Upregulation of Nox1 in vascular smooth muscle leads to impaired endothelium-dependent relaxation via eNOS uncoupling

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ELEVATIONS IN REACTIVE OXYGEN species (ROS) are present in many models of hypertension, including spontaneously hypertensive rats (30), DOCA-salt administration (1, 19), one- or two-kidney one-clip rats (6), and animals subjected to infusion of angiotensin II (ANG II) (27) or norepinephrine (22). Both superoxide and hydrogen peroxide (H2O2) are increased, whereas bioavailable nitric oxide (NO) is reduced (17, 19, 27). Increased tissue ROS levels mediate a number of pathologies associated with hypertension, including blood pressure regulation (18), endothelium-dependent control of vasomotor tone (26), renal cortical and medullary microvascular function (29), and end-organ damage (34).

There is some controversy about the source of the increased ROS production in hypertension, with evidence for involvement of NADPH oxidases (18), uncoupled endothelial nitric oxide synthase (eNOS) (19), xanthine oxidase (31), and mitochondria (7). Recent work suggests that substantial cross talk occurs among ROS-generating pathways. For example, in endothelial cells exposed to oscillatory shear stress, NADPH oxidase activity is required to maintain expression of xanthine oxidase, which then contributes to ROS production (24). Moreover, in ANG II-induced hypertension, blockade of NADPH oxidases using apocynin prevents subsequent mitochondrial H2O2 production (7). Finally, NADPH oxidase-derived superoxide contributes to peroxynitrite formation, leading to oxidation of tetrahydrobiopterin (BH4) and uncoupling of eNOS (19).

Most studies concerning cross talk among ROS pathways focus on the interaction of oxidant systems within endothelial cells. It is unclear whether ROS derived from medial smooth muscle cells (SMCs) can also influence endothelial sources of ROS and, indeed, if smooth muscle NADPH oxidases influence endothelial function. SMCs from large arteries express two NADPH oxidases, Nox1 and Nox4, but, of these, only Nox1 has been associated with hypertension (5, 9, 23, 34). Therefore, we investigated the possibility that ROS derived from Nox1 might uncouple eNOS, leading to endothelial dysfunction. To test this hypothesis, we used a transgenic mouse in which Nox1 is overexpressed specifically in SMCs. We found that these mice do in fact exhibit eNOS uncoupling and impaired endothelium-dependent relaxation, emphasizing the importance of cross talk among cell types, as well as enzyme systems, within the vessel wall.

MATERIALS AND METHODS

Animals. Mice used in this study were 6–7 mo old. TgSMCnox1 mice were generated on a C57BL/6 background and have significantly increased Nox1 levels in vasculature (5). Animals were genotyped by polymerase chain reactions (PCR) using DNA prepared from ear notch biopsies. Procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Male TgSMCnox1 and C57BL/6 wild-type (WT) mice were divided into the following two groups: control (saline infusion) and ANG II infused. The mice were anesthetized, and micro-osmotic pumps were implanted subcutaneously in the midscapular region. Pumps delivered either 0.9% saline or ANG II at a rate of 0.7 mg·kg−1·day−1. After 14 days, the animals were killed by CO2 inhalation, and their aortas were harvested.

For experiments involving BH4 administration, BH4 was compressed into rodent chow pellets, taking care to prevent oxidation of the compound by storing the pellets at −20°C and providing fresh pellets daily. The concentration of BH4 in the pellets (1 mg/g) was calculated to provide a dose of 5 mg/day based on an average mouse

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intake of 4–6 g of diet daily. BH₄ was administered 48 h before ANG II treatment and continued for the entire period of ANG II infusion.

Systolic blood pressure measurements. Systolic blood pressure was measured with the use of tail-cuff plethysmography (Visitech Systems). Blood pressure was measured two times before the implantation of osmotic pumps and on day 14 after pump placement. A set of 10 to 20 measurements was obtained for each animal, and the mean blood pressure was calculated.

Western blotting. Aortas were harvested and cleaned of fat and connective tissue. Proteins were extracted and analyzed for total eNOS by Western blotting as described previously (15). With the use of nonboiled lysates and low-temperature SDS-PAGE, eNOS dimers/monomers were immunoblotted (eNOS antibody 1:2,500; BD-Transduction Laboratory) as described elsewhere (15).

Detection of intracellular superoxide with high-performance liquid chromatography. To evaluate intracellular production of superoxide, we measured the formation of 2-hydroxyethidium from dihydrolipoic acid using high-performance liquid chromatography (HPLC) analysis (5). Hydroxyethidium was expressed per milligram protein. In some samples, polyethylene glycol (PEG)-superoxide dismutase (SOD, 100 U/ml) was added 1 h before addition of dihydrolipoic acid. PEG-SOD inhibited the dihydroethidium signal by 60%.

Measurement of NADPH oxidase activity. Aortas were dissected free of adventitia and then used to prepare membrane fractions, as described previously, with minor modifications (4). Protein aliquots (20 μg) were subjected to electron spin resonance spectroscopy (ESR) for quantitative measurement of NADPH (200 μmol/l)-dependent generation of superoxide radicals with 1 mmol/l 1-hydroxy-3-carboxypyrrolidine and 0.1 mmol/l diethylenetriamine pentaacetic acid in Chelex-treated phosphate-buffered saline. ESR spectra were recorded with an EMX ESR spectrometer (Bruker) and a super-high-Q microwave cavity exactly as described (4). SOD completely inhibited the NADPH-dependent signal.

Measurement of H₂O₂. H₂O₂ was measured using a fluorometric horseradish peroxidase-linked assay (Amplex red assay; Molecular termin content were performed using HPLC analysis and a differential reference transcribed with Superscript II enzyme (Invitrogen) using random primers. Message expression was quantitated on a Lightcycler instrument (Roche) with SYBR green dye and specific mouse Nox2, Nox4, or p22phox primers and normalized to luciferase and 18S RNA.

Statistics. Results are expressed as means ± SE. For multiple treatment groups, repeated-measures, two-way, or Latin-square design ANOVA followed by a Tukey-Kramer test was applied. For endothelium-dependent relaxation studies, one-way ANOVA with repeated measures followed by the Newman-Keuls test was used.

RESULTS

NADPH-dependent superoxide generation and H₂O₂ production are elevated in aortas from Tg℠SCMCnox1 mice. We previously showed that aortic Nox1 expression in Tg℠SCMCnox1 mice is increased four- to fivefold (5), similar to that observed in hypertensive mice or in injured carotid arteries (25, 32). To confirm that overexpression of Nox1 in SMCs increased NADPH oxidase-dependent ROS production in the aorta, we measured NADPH-dependent superoxide generation and production of H₂O₂, the more stable ROS that is formed immediately from superoxide by SOD. Basal NADPH oxidase activity was significantly elevated in transgenic mice (50 ± 10% over the level in WT mice) (Fig. 1A). Infusion of ANG II increased NADPH-dependent superoxide generation by twofold in WT mice and threefold in transgenic animals. Basal Nox1 and Nox4 expression were unchanged in Tg℠SCMCnox1 mice, whereas Nox4 was decreased following ANG II infusion in both sets of animals (Table 1).

These findings agree well with our previous measurements of superoxide production in aortas from these animals (5). Consistent with these observations, H₂O₂ production was also enhanced in aortas from Tg℠SCMCnox1 mice following ANG II infusion (Fig. 1B).

Nox1 overexpression impairs endothelium-dependent vasorelaxation: Improvement by apocynin. To determine if the increase in ROS production induced by Nox1 overexpression in smooth muscle impairs endothelium-dependent relaxation and increases blood pressure, we examined relaxation evoked by acetylcholine, which in the mouse thoracic aorta is entirely dependent on endothelial NO bioavailability (14), and measured blood pressure by tail cuff plethysmography. Consistent with our previous observations, a 2 wk infusion of ANG II increased blood pressure in WT mice from 101 ± 3 to 148 ± 4 mmHg (n = 20, P < 0.01), a response that was exacerbated in Tg℠SCMCnox1 (from 103 ± 3 to 174 ± 5 mmHg, n = 20, P < 0.01). Acetylcholine-induced endothelium-dependent relaxation in Tg℠SCMCnox1 mice was also impaired, with a maximal relaxation of 70 ± 3% compared with 90 ± 3% in WT mice (Fig. 2A). ANG II infusion inhibited endothelium-dependent vasodilatation in WT mice and worsened the impairment already present in Tg℠SCMCnox1 mice (P < 0.001, Fig. 2A). The NO donor, nitroglycerin, was used to test endothelium-independent relaxation. Endothelium-independent relaxations to nitroglycerin were similar in WT and Tg℠SCMCnox1 mice at baseline and were not altered by ANG II (Fig. 2D). The impairment in endothelium-dependent vasodilatation caused by Nox1 overexpression was completely prevented in aortas pretreated with the NADPH oxidase inhibitor apocynin, which inhibits assembly of the active NADPH oxidase complex. Aortic rings obtained from WT and Tg℠SCMCnox1 mice treated with apocynin showed virtually identical vascular reactivity to acetylcholine in the saline control and ANG II-treated groups (Fig. 2B). Preincubation with catalase also showed an improved relax-
by 20% in aortas of TgSMCnox1 mice under basal conditions (Fig. 3). ANG II decreased aortic NO levels in WT mice by 50 ± 4%, a response that was exacerbated in TgSMCnox1 mice (63 ± 5% decrease in bioavailable NO).

Decreased NO bioavailability could be caused by either oxidative inactivation of NO, presumably by Nox1-derived superoxide, or uncoupling of eNOS. Based on previous data showing eNOS uncoupling in hypertension (19), we measured eNOS dimers to estimate eNOS uncoupling. As shown in Fig. 4, compared with WT mice, TgSMCnox1 mice have a significantly smaller amount of dimerized aortic eNOS, indicating possible uncoupling of eNOS in these mice. Total eNOS was unchanged.

eNOS uncoupling can be caused by loss of BH4 due to oxidation (19), suggesting that TgSMCnox1 mice may have lower levels of BH4. To test this possibility, we measured reduced and oxidized aortic BH4 levels using HPLC. Indeed, reduced BH4 levels were decreased in TgSMCnox1 mice compared with WT mice, and ANG II decreased reduced BH4 to a greater extent in TgSMCnox1 mice than in WT mice (Fig. 5). Oxidized BH4 behaved in a reciprocal manner.

Effect of BH4 supplementation and N^G-nitro-L-arginine methyl ester on NO, superoxide production, eNOS uncoupling, and endothelium-dependent relaxation. Previous work has shown that oxidation of BH4 leads to eNOS uncoupling and generation of superoxide at the expense of NO. The loss of eNOS dimers in TgSMCnox1 mice thus suggests that superoxide derived from Nox1 may oxidize BH4 and uncouple eNOS, creating an additional source of superoxide in TgSMCnox1 mice. We tested this hypothesis by treating mice with oral BH4. BH4 administration beginning 2 days before ANG II had no effect on basal superoxide production but reduced superoxide in aortas of ANG II-infused TgSMCnox1 mice by 32 ± 4%, and by a smaller but significant amount in WT hypertensive mice (17 ± 5%, Fig. 6) without affecting Nox subunit expression (Table 1). As expected, this was accompanied by an increase in the amount of bioavailable NO (Fig. 3) and a partial reversal of the reduction in eNOS dimers caused by ANG II, indicative of improved eNOS coupling (Fig. 4). BH4 treatment restored NO levels in TgSMCnox1 mouse aortas to 97 ± 4% of the values observed in WT untreated mice. It also partially restored bioavailable NO in aortas of both WT (33 ± 5%) and TgSMCnox1 mice treated with ANG II (48 ± 6%) and significantly improved endothelium-dependent relaxation in TgSMCnox1 mice both basi-

![Fig. 1. Production of aortic reactive oxygen species (ROS) in transgenic mice overexpressing NADPH oxidase (Nox) 1 in smooth muscle cells (TgSMCnox1) and wild-type (WT) mice. A: NADPH-dependent superoxide generation in aortas of TgSMCnox1 and C57BL/6 mice measured by electron spin oxidase microscopy (ESR) with 1-hydroxy-3-carboxypyrrolidine (CPH) as a spin probe. Mice were infused with saline or ANG II (0.7 mg·kg⁻¹·day⁻¹) for 14 days. Aortas were harvested, and membrane fractions were prepared by differential centrifugation. Superoxide production was measured using ESR after stimulation with 200 μmol/l of NADPH. Values were calculated as the difference of signals obtained from membranes in the presence and absence of NADPH. Values represent means ± SE for 6 animals/group. *Significant increase in superoxide level vs. saline-infused WT (P < 0.05); †significant increase in superoxide level vs. saline-infused WT mice (P < 0.001); ‡significant increase in superoxide level vs. ANG II-infused WT mice (P < 0.001). B: aortic H2O2 release in TgSMCnox1 and C57BL/6 mice. Amplex Red was used to measure the release of H2O2 in C57BL/6 mice (open bars) and TgSMCnox1 mice (filled bars) following infusion with saline or ANG II for 14 days. Values represent means ± SE for a minimum of 6 animals/group. *Significant increase in H2O2 level vs. saline-infused WT (P < 0.001); †significant increase vs. saline-infused TgSMCnox1 mice (P < 0.001); ‡significant increase vs. ANG II-infused WT mice (P < 0.001).

Table 1. Expression of Nox subunit mRNA in WT and TgSMCnox1 mice treated with ANG II and tetrahydrobiopterin

<table>
<thead>
<tr>
<th></th>
<th>p22^Nox^</th>
<th>Nox2</th>
<th>Nox4</th>
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<tbody>
<tr>
<td>WT</td>
<td>200 ± 20</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>TgSMCnox1</td>
<td>190 ± 10</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>WT + BH4</td>
<td>200 ± 10</td>
<td>2.1</td>
<td>0.2</td>
</tr>
<tr>
<td>TgSMCnox1 + BH4</td>
<td>210 ± 20</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td>WT + ANG II</td>
<td>190 ± 10</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>TgSMCnox1 + ANG II</td>
<td>380 ± 50</td>
<td>4.1</td>
<td>0.3</td>
</tr>
<tr>
<td>WT + ANG II + BH4</td>
<td>400 ± 30</td>
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<td>0.3</td>
</tr>
<tr>
<td>TgSMCnox1 + ANG II + BH4</td>
<td>360 ± 30</td>
<td>3.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Values are means ± SE for 5 independent experiments. Units are copies/10^6 copies of 18S. Nox, NADPH oxidase; WT, wild type; TgSMCnox1, transgenic mice expressing Nox1 in smooth muscle cells; BH4, tetrahydrobiopterin. *P &lt; 0.05 compared with corresponding control; †P &lt; 0.05 compared with WT ANG II.</td>
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Fig. 2. Nox1 overexpression impairs endothelium-dependent vasorelaxation in a NADPH oxidase-dependent manner in mice. After the infusion of vehicle or ANG II for 13 days, vascular reactivity to the endothelium-dependent agonist acetylcholine (A–C) or endothelium-independent NO donor nitroglycerin (D) was measured in aortic rings from TgSMCnox1 and C57BL/6 mice. [ACh], acetylcholine concentration. Rings were preconstricted with PGF2α (1 μmol/l). To study the effect of the NADPH oxidase inhibitor apocynin, rings from the same aortas were preincubated for 30 min with apocynin (0.05 mmol/l), which was added to the organ bath (B). In C, mice were treated with tetrahydrobiopterin (BH4) in the food during ANG II or saline infusion before vasodilator measurements. Data are expressed as means ± SE. Lack of error bar indicates the error was smaller than the symbol; n = 4–10 mice/group. A: *P < 0.001, TgSMCnox1 with ANG II vs. WT and WT with ANG II; #P < 0.05, TgSMCnox1 with ANG II vs. TgSMCnox1; †P < 0.05 vs. WT; NS indicates not significant. C: #P < 0.05, TgSMCnox1 with ANG II vs. TgSMCnox1; *P < 0.001, TgSMCnox1 with ANG II vs. WT; NS indicates not significant. C: #P < 0.05, TgSMCnox1 with ANG II vs. TgSMCnox1; *P < 0.05, TgSMCnox1 vs. WT; NS indicates not significant. C: #P < 0.05, TgSMCnox1 with ANG II vs. TgSMCnox1; *P < 0.0001, TgSMCnox1 with ANG II vs. TgSMCnox1; †P < 0.0001, TgSMCnox1 with ANG II plus BH4; †P < 0.0001, TgSMCnox1 treated with BH4.

Fig. 3. Nitric oxide (NO) levels in TgSMCnox1 mice after ANG II-induced hypertension: effect of BH4 supplementation. Calcium ionophore-stimulated NO production was estimated by ESR using the spin-trap iron diethyldithiocarbamate (Fe[DETC]2). Four to five 2-mm segments of thoracic aorta were incubated with Fe[DETC]2 and A-23187 (10 μmol/l) for 60 min, snap-frozen in liquid nitrogen, and subjected to ESR analysis. Mean data for 5–10 animals/group are shown. *Significant decrease in NO vs. saline-infused mice of the same genotype (P < 0.001); # significant increase in NO vs. saline-infused TgSMCnox1 (P < 0.01); †P < 0.05 vs. WT mice with the same treatment; +P < 0.05 vs. ANG II alone in mice of the same genotype.

Fig. 4. Endothelial NO synthase (eNOS) protein expression. Representative Western blots (A) for dimer, monomer, and total eNOS using a low-temperature gel (3 different gels, but proteins from the same aortic homogenates). B: densitometry values for eNOS dimer/total eNOS ratio (n = 3). +P < 0.05 vs. untreated WT mice; *P < 0.001 vs. untreated WT mice; **P < 0.05 vs. WT ANG II; −P < 0.05 vs. WT ANG II; and #P < 0.01 vs. TgSMCnox1 mice with ANG II.
measured superoxide production using dihydroethidium. L-NAME treatment reduced superoxide production in response to ANG II by 20 ± 5% in aortas from WT mice and by 68 ± 9% in aortas from TgSMCnox1 mice (Fig. 7). These data suggest that superoxide production caused by overexpression of Nox1 is derived in part from subsequent uncoupling of eNOS.

DISCUSSION

In this study, we provide evidence that ROS production by Nox1 in medial SMCs can uncouple eNOS, leading to a self-perpetuating cycle of superoxide production and impaired endothelium-dependent relaxation. This finding is similar to that reported in p47phox knockout mice but is the first to implicate smooth muscle sources of ROS as an initiating signal.

Our results show that BH4 supplementation decreases aortic ROS production in aortas of TgSMCnox1 mice, in which the initial imbalance is NADPH oxidase overexpression. The kindling radical hypothesis originally proposed by Landmesser et al. (19) suggests that physiological levels of ROS can become pathological by creating a positive feedback loop in which ROS generation is enhanced by activation of another source of ROS. In addition to leading to eNOS uncoupling, NADPH oxidase activation is upstream of xanthine oxidase in endothelial cells exposed to shear stress, as well as mitochondrial ROS generation in aortic endothelial cells stimulated with ANG II (7, 24). The fact that apocynin corrects the impairment in endothelium-dependent relaxation confirms that the primary defect in TgSMCnox1 mice is overactivation of Nox1, but the correction of superoxide production and endothelium-dependent relaxation, as well as the partial reversal of hypertension, by BH4 supplementation indicates that uncoupled eNOS, or possibly uncoupling of other NOS isoforms, also plays a role. Thus this system is an additional example of cross talk among oxidant systems and, uniquely, even across cell types.

Nox1 is upregulated in a number of pathological conditions, including hypertension (25, 33) and neointimal formation after vascular injury (32). Increased expression and activity of this protein appears to contribute to blood pressure regulation. In mice in which Nox1 has been genetically deleted, ANG II infusion leads to an initial elevation in blood pressure similar to that observed in WT mice but does not cause sustained hypertension (9, 23). In contrast, TgSMCnox1 mice exhibit an enhanced blood pressure response (5). It is interesting to speculate that the failure to sustain blood pressure in Nox1 knockout mice may result from the secondary eNOS uncoupling demonstrated here. It should be noted, however, that, because BH4 treatment reduces blood pressure, this reduction in blood pressure itself could contribute to the observed responses.

Nox1 also contributes to the increased medial thickness that is often observed in large arteries of hypertensive animals. ANG II-induced medial hypertrophy is impaired in Nox1 knockout mice but enhanced in TgSMCnox1 mice (5, 9). Some controversy exists over the mechanism by which Nox1 regulates medial hypertrophy, because effects on both extracellular matrix and smooth muscle proliferation are observed in Nox1-deficient animals (9, 10, 21, 23). ROS regulate extracellular matrix integrity by upregulating a tissue inhibitor of metalloproteinases (10) and by inactivating matrix metalloproteinases (28). In the latter case, the extracellular ROS produced by uncoupled eNOS observed in this study is a likely source of oxidizing equivalents.
Our observation that endothelium-dependent relaxation is impaired in Tg^\text{SMCNOX1}^+ mice is consistent with similar findings in previous studies using hypertensive mice with altered NADPH oxidase activity (18, 20). However, the difference between previous studies and the model used here is that ROS produced by Nox1 in SMCs is largely intracellular and therefore might not be expected to reach the endothelium. One explanation may be that NO produced by eNOS is inactivated upon entering SMCs, thus preventing relaxation. This does not explain, however, the observation that eNOS itself becomes uncoupled. It is possible that the peroxynitrite formed upon NO interaction with Nox1-derived superoxide may exit SMCs and act to uncouple eNOS. Peroxynitrite has a low pKa, which allows the neutral/acid form of peroxynitrite to cross membranes (11). Indeed, extracellular supplementation of peroxynitrite explains, however, the observation that eNOS itself becomes uncoupled. Peroxynitrite has a low pKa, which allows the neutral/acid form of peroxynitrite to cross membranes (11). Indeed, extracellular supplementation of peroxynitrite leads to eNOS uncoupling (16). Alternatively, because H2O2 does not oxidize BH4 directly (16), Nox1-derived H2O2 may act on endothelial cells, or promote inflammatory cell infiltration, by initiating a ROS cascade that uncouples eNOS. This mechanism was recently demonstrated for H2O2 treatment of endothelial cells, which results in activation of NADPH oxidase and eNOS uncoupling (2). Another potential explanation is that the ROS produced by SMC Nox1 is either transported extracellularly, perhaps via activation of an ion channel, or stimulates the production of ROS by another source, such as xanthine oxidase. Finally, it has been suggested that H2O2 can induce eNOS protein expression (20), which conceivably contributes to enhanced uncoupling. However, we did not observe any change in eNOS expression in Tg^\text{SMCNOX1}^+ mice.

Based on these observations, we suggest that cross talk between oxidant systems occurs at multiple levels within the vessel wall. Not only can ROS produced within a cell type induce ROS formation by other enzymatic pathways, but ROS produced by cells in adjacent tissue can affect redox signaling as well. Such information may explain why in vitro studies of isolated cells do not always recapitulate in vivo responses. ROS generation in the vasculature regulates many aspects of cardiovascular diseases, and understanding the mechanisms controlling the generation of these important signaling molecules is of utmost importance in developing therapies that target pathophysiological mechanisms while keeping intact critical physiological signals.

ACKNOWLEDGMENTS

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DISCLOSURES

K. K. Griendling and J. D. Lambeth share a patent on Nox1.

REFERENCES


