Endothelial Nox2 Overexpression Potentiates Vascular Oxidative Stress and Hemodynamic Response to Angiotensin II
Studies in Endothelial-Targeted Nox2 Transgenic Mice

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Abstract—Vascular disease states are associated with endothelial dysfunction and increased production of reactive oxygen species (ROS) derived from vascular NADPH oxidases in both vascular smooth muscle cells (VSMCs) and endothelial cells. Recent evidence suggests an important role for VSMC NADPH oxidases in vascular ROS production. However, it is unclear whether increased NADPH oxidase activity in endothelial cells alone is sufficient to alter overall vascular ROS production and hemodynamics. We sought to address these questions using transgenic mice with endothelial-targeted overexpression of the catalytic subunit of NADPH oxidase, Nox2. Aortas of Nox2 transgenic (Nox2-Tg) mice had increased total Nox2 mRNA and protein levels compared with wild-type littermates. Both p22phox mRNA and protein levels were also significantly elevated in Nox2-Tg aortas. Aortic superoxide production was significantly increased in Nox2-Tg mice compared with wild-type, but this difference was abolished by endothelial removal. Superoxide dismutase inhibition increased superoxide release and levels of Mn superoxide dismutase protein were significantly elevated in aortas from Nox2-Tg mice compared with wild type. Increased ROS production from endothelial Nox2 overexpression led to increased endothelial nitric oxide synthase protein and extracellular signal-regulated kinase 1/2 phosphorylation in transgenic aortas. Basal blood pressure was similar, however the pressor responses to both acute and chronic angiotensin II administration were significantly increased in Nox2-Tg mice compared with wild type. These results demonstrate that endothelial-targeted Nox2 overexpression is sufficient to increase vascular NADPH oxidase activity, activate downstream signaling pathways, and potentiate the hemodynamic response to angiotensin II, despite compensatory increases in vascular antioxidant enzymes. Endothelial cell Nox2-containing NADPH oxidase plays an important functional role in vascular redox signaling.

Key Words: NADPH oxidase ■ oxidative stress ■ endothelium ■ hemodynamics

Many vascular disease states are associated with an increase in the production of reactive oxygen species (ROS) in the vessel wall, which is associated with reduced nitric oxide (NO) bioavailability, leading to endothelial dysfunction.1,2 The production of ROS, in particular superoxide (O$_2^-$), reduces vascular NO bioavailability by scavenging. In addition, ROS are involved in the activation of important intracellular signaling molecules, such as mitogen-activated protein kinases (MAPKs), that regulate numerous signaling pathways involved in cell growth, gene expression and apoptosis.3,4

Recent studies have shown that the phagocyte-type NADPH oxidases are a major source of ROS in the vasculature.5-7 These oxidases are expressed in several different cell types in the vessel wall, including endothelial cells, vascular smooth muscle cells (VSMCs), adventitial fibroblasts, and inflammatory cells. NADPH oxidases are multisubunit flavoprotein complexes that, in phagocytic cells, consist of the membrane-bound cytochrome b$_{558}$, comprising the catalytic gp91$^\text{phox}$ subunit and the p22$^\text{phox}$ subunit, as well as 4 regulatory subunits; p47$^\text{phox}$, p67$^\text{phox}$, p40$^\text{phox}$, and rac1. Recently, novel gp91$^\text{phox}$ (renamed Nox2) homologs have been identified in nonphagocytic cells, named Nox1 to 5, including Nox1, Nox2, and Nox4 in the vasculature.8,9 NADPH oxidases in endothelial cells use Nox2 and Nox4, whereas VSMCs express only low levels of Nox2 and predominantly use Nox1 and Nox4.10

Compelling evidence implicates NADPH oxidase-derived ROS from VSMCs in various forms of hypertension, including angiotensin II (Ang II)-mediated hypertension.5,11,12 Mice deficient in Nox2 have reduced basal blood pressure compared with wild-types,9,13 whereas Nox1-deficient mice have...
similar basal blood pressure yet significantly suppressed pressor responses to Ang II. Furthermore, mice with vascular smooth muscle-targeted overexpression of either Nox1 or p22phox have increased vascular ROS production, vascular smooth muscle hypertrophy, and enhanced pressor responses to Ang II.

Although these studies establish an important role for NADPH oxidase-derived ROS in VSMCs, whether NADPH oxidase(s) in endothelial cells have specific functional importance in hypertension and other vascular disease states remains unclear. This is an important question, because ROS production in endothelial cells may have specific pathophysiologic roles in vascular diseases, for example, through NO scavenging and through oxidation of tetrahydrobiopterin, the essential endothelial nitric oxide synthase (eNOS) cofactor.16 Furthermore, VSMC and endothelial cell NADPH oxidases appear to have distinct intracellular characteristics that may lead to cell- and isoform-specific roles. In VSMCs, Nox1-containing NADPH oxidase is colocalized with caveolin on the cell surface and is involved in cell growth, whereas Nox4-containing NADPH oxidase is localized in focal adhesions and is implicated in cellular senescence.17 Endothelial NADPH oxidase is associated mainly with the cytoskeleton in a perinuclear distribution. Finally, it is unclear how vascular antioxidant defenses may respond to a primary increase in endothelial cell ROS production.

Accordingly, we aimed to determine the specific importance of endothelial Nox2-containing NADPH oxidase in regulating vascular oxidative stress and blood pressure by generating transgenic mice with targeted overexpression of Nox2 in endothelial cells.

Materials and Methods

Generation of Nox2 Transgenic Mice

To target Nox2 gene expression to the vascular endothelium, we constructed a human Nox2 transgene incorporating the murine Tie2 promoter and intronic enhancer (Figure 1A).19 The Tie2-Nox2 transgene underwent promiscuous microinjection into fertilized eggs from superovulated C57BL/6xCBA mice. Transgenic founders were then back-crossed on to the C57BL/6J strain. Fluorescent in situ hybridization was performed to ascertain the chromosomal site(s) of transgene integration into the mouse genome. For more details, see the online data supplement, available at http://circres.ahajournals.org. Experiments were performed in accordance with the Guidance on the Operation of Animals (Scientific Procedures) Act, 1986/UK) on mice housed in individually ventilated cages with 12-hour light/dark cycle and controlled temperature (20-22°C) and fed normal chow and water ad libitum.

Isolation of Murine Endothelial Cells

Primary endothelial cells were isolated from lungs by immunoselection with CD31 antibody–coated magnetic beads. See the online data supplement.

Quantitative Real-Time RT-PCR

Quantitative RT-PCR was performed on RNA extracted from lung, aorta, and spleen and on CD31-positive and -negative cells isolated from lung (endothelial and nonendothelial cells, respectively; n=3 per group) to measure human, murine, or total Nox2 mRNA expression. See the online data supplement.

Western Blot Analysis

Western blot analysis was performed to measure protein levels of Nox2, p22phox, (phospho-)extracellular signal-regulated kinase (ERK)1/2, (phospho-)p38 MAPK, (phospho-)JNK, eNOS, Cu/Zn superoxide dismutase (Cu/ZnSOD), extracellular superoxide dismutase (ecSOD), Mn superoxide dismutase (MnSOD), and catalase in aortas from wild-type and Nox2-Tg mice (n=4 per group; see the online data supplement).

Lucigenin-Enhanced Chemiluminescence

O$_2^-$ production was measured in both left ventricular (LV) homogenate (n=5 to 11 per group) and from intact whole or endothelium-denuded aortas using lucigenin-enhanced (5 to 20 μmol/L) chemiluminescence according to methods previously described.20 See the online data supplement.

Oxidative Fluorescent Microtopography

O$_2^-$ production was detected in tissue sections of mouse aorta (n=3 to 4 per group) using the fluorescent probe dihydroethidium (DHE), as previously described.20 See the online data supplement.
Isometric Tension Vasomotor Studies
Aortic vasomotor function was assessed using isometric tension studies in a wire myograph (Multi-Myograph 610M, Danish Myo Technology, Aarhus, Denmark); see the online data supplement.

Measurement of Arterial Blood Pressure
Blood pressure was measured in anesthetized wild-type and Nox2-Tg mice (n=5 to 8 per group) using the Millar catheter system; see the online data supplement.

In Vivo Ang II Infusion and Measurement of Systemic Blood Pressure
Wild-type and Nox2-Tg mice were implanted with osmotic minipumps containing Ang II (infusion rate 0.4 mg/kg per day) as previously described,21 and systolic blood pressure was obtained using the tail-cuff system in conscious animals; see the online data supplement.

Statistical Analysis
One-way ANOVA tests were used to compare data sets, with appropriate post hoc correction for multiple comparisons. P<0.05 was considered significant. Data are expressed as means±SEM.

Results

Generation and Characterization of Endothelial-Targeted Nox2 Transgenic Mice
Generation of Nox2-Tg mice was confirmed by PCR, RT-PCR, and fluorescent in situ hybridization analysis (Figure 1). Two independent Nox2-Tg founders were identified: Nox2-Tg1 and Nox2-Tg2. Both transmitted the transgene to offspring (Figure 1B) and expressed human Nox2 mRNA, as confirmed by RT-PCR using human-specific Nox2 primers (Figure 1C). Fluorescent in situ hybridization revealed a single transgenic integration site for both colonies (Figure 1D): on chromosome 17 for Nox2-Tg1 and on chromosome 12 for Nox2-Tg2 mice. The endogenous signal for Tie2 was observed on both chromosome 4 homologs for both colonies. Compared with littermate wild types, Nox2-Tg1 and Nox2-Tg2 mice were overtly normal.

Neither body weights (BWs) nor heart-weight-to-BW ratios were significantly different between Nox2-Tg mice and wild types (heart-weight-to-BW ratios, 4.61±0.1 versus 4.66±0.1, respectively).

Expression of Nox2 mRNA and Protein Production
We verified the endothelial specificity of human Nox2 transgene expression by measuring human Nox2 mRNA in primary endothelial cells and nonendothelial cells, isolated by immunomagnetic bead selection, from wild-type and Nox2-Tg mice. Importantly, human Nox2 transgene expression was present in only the endothelial cell population from Nox2-Tg mice; none was detected in the nonendothelial cell population nor in either the endothelial or nonendothelial cells from wild types (Figure 2A). As expected, mouse Nox2 mRNA was expressed in both the endothelial and nonendothelial populations from both groups.

We next quantified the relative levels of transgene expression between the 2 colonies of founder lines using fluorescence quantitative RT-PCR and primers specific for the transgenic human Nox2 mRNA in total RNA extracted from lung. Human Nox2 mRNA, not detected in wild types, was detected in all samples from Nox2-Tg1 and Nox2-Tg2 mice, but expression was significantly greater in Nox2-Tg1 compared with Nox2-Tg2 mice (16.8±1.8 versus 5.9±0.2 arbitrary units, respectively; n=6 per group, P<0.05). Nox2-Tg1 mice were selected for further experiments.

We next determined the levels of transgenic (human), native (mouse), and total (both) Nox2 mRNA expression in organs with different proportions of endothelial cells (lung, aorta, and spleen). Total Nox2 mRNA expression was ~2-fold higher in lung and ~3.5-fold higher in aorta from Nox2-Tg mice compared with wild types (P<0.05 for both; Figure 2B). In spleen, there was no significant difference in total Nox2 expression between Nox2-Tg and wild-type mice, reflecting the high contribution of native Nox2 expression in spleen and the proportionately fewer endothelial cells. Native
from Nox2-Tg mice compared with wild types \((P<0.05\); Figure 4A). Subcellular fractionation into particulate (membrane) and soluble (cytosolic) fractions revealed that the majority of the NADPH-stimulated \(O_2^-\) production was localized to the membrane in both Nox2-Tg and wild-type mice \((P<0.001\); Figure 4A). \(O_2^-\) production remained significantly elevated in LV membrane fractions from Nox2-Tg mice \((>2\)-fold; \(P<0.05\); Figure 4A). Importantly, \(O_2^-\) production was also increased, although to a lesser extent, in Nox2-Tg mice compared with counterpart wild types \((26.4\pm8.6 \text{ versus } 13.8\pm5.8 \text{ relative light units per second per microgram of protein, respectively})\), correlating with the relative levels of transgene expression between the 2 colonies. The NADPH oxidase flavoprotein inhibitor, diphenylene iodonium, abolished the membrane-derived \(O_2^-\) signal in both colonies \((P<0.001)\).

We next measured NADPH-stimulated \(O_2^-\) production in LV membrane fractions in the presence of the SOD inhibitor, diethyl-dithiocarbamate (DETC), to investigate the effects of potential changes in SOD activity in transgenic animals. SOD inhibition significantly increased the \(O_2^-\) signal in both groups but to a greater extent in Nox2-Tg mice, thereby further enhancing the difference between Nox2-Tg and wild types. This suggests that endogenous SOD activity was increased in Nox2-Tg animals (Figure 4B). Notably, \(O_2^-\) production was also significantly elevated in Nox2-Tg mice compared with counterpart wild types in the presence of DETC \((P<0.05\); data not shown).

To further evaluate the effects of Nox2 overexpression on endothelial NADPH oxidase activity, we measured \(O_2^-\) production in intact aortas using both lucigenin-enhanced chemiluminescence and DHE fluorescence. In intact aortas, NADPH-stimulated \(O_2^-\) production was significantly increased in Nox2-Tg mice compared with wild types \((P<0.05\); Figure 4C). This \(O_2^-\) signal was almost completely abolished by diphenylene iodonium \((P<0.001)\). Endothelial denudation abolished the difference in both basal and NADPH-stimulated \(O_2^-\) production between wild-type and Nox2-Tg aortas (Figure 4C). Furthermore, oxidative confocal microtopography revealed that endothelial DHE fluorescence in aortic tissue sections was increased 2-fold in Nox2-Tg compared with wild-type mice (Figure 4D). Together with the RT-PCR data demonstrating Nox2 transgene expression exclusively in endothelial cells, these results demonstrate the endothelial specificity of Nox2 overexpression and increased Nox2-derived \(O_2^-\) production in Nox2-Tg mice.

**Antioxidant Enzymes, eNOS, and MAPK Activation**

To investigate whether increased vascular ROS production in Nox2-Tg mice altered antioxidant defenses, we measured protein levels of antioxidant enzymes by Western blot. There was no change in protein levels of Cu/ZnSOD, ecSOD, or catalase between Nox2-Tg and wild-type aortas (Figure 5A, 5B, and 5D). However, MnSOD protein was significantly increased in Nox2-Tg aortas \((P<0.01\); Figure 5C). eNOS protein levels were also significantly elevated in Nox2-Tg aortas compared with wild-type \((P<0.05\); Figure 6A).

**Levels of p22phox Protein and mRNA Expression**

We then determined the effects of endothelial Nox2 overexpression on the levels of p22phox, the other NADPH oxidase catalytic subunit required to form the active multimeric enzyme. Western blot analysis revealed that p22phox protein was significantly elevated in lung homogenates from Nox2-Tg mice compared with wild types \((P<0.05\); Figure 3A and 3B). RT-PCR revealed that p22phox mRNA expression was also significantly increased in Nox2-Tg mice compared with wild types \((P<0.05\); Figure 3C).

**Superoxide Production**

To investigate whether endothelial Nox2 overexpression, and the associated increase in p22phox, would increase overall NADPH oxidase activity, we first measured \(O_2^-\) production in tissue lysates. NADPH-stimulated \(O_2^-\) production was significantly increased in unfraccionated (total) LV lysates...
To determine the effects of Nox2 overexpression on downstream signaling pathways, we examined MAPK phosphorylation. Phospho-ERK1/2, as a proportion of total ERK1/2, was significantly increased in Nox2-Tg animals compared with wild-type ($P<0.05$; Figure 6B). These data indicate that increased endothelial $O_2^{•-}$ production in Nox2-Tg mice is sufficient to activate downstream ROS-sensitive signaling pathways despite increased expression of vascular antioxidant enzymes. However, phospho-JNK and phospho–p38 MAPK levels were not significantly increased in Nox2-Tg mice (Figure 6C and 6D), suggesting that nonendothelial cell types dominate levels of these kinases in the vascular wall.

**Endothelial Vasomotor Function**

To determine whether increased endothelial $O_2^{•-}$ production would alter NO-mediated endothelial function in Nox2-Tg animals, we measured endothelium-dependent vasorelaxation of aortic rings. Contraction responses to phenylephrine were similar between wild-type and Nox2-Tg aortas. Endothelium-dependent and -independent relaxations to acetylcholine and sodium nitroprusside, respectively, were similar between the 2 groups (Figure I in the online data supplement).

**Hemodynamic Response to Acute Ang II Infusion**

To determine the functional importance of increased endothelial $O_2^{•-}$ production in Nox2-Tg mice, we first measured systolic blood pressure using a Millar catheter in the left carotid artery. Heart rates were similar between Nox2-Tg and wild-type animals (496±20 versus 538±32 bpm, respectively). Basal systolic blood pressure was similar between Nox2-Tg and wild-type mice (100.4±2.1 versus 101.1±1.4 mm Hg, respectively). Acute administration of Ang II (10 μg/kg) caused a significantly greater increase in systolic blood pressure in Nox2-Tg mice compared with wild types ($P<0.01$; Figure 7A and 7B), although heart rates remained no different (482±24 versus 545±35 bpm, respectively).

**Hemodynamic Response to Chronic In Vivo Ang II Infusion**

We then measured systemic blood pressure in nonanesthetized Ang II–infused (0.4 mg/kg per day) wild-type and
Nox2-Tg mice using the tail-cuff method. BW, heart rates, and heart-weight-to-BW ratios were similar between Ang II–infused Nox2-Tg and wild-type mice (heart rates, 736 ± 110 versus 704 ± 110 bpm, respectively; heart-weight-to-BW ratios, 5.6 ± 0.3 versus 5.5 ± 0.3, respectively). We again found no difference in basal systemic blood pressure, however chronic Ang II infusion significantly increased blood pressure in Nox2-Tg mice compared with wild types after just 3 days (P < 0.05; Figure 7C).

Superoxide Production in Ang II–Infused Mice
Ang II infusion caused a marked increase in O$_2^-$ production, measured by lucigenin chemiluminescence, in both wild-type and Nox2-Tg aortas compared with untreated animals (P < 0.05 for both; Figure 8A) but to a similar extent. It is likely that Ang II is activating VSMC NADPH oxidases, as well the endothelial oxidase, potentially masking any difference in endothelial NADPH oxidase-derived O$_2^-$ production between Nox2-Tg and wild-type aortas.

To test this hypothesis, we also measured O$_2^-$ production in aortic endothelium from Ang II–infused mice using DHE fluorescence. Importantly, endothelial fluorescence was significantly increased in Ang II–infused Nox2-Tg mice compared with wild types (Figure 8B), whereas total wall fluorescence was similar between the 2 groups.

To determine the effects of chronic Ang II infusion on downstream signaling pathways in Nox2-Tg mice, we measured aortic MAPK phosphorylation. Ang II infusion significantly increased phospho–p38 MAPK and phospho-JNK protein in both Nox2-Tg and wild-type mice compared with counterpart non–Ang II–infused animals (P < 0.01 for all; data not shown) although to a similar extent in both groups. Ang II infusion also significantly increased phospho-ERK1/2 protein levels in Ang II–treated wild-type mice compared with untreated counterparts but not in the Nox2-Tg group. However, as reported above, non–Ang II–treated Nox2-Tg mice had significantly increased phospho-ERK1/2 levels compared with corresponding wild types (Figure 6B).

Discussion
In this study, we describe a novel transgenic mouse model with targeted overexpression of Nox2 in the endothelium to investigate the specific role of endothelial Nox2-containing NADPH oxidase in regulating vascular oxidative stress and hemodynamic responses. The major findings of this study are as follows. (1) Endothelial-specific Nox2 overexpression leads to an increase in total Nox2 mRNA and protein in Nox2-Tg mice compared with wild-type littermates. (2) Endothelial Nox2 overexpression is associated with an increase in p22phox mRNA and protein levels, and these are together sufficient to augment endothelial NADPH oxidase activity. (3) This increase in vascular O$_2^-$ production leads to a compensatory upregulation of MnSOD and eNOS protein and activates downstream vascular signaling pathways, as evidenced by ERK1/2 phosphorylation. (4) Increased activity of endothelial Nox2-containing NADPH
oxidase does not alter basal blood pressure but significantly potentiates the pressor response to both acute and chronic Ang II stimulation.

These findings provide important insights into the role and importance of endothelial Nox2 in vascular ROS production. Previous studies have demonstrated that vascular NADPH oxidase-derived ROS play important roles in vascular diseases such as hypertension and atherosclerosis. However, the recent discovery of multiple Nox homologs, expressed in different vascular cell types, has raised critical questions about the specific roles of these enzymes in vascular function and blood pressure regulation. Because endothelial cells express Nox2 and Nox4, but not Nox1 or Nox3, we generated a novel transgenic mouse model directing human Nox2 transgene expression to the endothelium under the control of the murine Tie2 promotor. This endothelial-specific Nox2-Tg mouse enabled us to assess the importance of endothelial-specific Nox2/NADPH oxidase responses, independently from those of the vascular smooth muscle and adventitia. As predicted, human Nox2 mRNA expression was detected in only endothelial cells, not in nonendothelial cells, from Nox2-Tg animals. Total Nox2 mRNA was increased in only endothelial-rich tissues in these mice (in lung and aorta but not in spleen), and Nox2 protein was elevated in Nox2-Tg aortas compared with wild types. Importantly, native Nox2 mRNA expression was unchanged in Nox2-Tg mice, indicating that transgene expression had no effect on transcriptional regulation of the native gene.

An interesting finding in this study is that overexpression of Nox2 was associated with marked upregulation of p22phox mRNA expression and protein levels. Previous studies in phagocytes have reported that when assembled as the transmembrane heterodimer b558, Nox2 and p22phox are significantly more stable than either uncomplexed protein subunit, suggesting that transgenic overexpression of Nox2 may be accompanied by increased p22phox levels through effects on protein stability. Indeed, Laude et al recently reported that mice overexpressing vascular smooth muscle p22phox had increased aortic Nox1 protein. We now demonstrate that in endothelial cells Nox2, overexpression is also accompanied by increased p22phox mRNA expression, suggesting that the increase in p22phox protein is likely attributable to effects on gene expression, in addition to the potential increase in protein stability. Although the mechanism for this remains unclear, it is possible that regulation of p22phox mRNA expression is redox-sensitive. In support of this, recent in vitro experiments in endothelial cells have demonstrated that ROS can upregulate p22phox mRNA and protein and that this
Important upregulation can be prevented by NADPH oxidase inhibition. These observations suggest the potential for a "positive feedback" mechanism in endothelial cells, where small initial increases in Nox2 may lead to larger changes in overall NADPH oxidase activity through associated changes in p22phox mRNA and protein levels.

We demonstrated that overexpression of the Nox2 subunit, in conjunction with the associated increase in p22phox levels, was sufficient to increase total NADPH oxidase activity, as determined by measuring O$_2^-$ production in both LV lysates and in fresh, intact aorta. Importantly, O$_2^-$ production was similar in endothelium-denuded aorta from wild-type and Nox2-Tg mice, further supporting the endothelial specificity of the Nox2 overexpression. The observation that the majority of NADPH oxidase activity was detectable in the membrane fraction rather than the cytosol of LV lysates is consistent with previous reports and the known localization of the active NADPH oxidase complex on the membrane. Importantly, 2 separate founder lines expressing different levels of Nox2 mRNA both demonstrated an increase in O$_2^-$ production from membrane fractions that correlated with their level of transgene expression. Nox2-Tg$^*$ mice, which had significantly lower human Nox2 mRNA expression compared with Nox2-Tg$^1$ animals, demonstrated only a modest increase in NADPH-dependent O$_2^-$ production that was significant only with SOD inhibition. However, a significant 2-fold increase in NADPH-dependent O$_2^-$ release was detectable in both LV membrane fractions and intact aorta from Nox2-Tg$^1$ animals, suggesting that a sufficiently large increase in Nox2 expression is required to increase overall NADPH oxidase activity and overcome antioxidant defenses. Indeed, we observed significant activation of ERK1/2 in aortas from Nox2-Tg mice compared with wild types, characteristic of activation of downstream signaling molecules typical of increased NADPH oxidase activity and ROS production. We did not observe increased levels of either JNK or p38 MAPK phosphorylation, suggesting either that these pathways are less important in mediating Nox2 redox effects in endothelial cells or that the levels of JNK and p38 MAPK phosphorylation in other cell types in the vascular wall are sufficient to mask changes within the endothelium. It is well known that the renin–angiotensin system plays an important role in the control of arterial blood pressure. Numerous studies have shown that Ang II induces hypertension, which is in part mediated by vascular NADPH oxidase–
derived ROS. \textsuperscript{5,11,12,28,29} For example, basal blood pressure is reduced in Nox2-deficient mice. \textsuperscript{13,21} In the present study, we found no change in either the basal blood pressure or in endothelium-dependent vasorelaxations in Nox2-Tg mice, despite clear evidence of increased vascular ROS production and activation of redox-sensitive targets in the vascular wall. However, increased endothelial O$_2^-$ release in Nox2-Tg mice is likely to lead to compensatory mechanisms that tend to balance vascular redox status and preserve normal hemodynamics. Indeed, we observed an increase in the protein levels of MnSOD and eNOS, both of which may contribute to the maintenance of normal basal blood pressure and endothelium-dependent vasorelaxation through enhanced O$_2^-$ removal and NO production. Although SOD enzymatic activity was not specifically measured, SOD inhibition led to a greater increase in NADPH-dependent O$_2^-$ release in LV membrane fractions from Nox2-Tg compared with wild-type mice, suggesting that overall SOD activity is elevated in transgenic animals. This notion is supported by recent studies reporting the effects of vascular smooth muscle-targeted Nox1 or p22\textsuperscript{phox} overexpression that found no change in basal blood pressure in transgenic animals\textsuperscript{14,15}; MnSOD and eNOS protein levels were also increased in these animals.

We clearly demonstrated that both acute and chronic Ang II stimulation led to a significant pressor response in Nox2-Tg animals but not in wild-type littermates. As expected, Ang II led to a significant increase in vascular O$_2^-$ release, as has been previously reported. \textsuperscript{5,11,12,28,29} However aortic O$_2^-$ production was similar between Ang II–treated wild-type and Nox2-Tg animals, likely because Ang II is well known to increase expression of a number of NADPH oxidase subunits in the endothelium, medial VSMCs, and adventitia. This substantial increase in total vascular NADPH oxidase activity is likely to have masked any difference in endothelial-specific O$_2^-$ release. However, when we measured in situ O$_2^-$ generation specifically in the endothelium, we did observe a significant increase in Nox2-Tg mice compared with wild types. In line with the increased vascular oxidative stress, we detected significant MAPK phosphorylation in both Ang II–infused wild-type and Nox2-Tg mice compared with untreated animals, which was similar between groups. Again, this is likely to reflect the global increase in ROS production throughout the vessel wall in both wild-type and Nox2-Tg animals subjected to Ang II.

Our findings have important implications for understanding how endothelial NADPH oxidases contribute to ROS-dependent signaling in the vascular wall. Whereas vascular NADPH oxidases in general, and VSMC oxidases in particular, are known to play key roles, the importance of endothelial cell NADPH oxidases is less clear. Importantly, endothelial cells express Nox2 and Nox4 rather than Nox1 that predominates in VSMCs. \textsuperscript{17,18} and these different NADPH oxidases appear to locate in different cellular compartments, suggesting cell- and Nox-specific signaling roles for vascular NADPH oxidase–derived ROS. We now demonstrate that endothelial Nox2-containing NADPH oxidase is sufficient to alter total vascular ROS production and modulate the hemodynamic response to Ang II. Future studies need to address how endothelial and VSMC NADPH oxidases together contribute to vascular ROS production in the pathogenesis of vascular disease states, and how distinct or complementary roles for these different oxidases might provide new targets for novel therapies.

To conclude, our studies using a novel transgenic mouse model overexpressing Nox2 specifically in endothelium provide valuable new insights into the role of this Nox homolog in vascular ROS production. Endothelial-targeted overexpression of Nox2 leads to upregulation of p22\textsuperscript{phox} and increases vascular NADPH oxidase–derived O$_2^-$ production. Basal blood pressure in Nox2-Tg mice is likely preserved by compensatory mechanisms, including changes in MnSOD and eNOS. However, these compensatory mechanisms are overcome by Ang II administration, leading to a potentiated pressor response. Together, these data suggest that endothelial Nox2-containing NADPH oxidase plays a specific and critical role in vascular oxidative stress and in mediating the hemodynamic response to Ang II.

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Disclosures
None.

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METHODS

Generation of Nox2 transgenic mice

To target Nox2 gene expression to the vascular endothelium, we constructed a Nox2 transgene incorporating the murine Tie2 promoter \(^1\). A human Nox2 cDNA was subcloned to incorporate SSe 5357 I and MluI sites at 5' and 3' ends, respectively. The plasmid pHHNS, comprising the murine Tie2 promoter, SV40 polyA signal and a 10-kb intronic enhancer from the murine Tie2 gene, was a generous gift from U. Deutsch \(^1\). The human Nox2 cDNA was directionally cloned into the pHNNS plasmid at the Sse 5357 I and MluI sites. The resulting plasmid, pTie2-Nox2, was confirmed by DNA sequencing.

The Tie2-Nox2 transgene (Figure 1A) was excised from pTie2-Nox2 by SalI digestion and purified using sucrose density-gradient ultra-centrifugation (10–30% wt/vol). The Tie2-Nox2 fragment was dissolved in sterile injection buffer (5 mM Tris/Cl [pH 7.5], 0.1 mM EDTA) at 2 ng/µl for pronuclear microinjection into fertilized eggs from superovulated C57BL/6 x CBA mice. Potential transgenic founders were screened by PCR of genomic DNA from tail tips, using primers specific for human Nox2 (forward, 5'-CAGGAGTTCCAAGATGCCTG-3' reverse, 5'-GATTGGCCTGAGATTCATCC-3'), producing a 225-bp PCR product (Figure 1B in manuscript). Founder mice on the C57BL/6 x CBA background were then back-crossed on to the C57BL/6J strain. Mice were housed in individually ventilated cages with 12-hour light-dark cycle and controlled temperature (20-22°C), and fed normal chow and water \textit{ad libitum}. In all experiments
mice heterozygous for Nox2 overexpression in endothelial cells and their wild-type littermate controls were used, between 12–20 weeks of age. All studies involving laboratory animals were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 (HMSO, UK).

**Fluorescent in situ hybridization**

Fluorescent in situ hybridization (FISH) was performed to ascertain the chromosomal site(s) of transgene integration into the mouse genome. Metaphase chromosome preparations were made from murine embryonic fibroblasts (MEFs) prepared from 15-day-old embryos by trypsin-EDTA digestion of embryonic tissue. A Tie2-Nox2 probe was obtained by linearizing the pTie2-Nox2 plasmid with SalI. This probe was DIG labeled (by nick translation), hybridized and then detected with anti-digoxigenin-rhodamine (Roche, UK). FITC chromosome paints (emitting green fluorescence; Cambio Ltd, Cambridge, UK) specific to each chromosome were used to confirm the chromosomal location of transgene integration.

**Isolation of murine endothelial cells**

Primary endothelial cells were isolated from lungs by immunoselection with CD31-antibody-coated magnetic beads. Sheep anti-rat-IgG Dynabeads were coated with monoclonal rat anti-CD31 antibody (BD Biosciences) according to the manufacturer’s instructions. Lungs were harvested, washed, finely minced and digested in DMEM containing 0.18U/ml Liberase Blendzyme 3 (Roche) and 0.1 mg/ml DNase I for 60 minutes at 37 °C. The digested tissue was filtered through a 100 µm cell strainer and
centrifuged at 1000 rpm for 10 minutes. The cell pellet was washed with DMEM/10% FCS, centrifuged, resuspended in 1 ml DMEM/10% FCS and incubated with 1 x 10⁶ CD31-Ab-coated beads at 4°C for 30 minutes. Bead-bound cells were separated from non-bead-bound cells using a magnet. Bead-bound (CD31 positive) and non-bead-bound cells were resuspended in 2 ml EGM-2-MV (Cambrex) supplemented with hEGF, hydrocortisone, VEGF, hFGF-B, R³-IGF-1, ascorbic acid, gentamicin, amphotericin-B and 5% FCS and plated out. Cells were reselected twice, after 6-7 and 11-13 days in culture, with CD31-Ab-bound beads to purify the populations. Only the endothelial cell population tested positive for a range of endothelial markers including eNOS, Tie2 and CD102 protein measured using immunoblotting.

**Quantitative real-time reverse transcriptase-PCR**

Total RNA was extracted from frozen samples of lung, aorta and spleen, and from CD31 positive and negative cells harvested and isolated from lung (endothelial cells and non-endothelial cells, respectively; n ≥ 3 per group) by homogenization in 1ml of Trizol solution. Total tissue RNA (50ng), measured using the ribogreen method (Qiagen), was used in RT-PCR (Quantitect SYBR Green RTPCR, Qiagen) using the following primers for mRNA transcripts of NADPH oxidase subunits (5’ - 3’): human Nox2 forward, CAGGAGTTCCAAGATGCCTG; reverse, GATTGGCCTGAGATTCATCC; murine Nox2 forward, TCCGTATTGTGGGAGACTGG; reverse, AAAGGGCGTGACTCCAATC; murine and human Nox2 forward, TTCAGCTATGAGGTGGTGATG; reverse, GGTGCACAGCAAAGTGATTG; murine p22phox forward, GCTCATCTGTCTGCTGGAGTATC; reverse, GCTCATCTGTCTGCTGGAGTATC; reverse,
CAGATAGATCACA CACTGGCAATGG. Quantitative fluorescent real-time RT-PCR analysis was used to compare relative quantities of mRNA using the Rotor-Gene system (Corbett Research Ltd). Samples were processed in triplicate with a reverse transcriptase negative control reaction for each. Standards were prepared using serial dilutions of mRNA extracted from the spleen of one Nox2-Tg mouse. Quantification was performed using proprietary software to generate standard curves, expressing relative quantities of PCR products in the sample in arbitrary units relative to the standard curve. Mean values were calculated from triplicates to produce n=1.

**Western blot analysis**

Freshly isolated and cleaned thoracic aortas from wild-type and Nox2-Tg mice (n ≥ 4 per group) were opened longitudinally and rocked in 80µl RIPA buffer (50mmol/L Tris pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1mmol/L PMSF, 1mmol/L DTT) containing protease inhibitors (Complete, Boehringer Mannheim, Germany) for 1 hour at 4°C. Protein lysates (20-50µg) were resolved using SDS-PAGE, transferred to PVDF membranes and incubated with a 1:500 dilution of rabbit anti-human Nox2 polyclonal antibody or a 1:1000 rabbit anti-p22phox polyclonal antibody (kind gifts from Dr F. Wientjes, University College London). Other proteins were detected using anti-(phospho) extracellular signal-regulated protein kinases (ERK1/2) antibody, anti-(phospho) p38 MAPK antibody, anti-(phospho) c-Jun NH(2)-terminal kinase (JNK) antibody (all Cell Signaling Technology), anti-eNOS monoclonal antibody (Transduction Laboratories), rabbit anti-Cu/Zn superoxide dismutase (Cu/ZnSOD) antibody, rabbit anti-extracellular superoxide dismutase (ecSOD) antibody, rabbit anti-manganese superoxide
dismutase (MnSOD) antibody (all Stressgen Bioreagents) or rabbit anti-catalase antibody (Calbiochem) followed by appropriate HRP-conjugated secondary antibodies (Promega). Protein bands were visualized by chemiluminescence.

**Lucigenin-enhanced chemiluminescence detection of superoxide**

Basal $O_2^-$ production was measured in both left ventricular (LV) homogenate (n = 5 to 11 per group) and from intact whole or endothelial-denuded aortas using lucigenin (5 or 20 µmol/L)-enhanced chemiluminescence according to methods previously described \(^3\)-\(^5\). In brief, hearts were flushed with ice-cold Krebs-HEPES buffer, the LV excised and snap frozen in liquid nitrogen. Samples were homogenized in Krebs-HEPES buffer containing protease inhibitors (Complete, Boehringer Mannheim, Germany) at pH 7.4 to obtain total LV lysate. In some experiments, LV lysates were separated into particulate (membrane-associated) and soluble (cytosolic) fractions by ultra-centrifugation at 100 000g for 30 minutes according to methods previously described \(^6\), \(^7\). Freshly-cleaned and harvested thoracic aortas were opened longitudinally and equilibrated in Krebs-HEPES buffer gassed with 95% oxygen/ 5% carbon dioxide for 30 minutes at 37°C. Chemiluminescence of both LV lysates and intact aortas was measured in a FB12 luminometer (Berthold Detection Systems, Germany) at 37°C under basal conditions and then after addition of NADPH (300 µmol/L), followed by the NADPH oxidase flavoprotein inhibitor diphenyleneiodium (DPI; 10 µmol/L). Some particulate fractions were incubated in the presence of the superoxide dismutase (SOD) inhibitor diethyldithiocarbamate (DETC; 1mmol/L) for >30 minutes prior to the assay in order to inhibit endogenous SOD activity.
Oxidative fluorescent microtopography

$\text{O}_2^-$ production in tissue sections of mouse aorta (n = 3 to 4 per group) was detected using the fluorescent probe dihydroethidium (DHE), as previously described $^2,^3,^8$. Fresh segments of thoracic aorta were frozen in optimal cutting temperature compound. Cryosections (30µm) were incubated with Krebs-HEPES buffer for 30 minutes at 37°C then for a further 5 minutes with DHE (2 µmol/L; Molecular Probes). Images were obtained using a Bio-Rad laser scanning confocal microscope, equipped with a krypton/argon laser, using identical acquisition settings for each section. DHE fluorescence was quantified by automated image analysis using Image-Pro Plus software (Media Cybernecitics UK). DHE fluorescence from high power (x60) images was measured throughout the total vessel wall and specifically in the endothelium by quantifying fluorescence on the luminal side of the internal elastic lamina. For each vessel, mean fluorescence (area x intensity of red) was calculated from four separate high power fields taken in each quadrant of the vessel to produce n=1, and all experiments were performed blinded and in a batch design. Endothelial fluorescence was corrected for the length of endothelium in the field of view and expressed as a proportion of total vessel wall fluorescence as an internal control.

Isometric tension vasomotor studies

Aortic vasomotor function was assessed using isometric tension studies in a wire myograph (Multi-Myograph 610M, Danish Myo Technology, Aarhus, Denmark). Thoracic aortic rings (2 mm; n = 5 to 7 per group) were mounted in organ bath chambers
containing 5 ml of Krebs-Henseleit buffer (KHB [in mmol/l]: NaCl 120, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, glucose 5.5, indomethacin 0.01) at 37°C, gassed with 95% O₂/5% CO₂. Dose-response curves to phenylephrine (PE, 10⁻⁹ to 10⁻⁵ M), acetylcholine (ACh, 10⁻⁹ to 10⁻⁵ M) and sodium nitroprusside in the presence of L-NAME (SNP, 10⁻¹⁰ to 10⁻⁶ M, L-NAME; 10⁻⁴ M) were performed. Responses were expressed as a percentage of the precontracted tension.

**Measurement of arterial blood pressure**

Blood pressure was measured in wild-type and Nox2-Tg mice (n = 5-8 per group) by direct invasive methods under general anaesthesia using the Millar catheter system. Animals were anaesthetised using inhalational isoflurane, and warmed to 36.5°C. The left carotid artery was isolated via a midline incision in the neck and a 1.5F Millar catheter was introduced into the aorta. Experimental anaesthesia was monitored to maintain a respiratory rate of 80/min. Blood pressure data were collected continuously for at least 15 minutes of equilibration and after administration of Ang II (10 µg/kg) by intra-peritoneal (IP) injection.

**In vivo angiotensin II infusion and measurement of systemic blood pressure**

Wild-type and Nox2-Tg mice (13 weeks old) were anesthetized by inhalation of 2% isoflurane, 98% oxygen. Osmotic minipumps (Alza Corp) containing Ang II (infusion rate 0.4 mg/kg/day) were implanted in the midscapular region as previously described 21. Measurements of systolic blood pressure were obtained using the Visitech® computerised tail-cuff system in conscious mice following 5 training periods.
FIGURE LEGEND

**Figure IS:** Isometric tension studies in aortic rings from Nox2-Tg (filled circles) and wild-type mice (empty circles). (A) Vessel contractions to phenylephrine, (B) endothelium-dependent relaxations to acetylcholine (ACh) and (C) endothelium-independent relaxations to the NO donor, sodium nitroprusside (SNP; n = 5 to 7 animals per group).
REFERENCES


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monitor vascular superoxide as well as basal vascular nitric oxide production.


Figure 1S.

A

WT  Nox2-Tg

% contraction

-log [phenylephrine] (M)

B

% contraction

-log [ACh] (M)

C

% contraction

-log [SNP] (M)