not by inducing translocation to the membrane, but by retaining the oxidase at the phagosome.<sup>15, 61, 64</sup> Depletion or mutation of p40phox impairs ROS

production in neutrophils<sup>65, 66</sup> and ECs.<sup>67</sup> While the PX domain is clearly essential to p40phox function, the target of the SH3 domain is unknown and its function is controversial.<sup>68, 69</sup> The C-terminal PB1 domain of p40phox interacts with the PB1 domain of p67phox, as shown using multiple approaches.<sup>13, 47</sup> In addition, phosphorylation of p40phox on serine and threonine by PKC is increased during stimulation,<sup>67, 70, 71</sup> particularly at threonine 154, which is usually,<sup>69</sup> but not always,<sup>72</sup> required for full oxidase activation. The significance of p40phox is emphasized by a single case of CGD known to date. In this patient a point mutation in the PX domain does not prevent extracellular superoxide production, but impairs oxidase activation following phagocytosis.<sup>73</sup>

Because Nox2 produces superoxide, a highly reactive and short-lived ROS, its subcellular localization is important for function. Superoxide can be released outside the cell when Nox2 is located at the plasma membrane, thus allowing it, for example, to intercept endothelial-derived nitric oxide (NO) before it reaches the adjacent smooth muscle cell layer in vessels.<sup>7</sup> However, Nox2 is also frequently found in intracellular compartments, around the nucleus and colocalized with the endoplasmic reticulum (ER) in vascular cells (Table 1).<sup>2, 74</sup> In ischemic cardiomyocytes, Nox2 is upregulated in the cytosol and targeted to the nuclear pore complex.<sup>75</sup> Production inside cells is consistent with a direct signaling role of superoxide or its metabolites, as will be seen later.

## Nox1

Nox1 is most highly expressed in colon epithelium, where it is thought to play a role in host defense.<sup>76</sup> It is also found in many other tissues and in all layers of the vascular wall, particularly after induction by agonists and in pathological conditions (Table 1). Nox1, like Nox2, associates with p22phox to form a membrane-bound cytochrome.<sup>77-79</sup> However, in colon the cytosolic subunits p47phox and p67phox are not expressed and are replaced by Noxo1 and Noxa1.<sup>80-82</sup> We now understand that Nox organizers include both p47phox and its homologue, Noxo1, while Nox activators comprise p67phox and the structurally similar Noxa1. Because Nox2 and Nox1 are closely related, both enzymes can be activated in transfected cells by various organizer and activator pairs.<sup>80</sup> Examples are also known in vivo, as in VSMC from mouse conduit arteries, where p47phox and Noxa1 activate Nox1.<sup>83, 84</sup> To understand the significance of these substitutions, a brief description of Noxo1 and Noxa1 is required.

Similar to p47phox, Noxo1 facilitates oxidase assembly by binding both an activator subunit and p22phox. The proline-rich region of Noxo1 binds to an SH3 domain of the activator, while the tandem SH3 domains of Noxo1 bind to the proline-rich region of p22phox.<sup>80, 87, 88</sup> Noxo1 also binds to the dehydrogenase domain of Nox1.<sup>89</sup> Furthermore, the PX domain of Noxo1 provides an essential affinity for membrane phosphoinositides.<sup>90</sup> However, unlike p47phox, because Noxo1 lacks an autoinhibitory domain, it is thought to constitutively bind the cytochrome.<sup>80, 81, 90</sup> Surprisingly, in spite of coexpression with Nox1 in colon and some vascular cells (Table 1), mice lacking Noxo1 apparently only present a deficiency in Nox3 activity during development of the inner ear,<sup>91</sup> perhaps because other organizers may compensate for its loss in other tissues. Besides p47phox, other possible organizers include Tks4 and Tks5, two Src substrates with a PX domain and multiple SH3 domains capable of binding p22phox and Noxa1, but not p67phox.<sup>92</sup>

Several important features of p67phox are conserved in Noxa1, allowing it to serve as an activator subunit. These include four Rac-binding TPR motifs, a Nox activation domain and an SH3 domain that interacts with the proline-rich region of an organizer subunit. However, the p40phox-binding PB1 domain is not well conserved and the SH3 domain in the middle of the molecule is missing.<sup>80, 81</sup> In contrast to its Noxo1 partner, Noxa1 activity appears to be tightly regulated. Phosphorylation of serine 461 by protein kinase A (PKA), serine 282

by ERK1/2 or p38 MAPK, or serine 172 by PKA or PKC, decrease ROS production by Nox1.<sup>93-95</sup> Phosphorylation of Noxa1 by PKA favors binding to 14-3-3 and dissociation from Nox1,<sup>93</sup> while other kinases appear to decrease Noxa1 affinity for Rac1 and Nox1.<sup>95</sup> In contrast, phosphorylation of Noxa1 by Src on tyrosine 110 increases Nox1 activity.<sup>96</sup> These regulatory mechanisms may be important in vessels that express Noxa1 (Table 1). The recent creation of a knockout model will likely allow future detailed in vivo studies of Noxa1 function.<sup>97</sup>

In addition to cytosolic organizers and activators, Nox1 also requires Rac1 for activity, as might be expected from its resemblance to Nox2. Nox1 is stimulated by constitutively active Rac1 and inhibited by Rac1 knockdown.<sup>87, 98</sup> In addition to binding an activator subunit, as mentioned above, Rac1 also has affinity for the plasma membrane due to prenylation<sup>99</sup> and interacts directly with the carboxy terminus of Nox1, even in the absence of Noxa1.<sup>12, 98, 100</sup> Another related small monomeric GTPase, Cdc42 cannot activate Nox1,<sup>98</sup> but could be a competitive inhibitor, as in the case of Nox2.<sup>101</sup> Interestingly,  $\beta$ Pix, a Rac1 guanine nucleotide exchange factor, appears to be constitutively bound to Nox1 and essential for its activity.<sup>89, 100</sup> These results suggest that Rac1 provides a crucial mechanism for activation by agonists, particularly in cells that exclusively express Nox1/Noxa1/Nox1, as colon epithelial HT29 cells.<sup>102</sup>

Although the structure of Nox1 is not known, it is expected to be very close to other Nox enzymes (Figure 1),<sup>1, 5</sup> with a dehydrogenase domain binding FAD and NADPH, as well as four conserved histidine residues in transmembrane helices III and V, thought to coordinate two heme molecules. Similar to Nox2, mutation of these histidines abolishes binding to p22phox.<sup>78</sup> Family resemblance is further substantiated by the creation of a functional chimera comprised of the transmembrane domain of Nox1 and the cytosolic moiety of Nox4 (abbreviated as Nox1-Nox4 below).<sup>103, 104</sup> However, the signal peptide at the N-terminus of Nox1 is important for localization at the plasma membrane, as it is prevented by replacement with the N-terminus of Nox4.<sup>103</sup> In VSMCs, Nox1 can be found at the plasma membrane, in caveolae<sup>105</sup> and in endosomes (Table 1).<sup>106</sup> Interestingly, agonists appear to stimulate Nox1 in specific locations, thus determining where superoxide is produced: extracellularly by muscarinic agonists and thrombin; in endosomes by IL-1 $\beta$  and TNF $\alpha$ ; both inside and outside cells by Ang II.<sup>106-108</sup> Of importance, Nox1 stimulation in endosomes is dependent on ClC-3, where this ion exchanger is required to balance the electrogenic activity of Nox1.<sup>106, 109</sup> Interestingly, Nox1 activity is also dependent on chaperones Hsp90<sup>104</sup> and PDI,<sup>110, 111</sup> which appear to be necessary not only for protein folding after synthesis, but also to maintain enzyme stability.

The primary biochemical function of vascular Nox1 is superoxide production, which is then rapidly converted to hydrogen peroxide. The moderate physiological activity of Nox1, compared to the phagocytic Nox2, can be attributed to its low expression as well as specific regulatory subunits and signaling cascades. However, in pathological conditions, upregulation of Nox1 can lead to oxidative stress in the cardiovascular system.

## Nox4

Abundant in kidney cortex where it was originally discovered,<sup>112, 113</sup> Nox4 appears to be expressed almost ubiquitously, including throughout the cardiovascular system and in macrophages important in atherosclerosis.<sup>114</sup> Nox4 is usually coexpressed with other homologues such as Nox1 and Nox2, but at significantly higher levels. Upregulation by prolonged exposure to agonists such as TGF- $\beta$  appears to be a major route of Nox4 regulation (Table 1).



Similar to Nox1 and Nox2, coprecipitation and colocalization studies showed that Nox4 binds to p22phox (Figure 1).<sup>74, 78, 115</sup> Furthermore, overexpression and siRNA experiments indicate that association with p22phox is required for Nox4 activity and that the two proteins stabilize each other.<sup>78, 79, 115-117</sup> As in Nox1 and Nox2, mutation of heme-binding histidine 115 also abolishes Nox4 interaction with p22phox.<sup>78</sup> In contrast, transfection of organizer and activator subunits does not increase ROS production, suggesting that Nox4 activity is constitutive.<sup>78, 79, 115</sup> This interpretation is supported by other observations in transfected cells: addition of cytosol to membrane fractions does not increase Nox4 activity;<sup>118</sup> mutation of the proline-rich domain of p22phox required for docking organizers does not affect Nox4 activity;<sup>79</sup> Nox1-Nox4 and Nox2-Nox4 chimeras are active without transfection of cytosolic subunits,<sup>103, 104, 119</sup> while the opposite Nox4-Nox2 chimera requires activation.<sup>117, 119</sup> However, ROS production is enhanced by the multifunctional Poldip2, which also interacts with p22phox,<sup>120</sup> presumably at the beginning of the cytosolic C-terminus, upstream of the region dispensable for Nox4 activity.<sup>121</sup> Unlike Nox1 and Nox2, Rac1 does not activate Nox4 in transfected cells,<sup>115, 122</sup> in spite of evidence compatible with an indirect effect.<sup>123-126</sup> A number of reports indicate that agonists can acutely increase Nox4 activity or signaling,<sup>122, 123, 127-134</sup> but only a few instances of possible coupling mechanisms are known: very rapid protein upregulation;<sup>134, 135</sup> control of NADPH availability;<sup>136, 137</sup> or direct interaction of the TLR4 receptor with the oxidase.<sup>127, 131, 138</sup> These results suggest that Nox4 can respond to agonist stimulation in addition to its widely accepted role as a constitutively active enzyme mostly regulated by transcription.

In the absence of a complete model, structural information about Nox4 can be inferred from comparison of its primary sequence to other oxidases. Similar to Nox1 and Nox2, the dehydrogenase domain of Nox4 requires FAD and NADPH.<sup>119</sup> A P437H mutation in the canonical NADPH binding motif, analogous to the Nox2 mutation of a CGD patient, abolishes activity,<sup>117, 139</sup> while deletion of the NADPH binding domain produces a dominant-negative Nox4.<sup>128, 140</sup> Similar to Nox2, Nox4 is inhibited by an R96E mutation in the cytosolic B loop, a region of the amino-terminal domain that interacts with the NADPH binding site.<sup>117, 141</sup> In spite of their sequence homology, Nox4 can produce a higher hydrogen peroxide to superoxide ratio than Nox1 and Nox2.<sup>104, 115, 117, 118, 142</sup> However, the structural determinants allowing hydrogen peroxide production by Nox4 are not well understood. The Nox4 carboxy-terminal dehydrogenase domain would seem responsible, considering that Nox1-Nox4 or Nox2-Nox4 chimeras produce only peroxide in some studies.<sup>104, 119</sup> But in other reports the Nox1-Nox4 chimera generates superoxide,<sup>103</sup> and Nox4-Nox2 peroxide.<sup>117, 119</sup> In addition, replacing the first transmembrane domain of Nox4 by that of Nox1,<sup>103</sup> or altering the last extracellular loop of Nox4, makes it produce superoxide, rather than peroxide.<sup>143</sup> Additional studies will be required to resolve these discrepancies and determine if Nox4 can catalyze superoxide dismutation (presumably via the extracellular region), or even directly produce hydrogen peroxide (possibly at the C-terminus).

Besides the type of ROS produced by Nox4 and equally important for signaling is the subcellular location in which they are released. The distribution of Nox4 is surprisingly wide, including endoplasmic reticulum, plasma membrane, nucleus and mitochondria (Table 1), perhaps because its location varies according to cell type. It is also possible that each antibody preferentially labels the enzyme in a specific compartment.<sup>144</sup> Alternatively, different Nox4 isoforms may be present in specific subcellular locations. Five splice variants, named Nox4A through E, are found in lung epithelial cells. The most intriguing isoform, also reported in ECs,<sup>145</sup> is Nox4D because it appears to be fully active in a DCFH-DA assay, although it lacks most of the transmembrane domain.<sup>140</sup> One could speculate, by analogy with dehydrogenase domain assays,<sup>119</sup> that Nox4D retains activity by coupling to electron acceptors, such as cytochrome c in mitochondria, which might also be an alternative

route of hydrogen peroxide formation by full-length Nox4. Regardless of its activity, because Nox4D is expected to be hydrophilic, it could be present in compartments devoid of membranes. However, the prevalence and functional significance of Nox4 isoforms remain to be explored.

## Nox5

Compared to that of its homologues described above, Nox5 expression is restricted to fewer tissues. Although found in human VSMCs, ECs and whole vessels (Table 1), Nox5 is unexpectedly absent in rodents.<sup>5</sup> The most striking structural difference with other Nox enzymes is the presence of an additional cytosolic N-terminal segment, containing four calcium binding EF-hands (Figure 1).<sup>146, 147</sup> However, this segment is missing in Nox5S, a short calcium-insensitive variant, which is the dominant isoform in carcinoma cells,<sup>148</sup> and expressed together with the long Nox5L in ECs.<sup>149, 150</sup> Nox5S may be constitutively active<sup>150</sup> or could be a competitive inhibitor of calcium-dependent activation when present in the same tetrameric complex as Nox5L.<sup>151</sup> Except for the N-terminus of the long isoform, Nox5 is similar to other Nox enzymes, with six transmembrane helices expected to bind two hemes and a cytosolic dehydrogenase domain including FAD and NADPH binding sites.<sup>146</sup> However, neither Nox5 isoform appears to require cytosolic subunits or p22phox. Thus, Nox5 is active in a cell-free system without addition of cytosol<sup>147</sup> and is unaffected by expression or knockdown p22phox in transfected cells.<sup>79, 150</sup> Furthermore, Rac1 may participate in Nox5 activation in some systems,<sup>152</sup> but not others.<sup>153</sup> Upon stimulation with agonists, such as PDGF, Ang II or endothelin-1 in VSMCs or ECs,<sup>153, 154</sup> or after addition of a calcium ionophore to transfected cells, an increase in cytosolic calcium concentration triggers high superoxide production by Nox5.<sup>146, 155</sup> Calcium induces binding of the N-terminal domain of Nox5 to the dehydrogenase domain, thus relieving autoinhibition.<sup>147, 156</sup> Moreover, two mechanisms may increase Nox5 sensitivity, allowing activation by resting resting calcium concentrations: 1) calcium-dependent binding of calmodulin to another site in the dehydrogenase domain,<sup>157</sup> and 2) phosphorylation of serine and threonine residues by PKC and calcium/calmodulin-dependent kinase II.<sup>155, 158, 159</sup> In addition, as Nox5 can be upregulated and activated by minute concentrations of hydrogen peroxide, ROS production may be sustained by a positive feed-back loop involving the tyrosine kinase c-Abl.<sup>160, 161</sup> Finally, binding of heat shock protein 90 to the C-terminus of Nox5 appears to stabilize the protein and enhance expression and activity.<sup>104</sup> Because Nox5 is found in intracellular compartments and at the plasma membrane, superoxide is expected to be produced both inside<sup>118, 146</sup> and outside<sup>155</sup> cells. Furthermore, a polybasic region in Nox5 with affinity for phosphoinositides may favor translocation to the plasma membrane and extracellular superoxide release.<sup>162</sup>

## p22phox

The foregoing sections highlight the catalytic subunits of NADPH oxidases and their cytosolic regulators. Because p22phox is an integral part of the Nox1, 2 and 4 complexes (as well as Nox3, which is not a vascular oxidase), it is worth a separate discussion. Among all NADPH oxidase subunits, p22phox is the most ubiquitous. It is highly expressed throughout the cardiovascular system, including heart and vessels and further upregulated by many stimuli (Table 1). Although the complete crystal structure of p22phox is not known, it is clearly an integral membrane protein and colocalizes with Nox proteins in numerous cell types and subcellular compartments. Current molecular models of p22phox indicate that two transmembrane helices are located in the middle of the molecule, leaving both N and C termini in the cytosol (Figure 1).<sup>163, 164</sup> The suggestion that histidine 94 in the first transmembrane region might coordinate a heme molecule in conjunction with a Nox subunit appears unlikely because this residue can be replaced without affecting the cytochrome.<sup>165</sup> How p22phox interacts with Nox catalytic subunits is still elusive. Maturation of Nox



requires the presence of p22phox,<sup>121, 164, 166</sup> and conversely, p22phox stability is increased by the presence of a Nox subunit.<sup>78</sup> Interestingly, a point mutation in p22phox at tyrosine 121, abolishes interaction with Nox2, but does not affect Nox4.<sup>117, 121, 167</sup> Many studies have demonstrated that the proline-rich region in the C-terminal tail of p22phox is a docking site for organizer subunits<sup>80, 87, 88</sup> and therefore required for activity of Nox1<sup>79</sup> and Nox2,<sup>164</sup> but not Nox4.<sup>121</sup> Point mutations at residues 152, 156 or 158 in this region of p22phox abolish cytosolic subunit translocation and activity. Interaction of p22phox with p47phox also appears to require phosphorylation on threonine 147 in the cytosolic tail.<sup>168</sup> Thus, p22phox is not only required for stability of the Nox complex, but also serves to regulate interaction with cytosolic regulators.

## CELL PHYSIOLOGY AND PATHOPHYSIOLOGY

At present, evidence suggests that under physiological conditions Nox proteins and their products superoxide and hydrogen peroxide participate in cardiovascular homeostasis by contributing to cell differentiation, repair of damaged tissue and vascular tone. They achieve all these functions through their roles as structural and signaling molecules. On the other hand, it is also well established that their dysregulation promotes and maintains pathological conditions, as outlined in the next section. Here, we will consider how NADPH oxidases signal distinct functional outcomes.

### Nox1

Much effort has been directed towards understanding the specific roles of Nox1 in specific functions of cardiovascular cells. Animal studies show that neointimal formation following femoral artery injury as well as hypertensive remodeling in conduit arteries is significantly reduced in Nox1 knockout mice<sup>169, 170</sup>, suggesting a role of this oxidase in the proliferative and migratory functions of phenotypically modulated VSMCs. Other studies implicate Nox1 in vascular inflammation, as oxidase activity and Nox1 expression are increased in VSMCs exposed to advanced glycation end-products (AGEs),<sup>171</sup> as well as in aortas of rats and mice with experimental diabetes<sup>172, 173</sup>. It should be noted that the involvement of Nox1 in these processes is not necessarily disease promoting: outward remodeling induced by high shear stress in a model of arteriovenous fistula, a positive adaptation, is likely mediated by Nox1,<sup>174</sup> and Nox1 has been implicated in angiogenesis, as necessary step in wound healing.<sup>175</sup>

A role for Nox1 in proliferation has been established on many fronts. First, Nox1 is activated by agonists that stimulate growth in cell culture, such as Ang II<sup>176</sup>, thyroid hormone,<sup>177</sup> PDGF,<sup>176</sup> urokinase plasminogen activator,<sup>178</sup> prostaglandin F<sub>2α</sub>,<sup>179</sup> thrombin<sup>83</sup> or oxLDL.<sup>180</sup> Serum- and PDGF-induced proliferation are increased in VSMCs from Nox1 transgenic mice;<sup>169</sup> conversely, targeting Nox1 with antisense or siRNA or genetic deletion of Nox1 in VSMCs inhibits proliferation induced by serum,<sup>181</sup> thyroid hormone<sup>177</sup> and PDGF.<sup>169</sup> In an interesting study, Stanic et al.<sup>182</sup> showed that a more oxidized extracellular environment increases Nox1 expression and induces Nox1-dependent proliferation of VSMCs by activation of EGF-receptor signaling. Conversely, in human abdominal aortic ECs, endothelin-1, via the ETB1 receptor, attenuates NADPH oxidase activity by inhibiting the Pyk2-Rac1-Nox1 pathway and thus inhibits Ang II-induced proliferation.<sup>183</sup>

The mechanisms by which Nox1 mediates proliferation are varied. In VSMCs stimulated with Ang II, p38MAPK and Akt are downstream of Nox1 and are required for the hypertrophic response.<sup>184, 185</sup> Similarly, shRNA against the Nox1 activator Noxa1 inhibits the activation of Janus kinase 2, Akt, and p38MAPK by thrombin.<sup>83</sup> In actively cycling mouse lung epithelial cells, Nox1 stimulates cell proliferation by reducing the requirement

for growth factors to maintain expression of cyclin D1, and stimulates transcriptional activation of Fos family genes required for induction of cell cycle re-entry.<sup>186</sup>

However, Nox1 may have a dual role in the regulation of the balance between cell proliferation and death. Although in cancer cells downregulation of Nox1 increases caspase-3 activity and cell death,<sup>187</sup> necrosis has been positively linked to Nox1 in mouse fibroblasts exposed to TNF- $\alpha$ .<sup>188</sup> Moreover, Nox1, together with Nox4, appears to mediate arrest in S phase and senescence, a hallmark of atherosclerosis and cause of vascular dysfunction, in ECs exposed to resveratrol.<sup>189</sup>

Nox1 has also been implicated in migration in different cell types. In VSMCs stimulated with thrombin, Nox1-derived ROS mediate migration in part through regulation of calcium influx via l-type Ca<sup>2+</sup> channels.<sup>190</sup> Similarly, Nox1 mediates PDGF and bFGF-induced migration.<sup>169, 191</sup> In the case of PDGF-induced migration, one role of Nox1 is to mediate the activation of the cofilin phosphatase, Slingshot,<sup>192</sup> an activity shown to be necessary for migration after PDGF stimulation in VSMC.<sup>193</sup> When bFGF is the stimulus, Nox1 is required for activation of JNK and subsequent phosphorylation of the focal adhesion protein paxillin.<sup>191</sup> Another mechanism by which Nox1 may affect migration is via regulation of matrix metalloproteinases. In fibroblasts, it has been demonstrated that Nox1-derived ROS induce the promoter activity of matrix metalloproteinase-9 by a NF $\kappa$ B-mediated mechanism.<sup>194</sup> Finally, in ECs, Nox1 mediates migration through PPAR- $\alpha$ -dependent pathways.<sup>175</sup>

A causal role for Nox1 in vascular inflammation has also been investigated. Exposure of VSMCs to thrombin increases IL-6 secretion, Nox1 expression and NF $\kappa$ B nuclear translocation.<sup>195</sup> These effects may be mediated by Nox1, as they were inhibited by atorvastatin, which is known to downregulate this oxidase.<sup>195</sup> Furthermore, stimulation of VSMCs with TNF $\alpha$ , IL-1 $\beta$  or AGEs activates NF $\kappa$ B via Nox1 stimulation, which, at least in the case of AGEs, leads to upregulation of inducible NO synthase (iNOS).<sup>171, 196</sup> Nox1 is also expressed in macrophages and is required for foam cell formation;<sup>197</sup> thus, Nox1 mediates the vascular inflammatory response via several independent mechanisms.

## Nox 2

Because of its expression pattern in the cardiovascular system, Nox2 functions mainly to regulate endothelial and cardiac function. It is upregulated and/or activated by a number of vasoactive stimuli, including Ang II, atrial natriuretic peptide, shear stress, activation of adenosine A(2A) receptors, deletion of  $\alpha$ 1-AMP kinase, and nonselective nonsteroidal anti-inflammatory drugs.<sup>122, 198-202</sup> Nox2 has been implicated in the control of vessel tone, inflammation, EC proliferation and migration (and therefore angiogenesis), as well as cardiac hypertrophy and remodeling.

Because superoxide produced in the endothelium can rapidly neutralize NO, Nox2 is expected to impair NO-mediated relaxation and lead to vessel contraction. Indeed, a negative correlation is observed between Nox2 expression and endothelium-dependent relaxation in isolated aorta.<sup>203, 204</sup> In CGD patients who have a specific mutation in Nox2, flow-mediated vasodilation (which is largely dependent on NO) is significantly higher than in normal controls, corresponding with higher levels of serum nitrite and nitrate and suggesting that these patients have more bioactive NO.<sup>205</sup> However, Nox2 also appears to affect vasodilatation via other mechanisms as well. For example, activation of Nox2 by high glucose leads to eNOS downregulation in human ECs.<sup>206</sup> Conversely, activation of NO production can downregulate Nox2 expression,<sup>198</sup> suggesting tight regulation between these two important molecules.

Nox2 has also been closely linked to inflammation. Hwang et al.<sup>207</sup> showed that in ECs exposed to oscillatory shear stress (a stimulus known to promote inflammation and lesion formation), nox2 mRNA expression correlates with superoxide production and monocyte binding. This suggests that Nox2 activation may be proinflammatory, a conclusion supported by its role in macrophage activation,<sup>208</sup> and by the observation that in VSMCs treated with IL-17, p38MAPK-mediated Nox2 activation is required for release of the proinflammatory cytokines IL-6, G-CSF, GM-CSF and MCP-1.<sup>209</sup> Moreover, shear stress-dependent activation of p47phox-dependent NADPH oxidases, presumably Nox2 in ECs, causes eNOS uncoupling and xanthine/xanthine oxidase activation, one of many examples of ROS-induced ROS release.<sup>210,211</sup>

In contrast to Nox1, the role of Nox2 in proliferation and cell cycle progression is more controversial. In human ECs, it has been demonstrated that alone or in coordination with Nox4, Nox2 contributes to cell proliferation by a mechanism that involves p38MAPK and Akt.<sup>122</sup> Similarly, in adventitial fibroblasts, Nox2-ds-tat (formerly gp91phox-ds-tat), a peptide that prevents the interaction of Nox2 and p47phox, inhibits serum-induced proliferation.<sup>212</sup> Along the same lines, it was suggested that Nox2 prevents apoptosis by inhibition of caspase 3/7 activity.<sup>213</sup> On the other hand, in human microvascular ECs, Nox2 mediates serum starvation-induced cell cycle arrest by a mechanism that targets the expression of p21cip1 and p53.<sup>214</sup> Similarly, Ang II/AT1R-induced senescence in endothelial progenitor cells (EPCs) was shown to require Nox2.<sup>215</sup> These disparate observations suggest that Nox2 regulation of the cell cycle is cell and context specific.

Like Nox1, Nox2 has been implicated in migration, but in this case almost exclusively in ECs and monocytes/macrophages.<sup>216-218</sup> It also plays a role in EPC mobilization.<sup>219</sup> In ECs, Nox2 accumulation at the leading edge of migrating cells is dependent upon an intact actin cytoskeleton, and associates with both actin and the scaffold protein IQGAP1.<sup>216</sup> Depletion of IQGAP1 prevents Nox2 localization at the leading edge, reduces ROS production in migrating cells, and impairs migration. During EPC mobilization by erythropoietin, Nox2-derived ROS inactivate the protein-tyrosine phosphatase SHP-2 that activates STAT5.<sup>219</sup> In macrophages, Nox2 is required for LPS-induced, ER1/2-mediated MMP-9, -10, and -12 expression and cell migration.<sup>218</sup>

In the heart, Nox2 has been shown to participate in hypertrophy and remodeling after injury or aging.<sup>220, 221</sup> Indeed, it has been demonstrated that after Ang II/AT1R activation in cardiomyocytes, Nox2-derived superoxide induces hypertrophy by a mechanism that requires Akt activation<sup>222</sup> and Wnt-dependent pathways.<sup>223</sup> The profibrotic effects of Ang II are also mediated by Nox2. In rats overexpressing renin, septal wall thickness and perivascular fibrosis are increased. These responses are attenuated when animals are treated with the mineralocorticoid receptor blocker spironolactone, in part due to reduction in Nox2-mediated ROS production.<sup>224, 225</sup> The increase in Nox2 that accompanies mineralocorticoid receptor activation depends upon apoptosis signal-regulating kinase 1 (ASK1) activation by a mechanism yet to be determined.<sup>226</sup>

#### Nox 4

Nox4 is the most highly expressed Nox family member in all cells of the cardiovascular system, but its role is perhaps the most controversial. Its expression is marginally higher in arteries than in veins,<sup>227</sup> and significantly higher in the cerebral than systemic vasculature.<sup>228</sup> Of relevance, in different cell types, Nox4 is thought to regulate basal ROS production,<sup>229, 230</sup> suggesting that it may function to control the redox set-point and thus influence cellular metabolism.

Nox4 is upregulated by a wide variety of agonists and cellular stresses. In ECs, Nox4 is sensitive to mechanical forces: it has been reported to be downregulated by cyclic strain or pulsatile flow and upregulated by oscillatory shear stress.<sup>231, 232</sup> The proinflammatory mediators TNF- $\alpha$  and oxidized 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine (Ox-PAPC) increase the expression or activity of Nox4.<sup>125, 145</sup> In both endothelial and VSMCs, serum withdrawal also increases Nox4 expression.<sup>229, 233</sup> While growth-promoting agonists and drugs such as Ang II, PDGF, interleukin-1 $\beta$ , thrombin and phorbol myristate acetate downregulate Nox4 in systemic VSMCs and adventitial fibroblasts,<sup>176, 230, 234</sup> the proliferative hormone urotensin upregulates Nox4 in pulmonary artery SMCs.<sup>116</sup> Only TGF- $\beta$  has been consistently shown to upregulate Nox4 in all cell types tested, and to activate it acutely.<sup>235-237</sup> In fact, TGF- $\beta$  is responsible for activation of Nox4 in response to other stimuli, such as hypoxia.<sup>238</sup> Nox4 expression is also increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGF- $\beta$ 1-induced fibroblast differentiation into myofibroblasts.<sup>239</sup> The ability of Nox4 to respond to such diverse agonists suggests that it mediates many different cellular responses. Indeed, the variety of reported functions of Nox4 in the vasculature is truly remarkable. Nox4 has been linked to differentiation,<sup>229, 236, 240, 241</sup> migration,<sup>234, 242</sup> growth,<sup>74, 178, 235</sup> apoptosis,<sup>243</sup> senescence,<sup>244, 245</sup> proinflammatory responses,<sup>125, 131, 246</sup> and oxygen sensing.<sup>243, 247, 248</sup>

Evidence linking Nox4 to differentiation is abundant. Correlative studies show that upregulation of Nox4 in the redifferentiating VSMCs in the neointima coincides with induction of SMC differentiation markers,<sup>249</sup> while Nox4 in human atherosclerotic lesions correlates with smooth muscle  $\alpha$ -actin expression.<sup>250</sup> Moreover, electron microscopic analysis of SMCs in human atherosclerotic lesions indicate that only those VSMCs that maintain the contractile phenotype continue to express Nox4, while those that become de-differentiated lose Nox4.<sup>251</sup> Treatment of vascular adventitial fibroblasts with TGF- $\beta$ , which leads to differentiation to myofibroblasts, upregulates Nox4.<sup>252</sup> Conversely, Nox4 expression is high in preadipocytes and goes down as these cells differentiate into adipocytes.<sup>253</sup>

A mechanistic link between Nox4 and differentiation was established in a number of studies using strategies to attenuate Nox4 expression or transfection to overexpress it. Nox4 depletion leads to a loss of differentiation marker gene expression in adult VSMCs and a reduction in the conversion of cardiac fibroblasts to myofibroblasts.<sup>229, 236</sup> Similar results were found in mouse embryonic stem cells, in which Nox4 overexpression increased SMC differentiation markers and Nox4 depletion decreased the ability of the cells to differentiate to VSMCs.<sup>254</sup> In both adult and embryonic cells, Nox4 appears to exert its effects by regulating SMC-specific transcription factors serum response factor (SRF) and/or myocardin, in some cases via a p38MAPK-dependent mechanism.<sup>229, 254, 255</sup> Differentiation of mouse embryonic stem cells into cardiomyocytes has also been shown to require Nox4.<sup>241</sup> During differentiation into embryoid bodies, Nox4 expression initially decreases (day 1), and then gradually increases by day 8. Depletion of Nox4 using ribozymes reduces cardiac commitment (as assessed by a decrease in Nkx2.5 and MEF2C transcripts and a consequent reduction in ventricular myosin regulatory light chain 2) and dramatically reduces the percentage of spontaneously beating cells.<sup>241</sup>

Although agonists such as TGF- $\beta$  do not generally induce migration, clear evidence has accumulated implicating Nox4 in this biologically critical function. Inhibition of Nox4 using siRNA decreases PDGF-induced VSMC migration and wounding-activated EC migration.<sup>120, 256</sup> Paradoxically, overexpression of Nox4 also decreases migration in VSMCs stimulated with PDGF and in adventitial fibroblasts exposed to Ang II.<sup>120, 234</sup> One potential explanation for these apparently opposing observations lies in the fact that the Nox4/Poldip2 complex activates RhoA in SMCs, leading to focal adhesion turnover and

stress fiber formation.<sup>120</sup> Thus, too little Nox4 prevents focal adhesion formation, while too much Nox4 prevents focal adhesion dissolution, both of which are required for cell motility. However, Nox4 can also inhibit motility by oxidizing the sarco-/ER Ca<sup>2+</sup> ATPase (SERCA) and blocking the antimigratory action of NO, as was shown in VSMCs from Zucker obese rats.<sup>257</sup> The opposite is true in ECs, where overexpression of Nox4 upregulates eNOS, leading to an enhanced migratory response.<sup>258</sup>

Nox4 has also been implicated in progression through the cell cycle. It has been shown that both Nox2 and Nox4 are required for basal ROS production and EC proliferation.<sup>74</sup> Moreover, knockdown of Nox4 impairs EGF- and HIV-tat induced EC proliferation, while overexpression of Nox4 enhances EC growth.<sup>256, 259</sup> Mechanistically, overexpression of Nox4 in HEK293 cells has been shown to activate ERK1/2 and JNK and to enhance insulin-induced phosphorylation of p38MAPK, Akt, and GSK3 $\beta$ , any of which might contribute to proliferation.<sup>122</sup> In pulmonary artery SMCs, urotensin-induced proliferation, TGF $\beta$ -mediated hypoxia-induced proliferation,<sup>238</sup> and TGF $\beta$  induced proliferation<sup>235</sup> all require Nox4. Nox4 also mediates hypoxia-induced proliferation in pulmonary artery adventitial fibroblasts.<sup>260</sup> Growth-related signaling pathways downstream of Nox4 are incompletely understood, but may involve ERK1/2, p38MAPK, JNK and Akt.<sup>116, 235, 256, 261</sup> In addition, Nox4 inhibits protein tyrosine phosphatase 1B (PTP1B) in the ER, leading to a constant activation of Akt and ultimately increased EC proliferation.<sup>128, 258, 259</sup>

With regard to apoptosis, reports of the role of Nox4 are again mixed. TNF- $\alpha$ -induced apoptosis in microvascular ECs is dependent upon Nox4,<sup>262</sup> but apoptosis in response to serum deprivation is inhibited by Nox4 overexpression.<sup>256</sup> Knockdown of Nox4 inhibits BMP4-induced ROS production, phosphorylation of p38MAPK and JNK, and ultimately apoptosis in ECs.<sup>263</sup> In the heart, transgenic overexpression of Nox4 leads to increased apoptosis by 13 -14 months of age.<sup>139</sup> In adventitial fibroblasts, however, Nox4 inhibits hypoxia-induced apoptosis.<sup>260</sup>

Nox4 has also been implicated in senescence in a number of cell types and tissues,<sup>264, 265</sup> and this function of Nox4 may be an important contributor to cardiovascular diseases as well. Correlative data show that Nox4 expression increases with aging, particularly in the aorta, renal cortex and medulla.<sup>266-268</sup> Functionally, Nox4 has been linked to senescence in ECs and polyploidy in VSMCs.<sup>268</sup> In human umbilical vein ECs, knockdown of Nox4 reduces lifespan-associated increases in DNA damage and extends replicative lifespan independent of telomere shortening.<sup>269</sup> Ang II-induced senescence of EPCs is also dependent on Nox4,<sup>270</sup> and resveratrol, an antioxidant compound found in red wine, causes eventual endothelial senescence in a Nox4-dependent manner.<sup>189</sup> Of interest, overexpression of miR-146a, an miRNA whose expression increases with age, inhibits Nox4 protein expression and decreases senescence, although a causal link between these two endpoints was not established in this study.<sup>271</sup> In VSMCs, overexpression of Nox4 increases polyploidy and decreases expression of the chromosome passenger protein, survivin, that has previously been implicated in polyploidization, suggesting that excess Nox4 impairs passage through the cell cycle.<sup>268</sup> In the heart, NADPH oxidase inhibition normalizes age-dependent deceleration of shortening/re-lengthening of ventricular myocytes, although the responsible NADPH oxidase homologue has not been identified.<sup>272</sup>

Another potential role of Nox4 is in regulating proinflammatory/profibrotic gene expression in the vasculature. Urotensin-mediated induction of the pro-fibrotic gene PAI-1 in pulmonary artery SMCs is dependent upon Nox4, and downregulation of Nox4 blocks IL-8, MCP-1, LDLR and ROS production in ECs stimulated with Ox-PAPC.<sup>116, 125</sup> Basal production of PAI-1 in human umbilical vein ECs is also dependent upon Nox4 via p38MAPK and NF $\kappa$ B.<sup>273</sup> Another lipid agonist, palmitate, activates NF $\kappa$ B in ECs via a



TLR-4, Nox4-dependent mechanism.<sup>274</sup> A similar relationship between Nox4 and inflammatory gene expression was found in cardiomyocytes, where both Nox2 and Nox4 siRNA significantly inhibited TNF- $\alpha$ -induced upregulation of IL-1 $\beta$  and IL-6.<sup>275</sup> Similarly, activation of the Fc $\gamma$ RIIIa by CRP leads to Nox4-dependent ROS production in VSMCs, and depletion of Nox4 attenuates AP-1 and NF $\kappa$ B activation, as well as MCP-1, IL-6, and ET-1 production.<sup>276</sup> These types of responses would support a proatherosclerotic response in intact vessels.

Finally, Nox4 has been proposed to be an oxygen sensor. The most convincing data in this regard was obtained in skeletal muscle, where Nox4-dependent ROS production is proportional to pO<sub>2</sub> and leads to oxidation of ryanodine receptor cysteine thiols to increase Ca<sup>2+</sup> release in isolated sarcoplasmic reticulum.<sup>248</sup> Another molecule proposed to be downstream of oxygen-dependent Nox4 activation is TASK-1, a pH-sensitive K<sup>+</sup>-channel. Hypoxia inhibits TASK-1 activity, a response abolished by Nox4 siRNA.<sup>247</sup> Further studies are needed to determine if Nox4 has similar functions in the cardiovascular system.

The apparent ability of Nox4 to participate in such disparate cellular functions suggests that Nox4 may regulate fundamental cellular processes that contribute to each of these responses. Candidate pathways include, for example, the actin cytoskeleton, microtubules or cellular metabolism. Although some data exist linking Nox4 to the cytoskeleton, further investigation is needed to understand Nox4's fundamental role in cell biology.

## Nox5

The role of Nox5 in the cardiovascular system has not been well studied, in part due to the lack of suitable animal models. Our knowledge of the potential functions of Nox5 is thus derived mainly from cultured cells and isolated tissue, as well as *Drosophila*, which express an ortholog of Nox5. In the latter system, proctolin, a neuropeptide that causes smooth muscle contraction to move eggs out of the ovary, utilizes Nox5 to stimulate calcium flux and subsequent contraction.<sup>277</sup> In cultured human VSMCs, Nox5 is required for PDGF-induced proliferation.<sup>154</sup> Knockdown of Nox5 using siRNA impairs the ability of PDGF to activate the JAK/STAT pathway, thus providing a potential mechanism for its growth-modulating effects. In microvascular ECs, overexpression of Nox5S or Nox5L leads to a modest increase in proliferation and the formation of capillary-like structures, while depletion of Nox5 partially reduces thrombin-stimulated growth and tube formation.<sup>150</sup> In these cells, Nox5 expression is increased by endothelin-1 and Ang II,<sup>153</sup> and mediates activation of ERK1/2, but not p38MAPK or SAPK/JNK.<sup>153</sup> In intact vessels, overexpression of Nox5 via adenovirus paradoxically increases eNOS activity, but as expected, reduces bioavailable NO via inactivation by superoxide.<sup>278</sup> As a result, Nox5 overexpression in the endothelium impairs acetylcholine-induced endothelium-dependent relaxation and potentiates the contractile responses to phenylephrine.

## PHYSIOLOGY AND PATHOPHYSIOLOGY IN VIVO

### Hypertension

Twenty years ago, the concept that ROS may play a role in hypertension was established by studies in which administration of heparin-bound SOD to spontaneously hypertensive rats reduced blood pressure.<sup>279</sup> Numerous reports since then have verified these initial observations and implicate NADPH oxidases as important sources of ROS in blood pressure regulation. Many investigators found upregulation of NADPH oxidase subunits in hypertensive animals and a reversal of blood pressure and vascular remodeling with agents such as the superoxide dismutase mimetic tempol or the putative NADPH oxidase inhibitor apocynin (for review see <sup>280, 281</sup>). Human studies are limited mostly to showing increased activity of NADPH oxidases in hypertensive subjects and association studies linking

p22phox polymorphisms to hypertension.<sup>282</sup> However, it was not until the development of animals with specific genetic modifications of Nox subunits that the function of each homologue in hypertension could be rigorously interrogated (Table 2).

The possibility that Nox1 might be implicated in hypertension is supported by descriptive data and correlative evidence. Indeed, in the kidney Nox1 is upregulated in various models of hypertension, including renin transgenic rats,<sup>283</sup> 2-kidney 2-clip rats,<sup>284</sup> and Dahl salt sensitive rats on a high salt diet.<sup>285</sup> In rats infused with Ang II for 7 days, increased aortic Nox1 expression and hypertension are both blunted by administration of the PKC antagonist chelerythrine, which may also block PKC-dependent phosphorylation of p47phox.<sup>286</sup> While a causal role for Nox1 in the acute response to hypertension is supported by a number of studies, its importance in chronic hypertension is controversial. Mice in which Nox1 is genetically deleted initially respond to Ang II with an increase in blood pressure, but sustained blood pressure elevation is reduced in these mice compared to their wild-type controls.<sup>170, 287</sup> Endothelium-dependent relaxation to acetylcholine is impaired in wild type mice exposed to Ang II, but is unaffected in Nox1 knockout mice, suggesting that it is Nox1-derived superoxide that inhibits endothelial function in Ang II-induced hypertension.<sup>287</sup> The effect of Nox1 deletion on vascular hypertrophy is less clear: it has been reported to have no effect after a moderate dose of Ang II<sup>287</sup> or to reduce hypertrophy by reducing matrix deposition after a high dose of Ang II.<sup>170</sup> This discrepancy may result from the extent of hypertension induced in these animals, as overexpression of Nox1 specifically in smooth muscle cells exacerbates the blood pressure response and enhances vascular hypertrophy to the lower dose of Ang II.<sup>288</sup> Mechanistically, Nox1 overexpressing animals exhibit impaired endothelium-dependent relaxation that is a consequence of NO inactivation, implying cross-talk between cell types.<sup>289</sup> In contrast to these results, when Nox1 knockout animals are crossed with animals that express human renin and so have a chronically activated renin angiotensin system, no effect on blood pressure is observed even though ROS levels are reduced in plasma.<sup>290</sup> These results are difficult to reconcile with the acute studies, although as in all work involving genetically modified animals, possible compensation must be considered. It should be noted, however, that deletion of Nox1 in this chronic model significantly reduced expression of the proinflammatory molecule VCAM-1.

Studies using recently designed Nox inhibitors or other therapeutic agents indirectly support the genetic studies implicating Nox1 in hypertension. Treatment of aged spontaneously hypertensive rats (which have increased Nox1 and Nox2 but not Nox4 compared to WKY animals) with VAS2870, a Nox1/Nox4 inhibitor, improved endothelium-dependent relaxation.<sup>291</sup>

Nox2 was also studied extensively with regard to its potential role in blood pressure control, and is implicated in hypertension by virtue of its ability to inactivate NO and affect vessel wall remodeling, as well as its ability to influence central and renal control of blood pressure. It is upregulated in the vessel wall, the kidney and brain in many forms of hypertension.<sup>280, 292-295</sup> In Nox2 knockout mice, basal blood pressure is reduced, but the response to Ang II is intact.<sup>295</sup> However, infusion of Nox2ds-tat, an inhibitor of Nox2 interaction with p47phox, or genetic deletion of p47phox reduces the hypertensive effect of angiotensin II.<sup>296, 297</sup> In Dahl salt-sensitive rats treated with Nox2ds-tat, ROS and endothelial-dependent relaxation are normalized, but blood pressure is not affected,<sup>298</sup> while in mice with smooth muscle-specific overexpression of p22phox, the blood pressure response to Ang II is exacerbated.<sup>299</sup> It should be noted that interfering with p47phox or p22phox likely affects other Nox homologues in addition to Nox2, but the predominance of Nox2 in resistance arteries, the site of blood pressure control, supports the concept that Nox2 may play a role in hypertension. More recent work has shown that endothelial-specific overexpression of Nox2 does not affect basal blood pressure, but exacerbates the

hypertensive response to Ang II.<sup>300</sup> Because this mimics the upregulation of Nox2 that occurs in hypertension, these observations suggest that vascular Nox2 may not be involved in the initiation of hypertension, but that once upregulated, it can exacerbate the disease.

Another potential contribution of Nox2 to hypertension lies in its ability to regulate central and renal function. Work from the Davissou group showed that intracellular superoxide production in the subfornical organ of the brain mediates the blood pressure response to Ang II.<sup>301</sup> Experiments involving administration of small interfering RNAs to Nox2 or Nox4 to this region showed that both Nox2 and Nox4 are required for the full vasopressor effects of Ang II, while only Nox2 regulates the dipsogenic response.<sup>302</sup> Similarly, administration of p22phox antisense systemically abolishes the contribution of renal ROS production to the slow pressor response to Ang II.<sup>303</sup> One mechanism that has been proposed to be the signal that integrates brain, vascular and renal control of blood pressure is the T-cell. Guzik et al.<sup>304</sup> showed that the blood pressure response to Ang II is reduced in mice lacking T- and B-cells (Rag1<sup>-/-</sup> mice), and that adoptive transfer of T cells lacking p47phox only partially restores Ang II-dependent hypertension, while wild-type T cells completely reverse the response. Adoptive transfer of T cells derived from either gp91phox<sup>-/-</sup> or wild-type mice into Rag-1<sup>-/-</sup> mice fully restore the prothrombotic phenotype induced by Ang II, suggesting that T-cell derived ROS are not involved in the thrombotic response that accompanies hypertension.<sup>305</sup>

A role for Nox4 in hypertension is quite controversial. Nox4 has been reported to be elevated in the vasculature in several models of hypertension,<sup>283, 306</sup> but not in others.<sup>286</sup> This increase in Nox4 may be compensatory, however, since Ray et al.<sup>307</sup> found that endothelial-specific Nox4 overexpression enhances agonist-mediated vasodilation by inducing hyperpolarization and decreases blood pressure by ~10 mmHg. In agreement with a potential pro-vasodilatory effect of Nox4, Paravicini et al.<sup>308</sup> showed that Nox4 expression in basilar arteries (which is 10-fold higher than in systemic arteries<sup>228</sup> and is even greater in SHR) is associated with enhanced vasodilation in response to NADPH due to H<sub>2</sub>O<sub>2</sub>-mediated activation of BK(Ca) channels. In the brain, however, Nox4 in the subfornical organ clearly plays a role in the pressor response to Ang II.<sup>302</sup> Nox4 in the kidney may also mediate hypertension or the renal injury that accompanies hypertension, as it is upregulated in the macula densa and distal nephron in the SHR model, in the kidney of transgenic hypertensive rats overexpressing the Ren2 gene and in the cortex of rats treated with aldosterone.<sup>283, 309, 310</sup> However, Nox4 is unlikely to be a major regulator of blood pressure, since knockout of Nox4 has no effect on basal blood pressure.<sup>311</sup>

Taken together, data from multiple genetically modified animal models confirms distinct roles for Nox homologues in hypertension. Depending on the cause of hypertension (genetic, diet-induced, hormone-regulated), its duration (acute vs. chronic), the contributing organ (brain, kidney, vasculature), and the endpoint (blood pressure per se vs. end organ damage), different homologues may play a predominant role. Translating these observations to human hypertension is obviously the next step, but may have to await the development of safe, homologue-specific Nox inhibitors.

### Atherosclerosis

Even more so than with hypertension, a significant body of literature implicates ROS in the development of atherosclerosis, at least in animal models. While early work focused on oxidation of LDL, more recent studies tested the notion that NADPH oxidases in the vessel wall or in infiltrating macrophages and T-cells can affect atherogenesis on multiple levels. Conflicting reports in the literature leave the question of the relative importance of NADPH oxidases in this disease unsettled at this time.

Direct and indirect evidence for a role of Nox1 in atherogenesis is just now emerging. Gavazzi et al.<sup>312</sup> showed that Nox1 deficient mice infused with the proatherogenic peptide Ang II were protected from aortic dissection, potentially because they express higher levels of tissue inhibitor of metalloproteinase 1 than their wild type controls, suggesting that Nox1 may regulate turnover of extracellular matrix. Mice in which Nox1 is deleted also exhibit an attenuated neointimal response to arterial injury, due in part to reduced VSMC proliferation and migration.<sup>169</sup> As all of these cellular processes contribute to atherosclerosis, one would predict that atherosclerotic lesions formation would be inhibited in the absence of Nox1. In fact, in the only study to directly assess the role of Nox1 in atherosclerosis, in which Nox1 knockout mice were crossed into the ApoE<sup>-/-</sup> background and fed a high fat diet for 18 weeks, just such an outcome was observed.<sup>313</sup> Compared with ApoE<sup>-/-</sup> mice, lesion area in the ascending and thoracic aorta was reduced by 20-30% in Nox1<sup>-/-</sup>ApoE<sup>-/-</sup> mice, and macrophage content was reduced by about 50%. Following carotid injury in mice fed a chow diet, cell proliferation was decreased but collagen content was increased in the double knockout mice compared to ApoE<sup>-/-</sup> controls. Moreover, Niu et al.<sup>83</sup> showed that adenovirus-mediated overexpression of the Nox1 activator Noxa1 enhanced neointimal hyperplasia in a model of mouse carotid injury. It is worth noting that Noxa1 is increased in atherosclerotic lesions in ApoE<sup>-/-</sup> mice and in humans.<sup>83</sup> Additional evidence comes from a study showing that GKT136901, a newly-developed inhibitor of Nox1 and Nox4 oxidases, attenuates ROS generation and expression of the adhesion molecule CD44 and its principal ligand, hyaluronan, in atherosclerotic lesions.<sup>314</sup> Finally, a study using the janus kinase-2 inhibitor tyrphostin AG490 found that in ApoE<sup>-/-</sup> mice fed a high fat diet, Nox1, 2 and 4 were elevated in the aorta, and that treatment with the inhibitor reduced expression and activity of all three homologues and reduced lesion area.<sup>315</sup>

The contribution of Nox2 to atherosclerosis is controversial. Because mononuclear cells express high levels of Nox2 and macrophages are critical to lesion development, it was originally assumed that Nox2-derived ROS in macrophages would contribute to atherogenesis. This notion was supported by the observation that p22phox and Nox2 expression is high in atherosclerotic lesions, especially in the shoulder region of the plaque,<sup>250, 316-318</sup> and the findings that risk factors for atherosclerosis such as hypertension, oscillatory shear stress, and diabetes increase Nox2 expression in the vasculature.<sup>317</sup> However, Kirk et al.<sup>319</sup> called this assumption into question when they observed no difference in lesion formation in the ascending aortas of WT and Nox2 knockout mice fed a high fat diet or crossed with ApoE<sup>-/-</sup> mice. In contrast, a more recent study found evidence for reduced atherosclerotic burden in the descending aortas of Nox2<sup>-/-</sup>ApoE<sup>-/-</sup> mice compared with ApoE<sup>-/-</sup> mice fed a high fat diet.<sup>320</sup> These observations are similar to those in p47phox<sup>-/-</sup>ApoE<sup>-/-</sup> mice, in which lesion area in the ascending aorta is identical between p47phox<sup>-/-</sup>ApoE<sup>-/-</sup> mice and ApoE<sup>-/-</sup> mice,<sup>321</sup> but lesions in the descending aorta are reduced.<sup>322</sup> Lesion formation is also reduced in Nox2<sup>-/-</sup> mice subjected to arterial injury due to reduced cellular proliferation and reduced leukocyte accumulation.<sup>323</sup> Further experiments are necessary to define the contributions of cell-specific Nox2 to atherogenesis.

The mechanism for increased Nox expression and activity in atherosclerotic arteries is unclear. There is some evidence for ROS-dependent upregulation of NADPH oxidase subunits, suggesting a potential feed-forward mechanism that could exacerbate disease development.<sup>324</sup> Moreover, proatherogenic cytokines that activate NFκB, such as TNF-α, upregulate Nox2 and its cytosolic activators.<sup>325</sup> Ox-PAPC has been shown to increase Nox4 activity in ECs.<sup>125</sup> Because the role of the various Nox isoforms in atherosclerosis is complex, their regulation is likely to be complicated as well, and measurements of different homologues in identical experimental conditions is needed.

Compared to Nox2 and Nox4, much less is known about the role of Nox4 in lesion formation because mice with genetically modified Nox4 alleles have only recently become available. As is the case for Nox2, risk factors such as diabetes<sup>173, 326, 327</sup> and hypertension (see above) increase Nox4 expression in the vasculature. ApoE<sup>-/-</sup> mice treated with a high fat diet also exhibit enhanced expression of Nox4,<sup>315</sup> and Nox4 expression is high in human atherosclerotic lesions.<sup>250</sup> Interestingly, treatment with statins, which has been shown to be beneficial in atherosclerosis, decreases Nox4 expression.<sup>327, 328</sup> Functionally, Nox4 has been implicated in neointimal formation after vascular injury. Adenoviral-mediated knockdown of Nox4 at the time of carotid injury in Zucker rats reduced the extent of oxidation of SERCA and inhibited the development of the neointima.<sup>257</sup>

Even less is known about the role of Nox5 in atherosclerosis, largely because it is not expressed in rodents, making genetic manipulation much more difficult. However, Guzik et al.<sup>329</sup> showed that Nox5 expression is higher in human coronary arteries that exhibit coronary artery disease than in those that are disease free. This increased expression was accompanied by a 7-fold increase in calcium-dependent NADPH oxidase activity. Nox5 was observed in ECs in early lesions and in VSMCs in advanced lesions. This is one of the few human studies linking any Nox homologue to atherosclerosis.

### Angiogenesis and collateral formation

Growth of new blood vessels and expansion of existing ones is important not only during development, but can also occur in response to ischemic injury or during tumor development. Thus, depending on the context, these processes can either be beneficial or detrimental to the health of the organism. ROS both promote and inhibit blood vessel formation, in part depending on the amount of ROS produced. Yun et al.<sup>330</sup> proposed the “redox window” hypothesis, which states that while collateral formation requires ROS, either excess or insufficient ROS can inhibit vessel growth. A series of recent studies support a role for several of the Nox homologues in vessel formation and expansion.

Direct evidence for a role of Nox1 in angiogenesis comes from Garrido-Urbani et al.,<sup>175</sup> who showed that Nox1 is upregulated by proangiogenic factors, that mice deficient in Nox1 exhibit impaired angiogenesis in the matrigel assay, and that tumor vascularization is inhibited either by genetic deletion of Nox1 in the host or by treatment with the Nox inhibitor GKT136901. They further demonstrated that Nox1 appears to promote tumor angiogenesis by inhibiting the anti-angiogenic factor PPAR $\alpha$ . This supports earlier work by Arbiser et al.,<sup>331</sup> who found that Nox1-expressing NIH 3T3 cells injected into athymic mice cause highly vascularized, aggressive tumors, due to upregulation of VEGF and its receptors as well as increased MMP-9 activity. Komatsu et al.<sup>332</sup> found that Nox1 is necessary, but not sufficient, for induction of VEGF, indicating other factors are also involved.

Strong data exist both supporting and refuting a role for Nox2 in revascularization following ischemia. Ushio-Fukai’s laboratory showed that Nox2 expression and superoxide production are increased in wild-type mice up to 7 days following femoral artery ligation, and that the neovascularization response is reduced in Nox2 knockout mice.<sup>333</sup> Subsequent work suggests that it is Nox2 in bone marrow-derived cells that mediates the neovascularization response. Transplantation of wild-type bone marrow into Nox2-deficient mice restores blood flow recovery.<sup>334</sup> In contrast, Nox2 knockout mice are protected against the loss of neovascularization induced by hypercholesterolemia or diabetes, suggesting that the increase in Nox2 in response to these conditions is responsible for the observed impaired blood flow recovery in wild-type mice.<sup>335, 336</sup> Moreover, in the tumor angiogenesis matrigel plug model, loss of Nox2 has no effect on vasculogenesis.<sup>175</sup> Some of these apparent inconsistencies may relate to the level of ROS present basally in these models, per the redox



window hypothesis, or to mechanistic differences between different types of vascular neogenesis.

The role of Nox4 in angiogenesis also seems to be context-specific. Subcutaneous implantation of a matrigel plug loaded with bFGF into Nox4<sup>-/-</sup> mice leads to vascularization indistinguishable from that observed in wild type mice.<sup>175</sup> However, the angiogenesis (defined as an increase in capillary density) that accompanies pressure overload-induced cardiac hypertrophy is impaired in Nox4 null mice and enhanced in cardiomyocyte-specific Nox4 overexpressing mice compared to wild type controls.<sup>337</sup> This appears to be a consequence of Nox4-mediated activation of hypoxia inducible factor-1 and the release of vascular endothelial growth factor from cardiomyocytes, and is supported by in vitro studies in which overexpression of Nox4 enhanced, whereas dominant negative Nox4 or knockdown of Nox4 by siRNA impaired, tube formation in ECs.<sup>256</sup> Similarly, Craige et al.<sup>258</sup> showed that endothelial-specific overexpression of Nox4 leads to eNOS-dependent accelerated recovery from hindlimb ischemia and enhanced aortic capillary sprouting. Finally, Bhandarkar et al.<sup>338</sup> found that formation of hemangiomas (endothelial-derived neoplasias) induced by grafting polyoma middle T-transformed brain ECs from angiopoietin 2 heterozygotes into nude mice is greatly reduced by lentiviral expression of siNox4. Thus, the bulk of the evidence suggests that Nox4 is pro-angiogenic, although its role in tumor formation and angiogenesis remains unclear.

### Ischemia/reperfusion in the brain, heart and lungs

Transient or sustained ischemia in the heart, brain or lung can lead to infarct, stroke, or pulmonary hypertension, respectively. NADPH oxidases have been implicated in the pathophysiology of all three diseases in animal models, with similar mechanisms.

In Nox1 knockout mice, the effects of ischemia have only been studied in the brain. Transient occlusion (30 minutes) of the middle cerebral artery results in similar neurological score, cerebral infarct volume and edema volume in wild-type and Nox1<sup>-/-</sup> mice, but cortical infarct volume is much higher in brains of Nox1<sup>-/-</sup> mice compared to wild-type mice after 24 hours.<sup>339</sup> This suggests that Nox1 may actually limit infarct development following cerebral ischemia. However, in another study, a 1-hour, but not a 2-hour, occlusion of the middle cerebral artery resulted in attenuation of lesion size at 24 hours after induction of ischemia, accompanied by a significant improvement of neurological outcome, preservation of blood-brain barrier integrity and reduced cerebral edema. Clearly, more studies are needed to understand the role of Nox1 in stroke.<sup>340</sup>

More is known about the role of Nox2 in ischemic injury. Using a similar experimental procedure to that described for Nox1, transient middle cerebral artery occlusion followed by 22 hours of reperfusion, two groups showed that male Nox2<sup>-/-</sup> mice had a significantly smaller infarct volume compared with controls.<sup>341, 342</sup> Bone marrow transplants of Nox2<sup>-/-</sup> cells into wild type mice fail to induce this protection, and if wild type cells are injected into Nox2<sup>-/-</sup> mice, infarct volumes are not reduced, suggesting that both circulating Nox2 and neural or endothelial Nox2 contribute to ischemic injury in this model. Similarly, in a Langendorff-perfused, isolated mouse heart model of ischemia/reperfusion injury, ischemic preconditioning confers protection against necrotic injury in wild type, but not Nox2<sup>-/-</sup> hearts, indicating that in the heart as well as the brain, Nox2 is activated during either ischemia or reperfusion.<sup>343</sup> Finally, administration of intermittent hypoxia to mice induces a phenotype of pulmonary hypertension, including elevated right ventricular systolic pressure, right ventricular hypertrophy, and increased muscularization of the distal pulmonary vessels.<sup>344</sup> These changes are accompanied by an increase in p22phox and Nox4 expression, but interestingly are attenuated in Nox2<sup>-/-</sup> mice, suggesting that Nox2 may in fact regulate

expression of other NADPH oxidases, and raise the possibility of a feed-forward mechanism that exacerbates pulmonary hypertension.

Emerging evidence strongly suggests a link between Nox4 and ischemic injury. In pulmonary hypertension induced by chronic intermittent hypoxia, Nox4 is upregulated as a consequence of Nox2 activation.<sup>344</sup> Cortical Nox4 is upregulated in neurons early (24 hours) after middle cerebral artery occlusion, as well as 7-14 days later in newly formed capillaries in the peri-infarct region.<sup>345</sup> Transient upregulation of Nox4 in the cortex is also observed after endothelin-induced stroke.<sup>346</sup> A functional role for Nox4 in ischemia-induced neurodegeneration was proposed based on the observation that compared to wild type mice, Nox4 knockout mice have less oxidative stress, less blood-brain barrier leakage and less neuronal apoptosis after either transient occlusion of the middle cerebral artery or permanent stroke induced by cortical photothrombosis.<sup>347</sup> Importantly, post-stroke treatment with the putative Nox1/Nox4 inhibitor VAS2870 improves function, suggesting that Nox4 may be a viable therapeutic target for stroke.

Nox4 has also been implicated in cardiac performance following myocardial infarction (MI). Infanger et al.<sup>348</sup> found that surgical ligation of the left anterior descending coronary artery induces a two-fold increase in Nox4 expression in the paraventricular nucleus (PVN) of the brain compared to sham animals, correlating with the increased sympathoexcitation and cardiac dysfunction that occurs following MI. Adenoviral-mediated silencing of Nox in the PVN improves both ejection fraction and fractional shortening two weeks post-MI, apparently by reducing sympathetic outflow. Surprisingly, these outcomes appeared to be a result of superoxide, rather than hydrogen peroxide, signaling in the PVN.

### **Cardiac hypertrophy, post-myocardial infarction remodeling and heart failure**

As noted, Nox2 and Nox4 are the most highly expressed Nox homologues in the heart. While both are important for normal physiology, they seem to regulate distinct aspects of cardiac function and thus have discrete roles in heart disease.

Early work by Ajay Shah's group implicated Nox2 in cardiac hypertrophy and fibrosis. Administration of a subpressor dose of Ang II to wild type mice increases heart/body weight ratio, myocyte area, and cardiac collagen content in wild-type mice, but these responses are attenuated in Nox2<sup>-y</sup> mice.<sup>349</sup> A similar reduction in cardiac hypertrophy is observed in mice with a cardiomyocyte-specific deletion of Rac1, which as expected, exhibit reduced ROS formation and reduced NADPH oxidase assembly after Ang II infusion compared with wild type mice.<sup>350</sup> Johar et al.<sup>351</sup> showed that in response to a pressor dose of Ang II, interstitial fibrosis, as well as upregulation of matrix proteins and matrix metalloproteinase 2 activity, is inhibited in Nox2<sup>-y</sup> animals compared with controls. This Nox2-dependent profibrotic effect can be reversed by the aldosterone antagonist spironolactone. Cardiac remodeling also occurs after MI, and Doerries et al.<sup>352</sup> found that cardiomyocyte hypertrophy and apoptosis, as well as interstitial fibrosis, are attenuated in mice lacking p47phox. Similarly, Nox2 knockout mice subjected to permanent left coronary ligation have less post-MI remodeling (as reflected by these same parameters) at 4 weeks despite no difference in infarct size.<sup>220, 353</sup> Thus, in multiple murine models, there is a clear link between Nox2, cardiac remodeling and fibrosis.

This link is not incontrovertible, however, and in some situations, Nox4 appears to be involved in cardiac remodeling. In contrast to its role in Ang II-induced cardiac hypertrophy, Nox2 does not appear to contribute to hypertrophy induced by pressure overload. Two separate groups reported no difference in myocardial hypertrophy, cardiac mass or atrial natriuretic factor expression in Nox2<sup>-y</sup> and wild type mice subjected to aortic constriction.<sup>354, 355</sup> However, Nox2 does appear to contribute to interstitial hypertrophy and

contractile dysfunction in this model.<sup>356</sup> Several studies have reported an increase in Nox4 expression in the heart in response to pressure overload,<sup>337, 354, 357</sup> but only two groups have directly addressed the role of Nox4 in cardiac hypertrophy in this model. Using Nox4 knockout mice and cardiomyocyte-specific Nox4 overexpressors, Zhang et al.<sup>337</sup> showed that there were no basal differences in cardiac function, but after pressure overload induced by aortic constriction, mice in which Nox4 is deleted exhibited greater cardiac hypertrophy, contractile dysfunction and ventricular dilatation, while Nox4 transgenic mice were protected from these pathologies due to Nox4-dependent preservation of capillary density. However, Kuroda et al.<sup>357</sup> found different results using mice with cardiac-specific Nox4 deletion or overexpression. In their hands, pressure overload-induced cardiac hypertrophy and contractile dysfunction were reduced in Nox4 knockout mice in parallel with improved mitochondrial function, but exacerbated in Nox4 overexpressing mice. With the exception of the Nox4 transgenic mice generated by Kuroda et al., mice used in both studies were on the C57/Bl6 background. However, the extent of pressure overload induced in the latter study was greater than that seen by Zhang et al., raising the possibility that the role of Nox4 may change with the severity of disease.

## INTERACTIONS AMONG NOX PROTEINS

From the above summaries, it is evident that cells and tissues express multiple Nox homologues (Figure 2). Because their ultimate products (superoxide and hydrogen peroxide) are the same, the question arises as to the need for multiple oxidases in the same system. In some cases, their function appears to be redundant, while in others, each oxidase appears to regulate a distinct function. Moreover, there is cross-talk among oxidase systems, so that activation of Nox enzymes leads to subsequent activation of other sources of ROS, creating a positive feedback loop that can lead to vascular pathology.

In ECs, which express primarily Nox2 and Nox4, agonists often activate both enzymes and each Nox contributes similarly to the resultant physiological response. As an example, Nox2 and Nox4 are both required for the angiogenic response, and both participate in serum-induced proliferation.<sup>74, 256, 358</sup> Both are upregulated by oscillatory shear stress and downregulated by pulsatile stimuli.<sup>232</sup> Interestingly, in these cells, Nox2 and Nox4 are perinuclear, and colocalize with ER markers, perhaps explaining their redundant functions.<sup>74</sup> Moreover, knockdown of either of these genes using siRNA leads to an increase in the other, further supporting the notion that their functions are overlapping.<sup>359</sup> One report, however, implicates both Nox2 and Nox4 in the regulation of cell proliferation, but provides evidence that only Nox2 prevents apoptosis and modifies the cytoskeleton in ECs.<sup>213</sup> In VSMCs from large arteries, where Nox1 and Nox4 are expressed in distinct subcellular compartments (caveolae and the nucleus and focal adhesions, respectively),<sup>105</sup> they mediate completely different physiological functions. Nox1 is required for hypertrophy and proliferation,<sup>176, 181</sup> while Nox4 mediates cell differentiation.<sup>229, 254</sup> Both participate in cell migration, although via different mechanisms.<sup>120, 169</sup> In this cell type, Nox1 and Nox4 are also differentially responsive to agonists<sup>176, 229</sup>, most likely a consequence of the differences in their subcellular localization. This hypothesis is supported by observations in HEK293 cells transfected with Nox2 or Nox4, in which each enzyme exhibits a specific subcellular localization (plasma membrane and ER, respectively) and each enzyme responds to specific agonists (Ang II and TNF- $\alpha$  vs. no stimulation and insulin) and activates distinct signaling pathways.<sup>122</sup> Ultimately, the subcellular localization of different Nox enzymes and their activation by specific external stimuli may dictate the integrated response to Nox activation.

It is also likely that Nox enzymes contribute to cardiovascular pathophysiology in a coordinated fashion. This has been best established in hypertension, where, as noted, T-cells

coordinate the rise in blood pressure. In Ang II-induced hypertension, and brain, vascular and kidney Nox enzymes contribute to blood pressure regulation.<sup>304</sup> A similar coordinated response may occur in the vessel wall in atherosclerosis, where macrophages release cytokines in a Nox2-dependent manner, which then activate Nox1 and Nox2 in VSMCs and ECs, respectively.

It should also be noted that ROS production by Nox enzymes often activates other oxidase systems, thus amplifying the response. For example, in ECs exposed to oscillatory shear stress, xanthine oxidase activation depends upon prior activation of NADPH oxidases.<sup>210</sup> In addition, Ang II stimulation of these cells increases mitochondrial ROS production in a Nox-dependent manner.<sup>360</sup> In many forms of hypertension, ROS production by Nox enzymes leads to the formation of peroxynitrite, which uncouples eNOS, greatly increasing superoxide production.<sup>211</sup> The converse is true as well. Hydrogen peroxide from these sources, or even from initial Nox activation, can feedback to further activate Nox enzymes and amplify ROS production. Thus, Nox enzymes appear to act as both initiators and integrators of redox signaling via cross-talk with other ROS producing systems. The notion of cross-talk leads to an important concept: inappropriate feed forward activation of NADPH oxidases can lead to self-reinforcing loops that maintain and worsen pathology.

## CONCLUSION

Tremendous progress since the discovery of the Nox family of enzymes has firmly established their essential role in cardiovascular physiology and pathophysiology in animal models. The field has progressed to a point where the next major challenge is to assess their role in human disease, and then translate these findings into treatments, while continuing to dissect the molecular mechanisms and pathways specific to individual Nox homologues. Several research groups and companies have developed homologue-specific inhibitors that are only now beginning to undergo clinical testing. Because of the far-reaching effects of Nox enzyme activation, the therapeutic potential of these compounds is enormous compared with previously used, non-specific antioxidants. It is likely that these new agents will enable, for the first time, a true test of the redox hypothesis of human disease.

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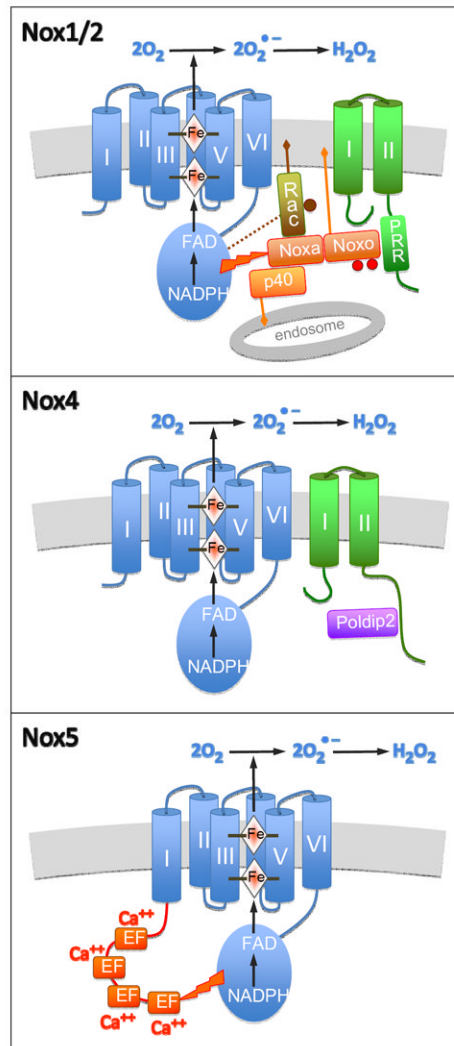
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## NON-STANDARD ABBREVIATIONS AND ACRONYMS

<b>AGEs</b>	Advanced glycation end-products
<b>Ang II</b>	Angiotensin II
<b>AP-1</b>	Activator protein 1
<b>ASK1</b>	Apoptosis signal-regulating kinase 1
<b>AT1R</b>	Angiotensin II type 1 receptor
<b>bFGF</b>	basic fibroblast growth factor
<b>BMP4</b>	Bone morphogenetic protein 4
<b>CGD</b>	Chronic granulomatous disease
<b>CRP</b>	C-reactive protein
<b>Duox</b>	Dual oxidase
<b>EGF</b>	Epidermal growth factor
<b>ECs</b>	Endothelial cells
<b>EPCs</b>	Endothelial progenitor cells
<b>eNOS</b>	endothelial nitric oxide synthase
<b>ER</b>	endoplasmic reticulum
<b>ERK</b>	Extracellular signal-regulated kinase
<b>ET-1</b>	Endothelin 1
<b>G/GM-CSF</b>	Granulocyte/granulocyte macrophage colony-stimulating factor
<b>GSK3</b>	Glycogen synthase kinase 3
<b>Hsp</b>	Heat shock protein
<b>IL</b>	Interleukin
<b>iNOS</b>	inducible NO synthase
<b>IQGAP1</b>	IQ motif-containing GTPase-activating protein 1
<b>JAK</b>	Janus kinase
<b>JNK</b>	c-Jun N-terminal kinase
<b>LDLR</b>	Low-density lipoprotein receptor
<b>LPS</b>	Lipopolysaccharide
<b>MAPK</b>	Mitogen-activated protein kinase
<b>MCP-1</b>	Monocyte chemotactic protein-1
<b>MEF2C</b>	Myocyte enhancer factor-2C
<b>MI</b>	myocardial infarction
<b>miRNA</b>	microRNA
<b>MMP</b>	Matrix metalloproteinase
<b>NF<math>\kappa</math>B</b>	Nuclear factor kappa B
<b>NO</b>	nitric oxide

<b>Nox</b>	NADPH oxidase
<b>Nox5S/L</b>	Nox5 short/long
<b>Noxa</b>	Nox activator
<b>Noxo</b>	Nox organizer
<b>PDI</b>	Protein disulfide isomerase
<b>oxLDL</b>	oxidized low-density lipoprotein
<b>Ox-PAPC</b>	oxidized 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine
<b>PAI-1</b>	Plasminogen activator inhibitor-1
<b>PVN</b>	paraventricular nucleus
<b>PDGF</b>	Platelet-derived growth factor
<b>PI3K</b>	phosphoinositide 3-kinase
<b>Poldip2</b>	Polymerase delta-interacting protein 2
<b>PKA/C</b>	Protein kinase A/C
<b>PPAR</b>	Peroxisome proliferator-activated receptor
<b>PTP1B</b>	Protein tyrosine phosphatase 1B
<b>ROS</b>	Reactive oxygen species
<b>SAPK</b>	Stress-activated protein kinase
<b>STAT</b>	Signal-transducer and activator of transcription
<b>SERCA</b>	sarco/endoplasmic reticulum calcium ATPase
<b>SRF</b>	serum response factor
<b>SH2/3</b>	Src homology 2/3
<b>SHP-2</b>	SH2 domain tyrosine phosphatase 2
<b>SHR</b>	Spontaneously hypertensive rat
<b>si/shRNA</b>	small interfering/short hairpin RNA
<b>TASK-1</b>	TWIK-related acid-sensitive potassium channel
<b>TGF</b>	Transforming growth factor
<b>TLR</b>	Toll-like receptor
<b>TNF</b>	Tumor necrosis factor
<b>TPR</b>	Tetratricopeptide repeat
<b>VSMCs</b>	Vascular smooth muscle cells
<b>VCAM</b>	vascular cell adhesion molecule
<b>WKY</b>	Wistar-Kyoto rats





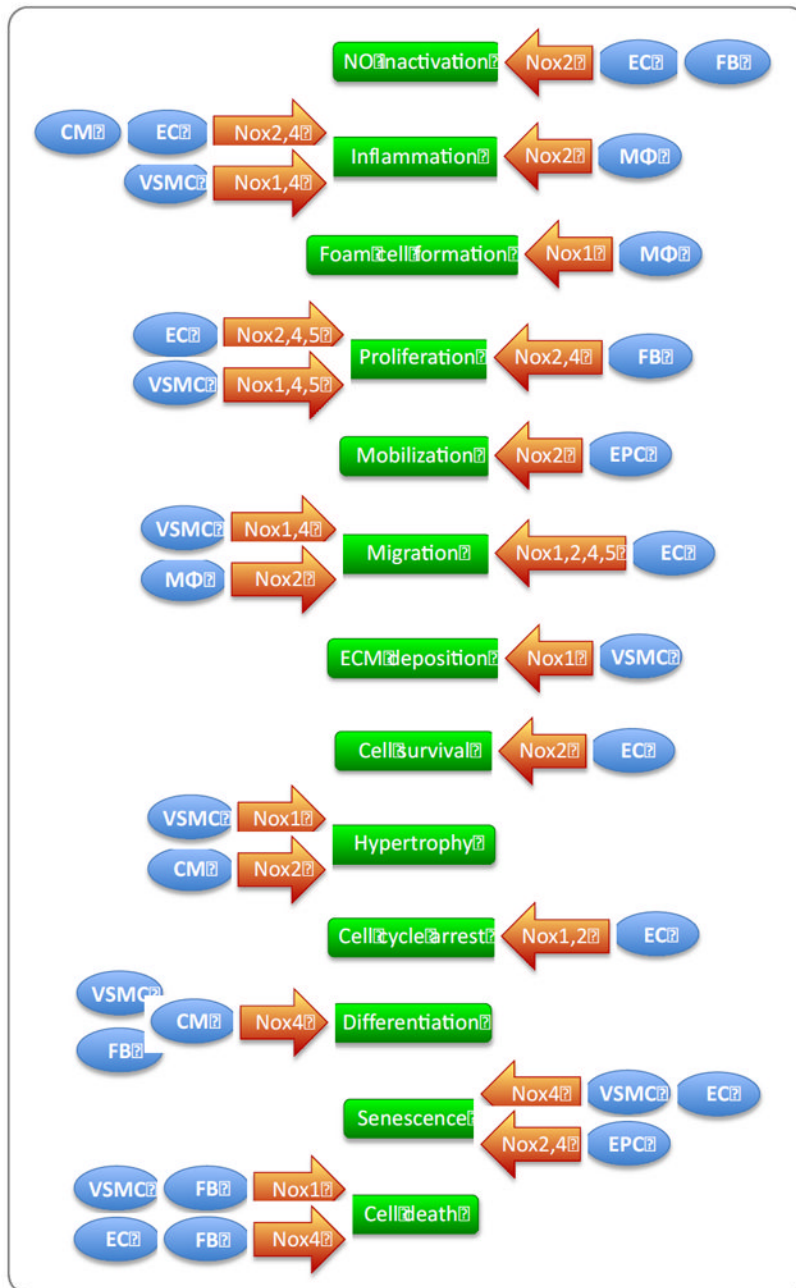
### Figure 1. Structure and activation of Nox enzymes

Catalytic Nox subunits, represented in blue, include an N-terminal domain composed of six transmembrane helices, numbered I-VI. Four histidine residues in helices III and V coordinate two heme iron atoms. A cytosolic C-terminal dehydrogenase domain includes an FAD cofactor and an NADPH substrate binding site. Upon activation, electrons are transferred from NADPH to FAD and across the membrane, via heme irons, to molecular oxygen, thus producing superoxide anion, which can be dismutated into hydrogen peroxide.

**Upper panel.** Both Nox1 and Nox2 (blue) form a complex with p22phox (green), with its two transmembrane domains and C-terminal proline-rich region (PRR). A cytosolic complex (orange), is composed of an organizer (Noxo1 or p47phox), an activator (Noxa1 or p67phox) and p40phox (only with p67phox). The organizer, stimulated by phosphorylation (red dots) in the case of p47phox, binds the proline-rich region of p22phox and membrane lipids. Likewise, p40phox binds lipids in endosomal membranes. Rac, activated by GTP (brown dot), binds membrane, Nox and Noxa. The latter subunit triggers FAD reduction.

**Middle panel.** Nox4 (blue) also forms a complex with p22phox (green). Its activity, constitutive in the absence of cytosolic subunits, can be increased by binding of Poldip2 to the cytosolic C-terminal of p22phox.

**Lower panel.** While Nox5S is composed of a catalytic subunit similar to the other oxidases (blue), Nox5L includes an additional N-terminal segment (red) with four EF-hands. Binding of cytosolic calcium to the EF hands triggers Nox5L activation.



**Figure 2. Physiological and pathophysiological responses mediated by Nox homologues in cardiovascular cells**

This summary of the roles of Nox enzymes in different cell types of the cardiovascular system illustrates the paradox of their mediating both specialized and redundant functions. VSMC, vascular smooth muscle cells; EC, endothelial cells; FB, fibroblasts; CM, cardiomyocytes, MΦ, macrophages; and EPC, endothelial progenitor cells.

Table 1

Selected examples of characterization and interventions affecting Nox subunits

	Cell/tissue distribution	Upregulators	Transcription factors	Subcellular localization	Genetic models	Inhibitors
<b>Nox1</b>	EC VSMC Adventitial fibroblasts Whole vessels <sup>4</sup> Cardiomyocytes <sup>361</sup>	Ang II PDGF PGF <sub>2α</sub> , LDL <sup>4</sup> TNF-α <sup>196</sup> Oscillatory shear stress BMP4 <sup>362</sup> Aldosterone+salt <sup>363</sup> IFN-γ <sup>364</sup> ET-1 <sup>365</sup> T3 <sup>177</sup> Urokinase <sup>78</sup> Oxidized LDL <sup>180</sup> Vascular injury <sup>4</sup>	AP-1 <sup>366</sup> ATF-1 <sup>367</sup> MEF2B <sup>368</sup> NFκB <sup>369</sup> STAT1/3 <sup>364</sup>	Plasma membrane Caveolae <sup>105</sup> Endosomes <sup>106</sup> Perinuclear <sup>74</sup>	Overexpressor <sup>289</sup> Knockout <sup>170, 287</sup>	Apocynin DPI GK-136901 ML171 Nox2dstat <sup>370</sup>
<b>Nox2</b>	EC VSMC Adventitial fibroblasts Whole vessels <sup>4</sup> Cardiomyocytes <sup>371</sup>	Ang II ET-1 TGF-β IFN-γ Oxidized LDL <sup>4</sup> Oscillatory shear stress <sup>232, 362</sup> Aldosterone +salt <sup>372</sup> Ischemia <sup>73</sup> Vascular injury <sup>4</sup>		Plasma membrane <sup>374</sup> Perinuclear <sup>2, 74, 375</sup> Nuclear pore <sup>75</sup>	Knockout <sup>376</sup>	Apocynin DPI Nox2dstat VAS2870 <sup>370</sup>
<b>Nox4</b>	EC VSMC Adventitial fibroblasts Whole vessels <sup>4</sup> Cardiac fibroblasts <sup>236</sup> Cardiomyocytes <sup>357</sup> Heart <sup>374</sup>	TGF-β <sup>235, 236, 255</sup> Thromboxane A2 TNF-α <sup>145, 377</sup> IFN-γ <sup>364</sup> Urokinase <sup>116</sup> Urokinase <sup>78</sup> Oscillatory shear stress <sup>232</sup> Hypoxia <sup>238</sup> Hyperoxia <sup>217</sup> Vascular injury <sup>4</sup>	E2F <sup>378</sup> NFκB <sup>369</sup> STAT1/3 <sup>364</sup> HIF-1α <sup>379</sup> Nrf2 <sup>380, 381</sup> Oct-1 <sup>381</sup>	ER <sup>259, 374</sup> Perinuclear <sup>74</sup> Nucleus <sup>105, 129, 217, 374</sup> Mitochondria <sup>139, 357, 374</sup> Focal adhesions <sup>105, 229</sup> Stress fibers <sup>229</sup> Cytoplasm <sup>382</sup>	Overexpressor <sup>139, 337</sup> Knockout <sup>337, 347, 357, 383</sup>	DPI GK-136901 Plumbag in VAS2870 <sup>370</sup>
<b>Nox5</b>	EC <sup>153, 377</sup> VSMC <sup>146, 154</sup> Cardiac FB <sup>236</sup>	Ang II ET-1 <sup>153</sup> Thromboxane A2 TNF-α <sup>377</sup> Atherosclerosis <sup>329</sup>		Plasma membrane Intracellular <sup>155, 162</sup>		DPI <sup>370</sup>

	Cell/tissue distribution	Upregulators	Transcription factors	Subcellular localization	Genetic models	Inhibitors
<b>p22phox</b>	EC VSMC Adventitial fibroblasts Whole vessels <sup>4</sup> Cardiomyocytes <sup>371</sup>	Ang II <sup>4, 384</sup> Thrombin <sup>385</sup> PDGF TGF- $\beta^4$ Urotensin <sup>116</sup> TNF- $\alpha^4, 145, 384$ IFN- $\gamma^386$ LPS <sup>387</sup> Pulsatile shear stress <sup>4</sup> Stretch + Ang II <sup>388</sup> Aldosterone + salt <sup>372</sup> Vascular injury <sup>4</sup>	AP-1 <sup>384</sup> STAT1/3 <sup>364</sup> NFkB <sup>389</sup>	Plasma membrane Caveolae Focal adhesions <sup>105</sup> Perinuclear <sup>2, 375</sup> Nucleus <sup>105, 217</sup> Nuclear pore <sup>75</sup>	Overexpressor <sup>299</sup> Point mutation <sup>121, 167</sup>	
<b>p47phox</b>	EC VSMC Adventitial fibroblasts Whole vessels <sup>4</sup> Cardiomyocytes <sup>371</sup>	Ang II <sup>4, 384</sup> Thrombin <sup>4</sup> TNF- $\alpha^4, 145, 384$ Aldosterone + salt <sup>372</sup>	HBPI <sup>67</sup> Ets-1 <sup>390</sup> STAT1/3 <sup>364</sup>	Perinuclear <sup>375</sup> Nuclear pore <sup>75</sup> Actin fibers <sup>32</sup>	Spontaneous null <sup>35</sup> Knockout <sup>36, 37</sup>	Apocynin Nox2dstat <sup>370</sup>
<b>p67phox</b>	EC VSMC Adventitial fibroblasts Whole vessels <sup>4</sup> Cardiomyocytes <sup>371</sup>	Ang II <sup>4, 384</sup> TNF- $\alpha^4, 145, 384$	STAT1/3 <sup>364</sup>	Perinuclear <sup>375</sup> Actin fibers <sup>32</sup>	Spontaneous mutant (requires confirmation) <sup>57</sup>	
<b>p40phox</b>	EC VSMC Whole vessels <sup>4, 67</sup>	Ang II <sup>25</sup>	HBPI <sup>67</sup>		Point mutation Knockout <sup>65</sup>	
<b>Nox01</b>	EC <sup>86</sup> Whole vessels <sup>85</sup>				Spontaneous null <sup>91</sup>	
<b>Noxal</b>	EC <sup>86, 145, 391</sup> VSMC <sup>83, 84</sup> Whole vessels <sup>85</sup>	Atherosclerosis <sup>83</sup>			Knockout <sup>97</sup>	



**Table 2**

Phenotypes of mice in which Nox enzymes are genetically modified

Genotype	Challenge	Phenotype	Reference
Nox1 <sup>-/-</sup>	Ang II	Acute increase of blood pressure & chronic reduction of blood pressure	170,287
Nox1 <sup>-/-</sup>	Ang II	No change in endothelium-dependent relaxation to acetylcholine	287
Nox1 <sup>-/-</sup>	High dose Ang II	Reduction in hypertrophy	170
Nox1 overexpression in VSMC	Ang II	Increased blood pressure & Increased hypertrophy	288
Nox1 overexpression in VSMC	Basal	Impaired endothelium-dependent relaxation	289
Nox1 <sup>-/-</sup>	30 min Brain ischemia	Unchanged neurological score, cerebral infarct volume and edema; higher cortical infarct volume	339
Nox1 <sup>-/-</sup>	1 hour Brain ischemia	Improved neurological outcome, preservation of blood-brain barrier integrity and reduced cerebral edema	340
Nox2 <sup>-/-</sup>	Ischemia/reperfusion	Smaller infarct volume	341, 342
Nox2 <sup>-/-</sup>	Ischemia/reperfusion	Loss of protection with ischemic preconditioning in the heart and brain	343
Nox2 <sup>-/-</sup>	Basal	Reduction in blood pressure	295
Nox2 <sup>-/-</sup>	Ang II	No change in blood pressure	295
p47phox <sup>-/-</sup>	Ang II	Reduced hypertensive effect	296
p22phox overexpression in VSMC	Ang II	Exacerbated hypertensive response	299
Nox2 overexpression in EC	Ang II	Exacerbated hypertensive response	300
p47phox deletion in T cells	Ang II	Reduction in Ang II-dependent hypertension	304
Nox4 overexpression in EC	Acetylcholine & histamine	Enhanced agonist-mediated relaxation	307
Nox4 <sup>-/-</sup>	Basal	No effect on blood pressure	311
Nox1 <sup>-/-</sup>	Ang II	Protected from aortic dissection	312
Nox1 <sup>-/-</sup>	Wire injury	Attenuated neointimal response	169
Nox1 <sup>-/-</sup>	ApoE <sup>-/-</sup>	Reduced lesion area and macrophage infiltration in the ascending and thoracic aorta	313
Nox2 <sup>-/-</sup>	ApoE <sup>-/-</sup> & high fat diet	No difference in lesion formation in the ascending aorta	319
Nox2 <sup>-/-</sup>	ApoE <sup>-/-</sup> & high fat diet	Reduced atherosclerosis in the descending aorta	320
Nox2 <sup>-/-</sup>	Femoral artery ligation	Reduced neovascularization	333
Nox2 <sup>-/-</sup> plus wt bone marrow	Femoral artery ligation	Restoration of collateral formation	334
Nox2 <sup>-/-</sup>	Arterial injury	Reduced lesion formation	323
Nox2 <sup>-/-</sup>	Suppressor & pressor dose of Ang II	Attenuation of the hypertrophic response	349, 351
Nox2 <sup>-/-</sup>	Left coronary ligation	Less post-MI remodeling & unchanged infarct size	220, 353
Nox2 <sup>-/-</sup>	Aortic constriction	No difference in myocardial hypertrophy	354, 355
Nox2 <sup>-/-</sup>	Aortic constriction	Less interstitial hypertrophy and contractile dysfunction	356
Nox2 <sup>-/-</sup>	Hypercholesterolemia or diabetes	Protection against the loss of neovascularization	335, 336

Genotype	Challenge	Phenotype	Reference
p47phox <sup>-/-</sup>	ApoE <sup>-/-</sup>	Unchanged lesions in the ascending aorta	321
p47phox <sup>-/-</sup>	ApoE <sup>-/-</sup>	Reduced lesions in the descending aorta	322
p47phox <sup>-/-</sup>	ApoE <sup>-/-</sup>	No effect on lesion formation in the aortic sinus	321, 322
p47phox <sup>-/-</sup>	Myocardial infarction	Attenuation of cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis	352
Nox4 <sup>-/-</sup>	bFGF	Unchanged neovascularization	175
Nox4 overexpression in cardiomyocytes	Pressure overload	Impaired angiogenesis	337
Nox4 overexpression in EC	Hindlimb ischemia	eNOS-dependent accelerated neovascularization	258
Nox4 <sup>-/-</sup>	Transient occlusion of middle cerebral artery	Less oxidative stress, blood-brain barrier leakage and neuronal apoptosis	347
Nox4 <sup>-/-</sup>	Aortic constriction	Greater cardiac hypertrophy, contractile dysfunction and ventricular dilatation	337
Nox4 overexpression in cardiomyocytes	Aortic constriction	Preservation of capillary density, protection against hypertrophy	337
Cardiac-specific Nox4 deletion	Aortic constriction	Reduced hypertrophy and contractile dysfunction	357