Endothelial Nox4 NADPH Oxidase Enhances Vasodilatation and Reduces Blood Pressure In Vivo

Robin Ray, Colin E. Murdoch, Minshu Wang, Celio X. Santos, Min Zhang, Sara Alom-Ruiz, Narayana Anilkumar, Alexandre Ouattara, Alison C. Cave, Simon J. Walker, David J. Grieve, Rebecca L. Charles, Philip Eaton, Alison C. Brewer, Ajay M. Shah

Objective—Increased reactive oxygen species (ROS) production is involved in the pathophysiology of endothelial dysfunction. NADPH oxidase-4 (Nox4) is a ROS-generating enzyme expressed in the endothelium, levels of which increase in pathological settings. Recent studies indicate that it generates predominantly hydrogen peroxide (H₂O₂), but its role in vivo remains unclear.

Methods and Results—We generated transgenic mice with endothelium-targeted Nox4 overexpression (Tg) to study the in vivo role of Nox4. Tg demonstrated significantly greater acetylcholine- or histamine-induced vasodilatation than wild-type littermates. This resulted from increased H₂O₂ production and H₂O₂-induced hyperpolarization but not altered nitric oxide bioactivity. Tg had lower systemic blood pressure than wild-type littermates, which was normalized by antioxidants.

Conclusion—Endothelial Nox4 exerts potentially beneficial effects on vasodilator function and blood pressure that are attributable to H₂O₂ production. These effects contrast markedly with those reported for Nox1 and Nox2, which involve superoxide-mediated inactivation of nitric oxide. Our results suggest that therapeutic strategies to modulate ROS production in vascular disease may need to separately target individual Nox isoforms. (Arterioscler Thromb Vasc Biol. 2011;31:1368-1376.)

Key Words: blood pressure ■ endothelial function ■ reactive oxygen species ■ vasodilation ■ NADPH oxidase

Endothelial dysfunction, in particular the impairment of endothelium-dependent vasodilatation, is involved in the pathophysiology of hypertension and atherosclerosis. Increased reactive oxygen species (ROS) production contributes to endothelial dysfunction through the inactivation of endothelium-derived nitric oxide (NO) by superoxide and by ROS-dependent modulation of intracellular signaling pathways.

See accompanying article on page 1255

Nox family NADPH oxidases are major vascular ROS sources. Five distinct NADPH oxidases based on separate Nox catalytic isoforms (NADPH oxidase-1 [Nox1] to Nox5) have been described. Vascular cells express multiple Nox isoforms. Endothelial cells coexpress Nox2 (also known as gp91phox) and Nox4, whereas vascular smooth muscle cells express mainly Nox4 and Nox1, at least in rodents. Although Nox1, Nox2, and Nox4 exist as membrane-bound heterodimers with a p22phox subunit, there are important differences between Nox4 and Nox1/2. Nox1 and Nox2 are normally quiescent and are activated by specific agonists (eg, angiotensin II [Ang II]) in a process that involves binding of regulatory cytosolic subunits. This initiates production of superoxide, which may react with NO or be converted to hydrogen peroxide (H₂O₂) in the presence of superoxide dismutases (SODs). In contrast, Nox4 constitutively generates ROS without requiring activating cytosolic factors. Furthermore, multiple recent reports indicate that Nox4 generates predominantly H₂O₂ rather than superoxide.

Nox1 and Nox2 are implicated in the development of endothelial dysfunction and hypertension in preclinical models and humans. Nox2 knockout mice have reduced endothelial dysfunction during experimental hypertension, whereas p47phox knockout mice (which have deficient Nox1 and Nox2 activation) exhibit reduced hypertension and preserved endothelial function after chronic Ang II treatment. Nox1 knockout mice have a reduced basal blood pressure (BP), preserved endothelial function, and a reduced hypertensive response to Ang II infusion. Vascular smooth muscle-targeted Nox1 transgenic mice have enhanced vascular hypertrophy, hypertension, and endothelial dysfunction. Nox4 levels in the endothelium and vessel wall...
increase in several disease settings, but the effects of Nox4 on vascular function in vivo remain unknown.

In this study, we investigated the effects of a targeted increase in endothelial Nox4 expression on vascular function in vivo.

Methods
For detailed Methods, please see the supplemental materials, available online at http://atvb.ahajournals.org.

Procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (United Kingdom Home Office).

Generation of Transgenic Mice With Endothelium-Targeted Nox4 Overexpression
Endothelium-targeted Nox4 overexpression was achieved using a Tie2 promoter/enhancer construct. We studied 8- to 16-week-old male transgenic mice with endothelium-targeted Nox4 overexpression (Tg) and matched wild-type (WT) littermate mice.

Real-Time Reverse Transcription–Polymerase Chain Reaction
mRNA quantitation was performed using the comparative Ct method. For primer sets, please see the supplemental materials.

Histology
Aortic wall thickness and cross-sectional area were measured from in vivo pressure-fixed specimens.

ROS Measurements
H2O2 production was measured using a homovanillic acid–based assay or the ferrous oxidation–xenol orange method. In situ H2O2 levels were assessed in coronary microvascular endothelial cells (CMEC) loaded with 5-(and 6)-chloromethyl-2',7'- dichlorodofluorescein diacetate. Endothelial cell superoxide production was measured using high-performance liquid chromatography–based detection of dihydroethidium oxidation products, ie, 2-hydroxyethidium and ethidium. SOD activities in aortic homogenates were assayed by zymography.

Vascular Tone
Isometric tension was measured in aortic segments and coronary vascular tone in isolated hearts perfused at constant flow.

Surgery and BP
Pressure transducers for BP telemetry were implanted via the left carotid artery. Ang II was administered via osmotic minipumps.

Statistics
Data are expressed as mean±SEM. Comparisons between Tg and WT were made by unpaired Student t test for 2 groups, 1-way ANOVA with Bonferroni post hoc testing for more than 2 groups, or repeated-measures ANOVA. Two-way ANOVA was used to compare treatment responses between Tg and WT. Concentration–response curves were compared by nonlinear regression analysis followed by the extra sum-of-squares F test. P<0.05 was considered significant.

Results
Endothelium-Specific Overexpression of Nox4
In Vivo
Two independent Tg lines were generated (lines 1 and 2). Results were similar for the 2 lines, and data reported herein are for line 1. Tg were born in the expected mendelian ratios and had body weights and organ weights similar to those of WT littersates (eg, body weight 26.6±0.48 g in WT versus 27.5±0.50 g in Tg at age 12 weeks; n=22 per group; P=not significant [NS]). Nox4 transgene expression in a range of organs tracked endothelial nitric oxide synthase (eNOS) mRNA level, as a marker of endothelial cell content, consistent with endothelial specificity—apart from heart, where cardiomyocytes also express eNOS (Supplemental Figure I). Immunolabeled aortic sections showed increased Nox4 staining in the endothelium of Tg compared with WT (Supplemental Figures II and III). Tg CMEC had significantly greater Nox4 protein levels than WT (2.60±0.45 versus 1.00±0.11 arbitrary units; n=4; P<0.05) (Figure 1A).

Endothelial Nox4 Generates Predominantly H2O2
Tg CMEC produced >2-fold more H2O2 than WT by a homovanillic acid assay (Figure 1B). H2O2 levels assessed by 5-(and 6)-chloromethyl-2',7'-dichlorodofluorescein diacetate fluorescence were also significantly higher in Tg CMEC (Figure 1C). H2O2 levels in aorta assessed by a xenol orange assay were significantly higher in Tg than WT (Figure 1D). To definitively address under carefully controlled conditions whether elevated endothelial Nox4 increases both superoxide and H2O2 levels or only H2O2, we studied the effect of acute Nox4 elevation in human umbilical vein endothelial cells (HUVEC) (Figure 1E). We studied HUVEC rather than CMEC because adenoviral overexpression in the latter cell type is poorly reproducible. Nox4-overexpressing HUVEC generated more H2O2 than control cells by homovanillic acid assay (Figure 1E). Using high-performance liquid chromatography–based detection of dihydroethidium oxidation products, there was no significant difference in levels of superoxide-specific 2-hydroxyethidium in Nox4-overexpressing versus β-galactosidase–overexpressing cells (Figure 1F). However, levels of the ethidium oxidation product, which is related to H2O2-dependent oxidation, were significantly higher after Nox4 overexpression, and this increase was abolished by catalase (Figure 1F).

A major difference between H2O2 versus superoxide production by endothelial cells in vessels is the interaction with NO. Whereas superoxide reacts rapidly with NO in a diffusion-limited reaction to generate peroxynitrite, H2O2 does not undergo this reaction. We quantified nitrotyrosine levels in aorta as a readout of nitrosative/nitrative stress resulting from NO/superoxide interaction but found no significant difference between Tg and WT (Figure 1G).

Nox Subunits, eNOS, and Antioxidant Status
We investigated whether Nox4 overexpression led to compensatory changes in other proteins that may influence the vascular response. Protein levels of p22phox and Nox2 were similar in Tg and WT aorta (Supplemental Figure IVA and IVB), whereas Nox1 expression was below the detection limit. Nox2 and p22phox levels in CMEC were also similar between groups (Supplemental Figure IB and IC). Neither
eNOS nor phospho-eNOS levels were different between Tg and WT (Supplemental Figure IVA and IVB). The expression of the antioxidant enzymes SOD1 to SOD3 and catalase, which scavenge superoxide and H$_2$O$_2$, respectively, was similar in WT and Tg aorta (Supplemental Figure IVC). Furthermore, SOD activity was no different between Tg and WT (Supplemental Figure IVD).

Enhanced Endothelium-Dependent Relaxation in Tg

We next studied vascular function in aortic rings. Vasoconstriction to phenylephrine or prostaglandin F2α was similar in Tg and WT, as was vasodilation to the NO donor sodium nitroprusside (Figure 2A–2C). Maximal constriction to 80 mmol/L KCl was similar between groups (0.64±0.03 g in Tg versus 0.65±0.03 g in WT; n=12; P=NS). However, vasodilation to the endothelium-dependent agonist acetylcholine was enhanced in Tg, with a significantly greater potency (Figure 2D). In isolated retrogradely perfused hearts, where a reduction in coronary perfusion pressure at constant flow reflects a decrease in resistance vessel tone, acetylcholine-induced coronary vasodilation was significantly enhanced in Tg versus WT (Figure 2E). Sodium nitroprusside–induced vasodilatation was similar between groups (53.2±10.2% in Tg versus 58.2±4.7% in WT; n=5; P=NS). The enhanced response to acetylcholine was not attributable to changes in M3-muscarinic receptors: mRNA expression by real-time polymerase chain reaction was 0.013±0.001 arbitrary units in WT versus 0.011±0.001 in Tg (n=3/group; P=NS).

Enhanced Endothelium-Dependent Vasodilatation in Tg Results From Increased H$_2$O$_2$

Because Nox4 elevation induced an increase in H$_2$O$_2$ levels (Figure 1) and H$_2$O$_2$ can evoke or enhance endothelium-dependent relaxation through multiple mechanisms, we investigated whether the enhanced vasodilatation in Tg was attributable to H$_2$O$_2$. In the presence of catalase to scavenge H$_2$O$_2$, the enhanced acetylcholine-induced relaxation in Tg aorta was abolished (Figure 2F). Vasodilatation to acetylcholine in isolated perfused hearts of Tg and WT aortae (n=4) was similar in the presence of catalase (Figure 2G). Vasodilatation to a different agonist, histamine, was also enhanced in Tg versus WT aorta, and this difference was abolished by catalase (Figure 2H and 2I).
Mechanism of H₂O₂-Dependent Enhancement of Vasorelaxation

In the presence of Nω-monomethyl-L-arginine (L-NMMA; 100 μmol/L) to inhibit NOS, the magnitude of acetylcholine-induced vasodilatation was reduced in both Tg and WT aorta, but residual vasodilatation remained significantly greater in Tg (Figure 3A). 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μmol/L), which inhibits soluble guanylate cyclase (the major downstream target of NO involved in vasodilatation), reduced acetylcholine-induced vasodilatation in both Tg and WT, but EC₅₀ remained greater in Tg (Figure 3B). An inhibitor of protein kinase G, KT5823 (2 μmol/L), which inhibits soluble guanylate cyclase, or protein kinase G activities.

Furthermore, the vascular response to L-NMMA in both Tg and WT involves increased H₂O₂ generation, which likely acts through hyperpolarization rather than enhanced NO, guanylate cyclase, or protein kinase G activities.

Tg Have a Reduced BP

Tg had a lower BP than WT littermates by tail cuff plethysmography (106.3 ± 0.7 mm Hg versus 116.7 ± 0.9 mm Hg; n ≥ 6; P < 0.05), and BP was also significantly lower in the second line of Tg (by ~12 mmHg; P < 0.05; n = 7). Telemetric ambulatory BP measurements confirmed a significantly lower BP in Tg (Figure 4A and 4B and Supplemental Figure V). This difference was present during night-time and daytime periods and was not attributable to differences in heart rate or activity level (Supplemental Figure V).

Plasma and urinary sodium and urinary volumes were similar in WT and Tg (Figure 4C), suggesting that the lower BP was not accounted for by altered renal sodium or fluid excretion. The lower BP in Tg was not attributable to vascular structural remodeling, as there was no difference in vessel wall thickness or cross-sectional area between groups (Figure 4D). We assessed whether the lower BP in Tg was the result of increased NO bioavailability. However, plasma nitrite/nitrate levels (as a marker of NO production) were similar between groups (Figure 4E). The levels of phosphorylated vasodilator-stimulated phosphoprotein in aorta, as a readout of bioavailable NO and protein kinase G activity, were similar between groups (Figure 4F). Vascular cGMP levels quantified by enzyme-immunoassay were also similar (Figure 4G). Furthermore, the vascular response to L-NMMA in lightly constricted aortic rings, as a functional readout of
basal NO bioactivity, showed no difference between Tg and WT (Figure 4H).

Acute in vivo inhibition of NO production with l-NMMA (10 mg/kg IV) in anesthetized mice increased systolic BP to a similar extent in Tg and WT (Figure 5A), and BP remained significantly lower in Tg such that the difference in BP between groups was attenuated such that the difference between groups was abolished; the difference in BP between groups was restored on removal of EUK-8 (Figure 5B). Chronic treatment with N-acetylcysteine reduced aortic nitrotyrosine levels in both WT and Tg (Figure 5C), consistent with a reduction in superoxide levels. N-Acetylcysteine abolished the elevation in H$_2$O$_2$ levels in Tg aorta (Figure 5D).

**Effect of Endothelial Nox4 Overexpression on Ang II–Induced Hypertension**

To assess the impact of Nox4 overexpression in a pathological setting, we examined BP during chronic Ang II infusion (1.1 mg/kg per day SC). The hypertensive response to Ang II was biphasic, with an initial rapid rise followed by a slower sustained increase (Figure 6A to 6C). The initial phase was absent in Tg (significant interaction by 2-way ANOVA), whereas the later phase appeared similar to that of WT, albeit at a lower level of BP. Ang II–induced increases in aortic wall thickness were similar between groups (Figure 6D).

**Discussion**

We have undertaken the first direct investigation of the in vivo effects of Nox4 on vascular function, using gene-modified mice with endothelium-targeted Nox4 overexpression. The principal findings are that an increase in endothelial Nox4 and associated H$_2$O$_2$ production results in enhanced vasodilator function and a lower basal BP. These effects contrast markedly with those previously reported for vascular Nox1 and Nox2 and suggest that increases in Nox4 may potentially be beneficial as compared with other Noxs.

Tg had increases in Nox4 levels in a similar range to that previously observed in various vascular disease settings. Increased Nox4 expression was not associated with significant changes in vascular expression of other Nox isoforms, SOD1 to SOD3, catalase, or eNOS, suggesting that no major compensatory changes occurred in these pathways, unlike previous studies in which p22phox was overexpressed in vascular smooth muscle. Previous work suggests that forced increases in Nox subunit expression may be accompanied by an increase in p22phox protein levels, because the 2 proteins stabilize each other in a complex. We did not observe increased p22phox levels which could be because the level of increase in Nox4 was modest. Tg had significantly enhanced aortic vasodilatation to the endothelium-dependent agonists acetylcholine and histamine, whereas smooth muscle relaxation to sodium nitroprusside was unaffected. This enhanced vasorelaxation was attributable to increased H$_2$O$_2$ generation, and Tg produced significantly more H$_2$O$_2$ than WT. EUK-8 abolished the elevation in H$_2$O$_2$ levels in Tg aorta (Figure 5B). On withdrawal of N-acetylcysteine, the baseline difference in BP between Tg and WT was restored. EUK-8 also caused a reduction in BP in WT but a slight increase in Tg such that the difference in BP between groups was abolished; the difference in BP between groups was restored on removal of EUK-8 (Figure 5B). Chronic treatment with N-acetylcysteine reduced aortic nitrotyrosine levels in both WT and Tg (Figure 5C), consistent with a reduction in superoxide levels. N-Acetylcysteine abolished the elevation in H$_2$O$_2$ levels in Tg aorta (Figure 5D).
of Nox4 with other Nox isoforms suggest that its activation mechanism is significantly different, such that H$_2$O$_2$ is generated without intermediate superoxide production.\textsuperscript{11–13} A different intracellular location of Nox4 compared with Nox1/2 could also be important, although a careful comparative study of superoxide versus H$_2$O$_2$ production by Nox1 and Nox4 in membrane fractions (where intracellular location is not a factor) also showed mainly H$_2$O$_2$ production by Nox4.\textsuperscript{10} Consistent with these data in other cell types, we found that Nox4 elevation in endothelial cells resulted in an increase in H$_2$O$_2$ production with hardly any detectable change in superoxide using a well-validated high-performance liquid chromatography--based method for the detection of dihydroethidium oxidation products. Consistent with predominant H$_2$O$_2$ rather than superoxide production in Tg vessels in situ, we found an increased level of oxidized peroxiredoxin (as a readout for chronic H$_2$O$_2$ production\textsuperscript{26}) but no difference in nitrotyrosine levels, as a readout of nitrosative stress resulting from the interaction of superoxide with NO to generate peroxynitrite.\textsuperscript{25}
**Figure 6.** Response to chronic Ang II (1.1 mg/kg per day) infusion. A to C, Mean, systolic, and diastolic BP in Tg (dashed lines) and WT (solid lines) (n=10 per group) (**P<0.01, ***P<0.001 for significant interaction by repeated-measures ANOVA). D, Aortic wall thickness (**P<0.01, ##P<0.01; n=4). MAP indicates mean arterial pressure.

\( \text{H}_2\text{O}_2 \) induces vasodilatation in various murine and human vessels, and endogenous \( \text{H}_2\text{O}_2 \) may act as an endothelium-derived vasodilator. \( \text{H}_2\text{O}_2 \)-induced vasodilatation involves different mechanisms, including altered eNOS expression/activation or the activation of soluble guanylate cyclase or protein kinase G, the downstream targets of NO responsible for vasodilatation. Our data suggest that the enhanced acetylcholine-induced vasodilatation in Tg did not result from these mechanisms because the difference between Tg and WT persisted after inhibition of NOS, soluble guanylate cyclase, or protein kinase G. However, enhanced acetylcholine-induced vasodilatation was abolished in the presence of high extracellular potassium or with \( \text{K}^+ \) channel blockers, consistent with \( \text{H}_2\text{O}_2 \)-induced hyperpolarization. \( \text{H}_2\text{O}_2 \)-dependent “endothelium-derived hyperpolarizing factor-type” activity could occur through several mechanisms. Some workers suggest that the response involves diffusion of \( \text{H}_2\text{O}_2 \) from endothelium to vascular smooth muscle, resulting in smooth muscle hyperpolarization. Others propose that the endothelium-derived hyperpolarizing factor phenomenon is underpinned by endothelial hyperpolarization, which spreads electrotonically through the vessel wall via myoendothelial and homocellular smooth muscle gap junctions. These authors showed that \( \text{H}_2\text{O}_2 \) potentiates vasorelaxation by enhancing \( \text{Ca}^{2+} \) release from endothelial endoplasmic reticulum stores, which in turn potentiates the opening of \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channels. Interestingly, with this mechanism, there is no basal effect of \( \text{H}_2\text{O}_2 \) in the absence of agonist-induced relaxation. Although we did not directly address this mechanism, it is intriguing in view of the reported endoplasmic reticulal location of endothelial Nox4.

Tg also had a significantly lower basal BP than WT, which was not attributable to alterations in renal sodium or fluid excretion or to vascular structural remodeling. Increased NO bioactivity could theoretically lead to reduced vascular tone and lower BP, but our studies did not support this mechanism on the basis of unaltered plasma nitrite/nitrate levels; levels of vascular phosphorylated vasodilator-stimulated phosphoprotein, phospho-eNOS, and cGMP; assessment of basal NO bioactivity in aortic rings; and the BP response to acute NOS blockade. Instead, the difference in BP between WT and Tg was attributable to ROS generation per se in that short-term treatment with either of 2 structurally distinct antioxidants attenuated the difference in BP between groups. The effects of systemic antioxidant treatment were complex in that both agents reduced BP in WT animals but increased it in Tg. An explanation for these findings may be that in WT mice the scavenging of superoxide from Nox1 and Nox2 (and possibly other sources) reduces BP, whereas in Tg this effect is outweighed by the scavenging of Nox4-derived vasodilator \( \text{H}_2\text{O}_2 \). Consistent with this possibility, we found that chronic \( \text{N} \)-acetylcysteine treatment reduced aortic nitrotyrosine levels in both groups, whereas \( \text{H}_2\text{O}_2 \) levels were reduced in the Tg group. It is tempting to link the effects of increased Nox4 expression on ex vivo vasodilator function with those on BP in vivo, but ROS-dependent pathways in the kidneys and brain are also important in BP regulation. Therefore, it is feasible that endothelium-targeted Nox4 overexpression could have an impact on pathways in those organs.

The role of Nox enzymes in hypertension has attracted significant attention. Good evidence supports a link between Nox1 and hypertension, whereas increased Nox2 activation is especially implicated in endothelial dys-
function.\textsuperscript{14,15} Nox4 levels increase in Ang II–induced hypertension,\textsuperscript{17} but there has so far been no direct data on possible functional effects. We found that during 2 weeks of Ang II infusion, Tg had a blunted initial hypertensive response but a similar pattern of later rise in BP to WT, albeit at a lower absolute level. The delayed rise in BP may be related to vascular structural remodeling, which was indeed similar between Tg and WT. The BP response to Ang II in Tg contrasts to previously reported effects of Nox2 and Nox1, which enhance hypertension.\textsuperscript{14–18} It therefore seems possible that increases in Nox4 could act to buffer rises in BP. Interestingly, Nox4 expression was reported to be elevated in basilar arteries of spontaneously hypertensive rats, and the application of NADPH to these vessels induced H2O2-dependent vasodilatation.\textsuperscript{35}

The importance of increased ROS production as a pathogenic mechanism in vascular diseases is well recognized, and the targeting of NADPH oxidases is considered to be a promising therapeutic approach. However, it has recently become apparent that Nox4 is different from other vascular Nox isoforms in terms of its biochemical activity. The current results suggest that an elevation of Nox4 levels in endothelial cells has quite different functional effects from Nox1 and Nox2 and that these may be potentially beneficial for vascular function. The key mechanism underlying the vasodilator effect of Nox4 is its propensity to produce predominantly H2O2 rather than superoxide which means that, on the one hand, there is little or no inactivation of NO by superoxide, whereas on the other, H2O2 induces vasodilatation through enhancement of hyperpolarization. In contrast, superoxide generated by Nox1 and Nox2 is much more likely to react with NO and inactivate it than to be converted to H2O2 because the rate constant for the former reaction is much greater than for the latter.\textsuperscript{23} The overall effects of changes in Nox levels will of course also depend on the different cell types within which Nox levels are altered. In conclusion, our results establish a novel paradigm for Nox4-dependent effects in the vasculature and indicate that therapeutic strategies to modulate ROS production in vascular disease need to take into account the potential for both beneficial and detrimental effects.

Sources of Funding

This work was supported by the British Heart Foundation (RG/08/011/25922, CH/99001, and RE/08/003), a Fondation Leducq Transatlantic Network of Excellence award, and in part by European Union FP6 Grant LSHM-CT-2005-018833 (EUGeneHeart). Drs Ray, Murdoch, and Alom-Ruiz were funded by British Heart Foundation PhD studentships.

Disclosures

None.

References


Endothelial Nox4 NADPH Oxidase Enhances Vasodilatation and Reduces Blood Pressure In Vivo

Robin Ray, Colin E. Murdoch, Minshu Wang, Célio X. Santos, Min Zhang, Sara Alom-Ruiz, Narayana Anilkumar, Alexandre Ouattara, Alison C. Cave, Simon J. Walker, David J. Grieve, Rebecca L. Charles, Philip Eaton, Alison C. Brewer and Ajay M. Shah

Arterioscler Thromb Vasc Biol. 2011;31:1368-1376; originally published online March 17, 2011;
doi: 10.1161/ATVBAHA.110.219238
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/31/6/1368

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/03/17/ATVBAHA.110.219238.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure \textit{in vivo}.
King’s College London British Heart Foundation Centre, Cardiovascular Division, London SE5 9PJ, UK.

Detailed Methods

All procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK Home Office).

Generation of Tg mice

Endothelium-targeted overexpression was achieved using a tie2 promoter/enhancer construct (1) containing the entire Nox4 cDNA, microinjected into fertilised oocytes of CBA/C57Bl6 mice. Tg lines were backcrossed >10 generations into a C57Bl6/j background. We studied male mice aged 8-16 weeks and matched wild-type littermates.

Real-time RT-PCR

Quantitation of mRNA levels was performed on an Applied Biosystems 7000 sequence detection system using Sybr Green. Data were analyzed using the comparative Ct method and normalized by GADPH or β-actin levels. Forward and reverse primer sequences were as follows (all 5'-3'): GAPDH CGTGCCGCCTGGAGAA, CCCTCAGATGCCTGCTTCAC; β-actin
ATVB/2010/219238 R1

CGTGAAAAGATGACCAGATCA, TGGTACGACCAGGCATACAG; Nox1
CATCCAGTCTCAAACATGACAG, GCTACAGTGGCAATCACACTCCAGTA; Nox2
ACTCCTTGGGTCGACTCGG, TGGTACGACCAGAGGCATACAG; Nox4
CCGGACAGTCCTGGCTTATC, TGCTTTTATCCAAACATCCTCTTTGTT; Nox4 Tg
AGCTTGATTCCAGGTATCGAT, AAAAAATGAGCTGGCTGAGGTGTT; p22^phox
TGGACGTTCACACAGTGGT, AAAGAGGAAAAAGGGGTCCA; eNOS
GGGAAAGCTGCAGGTATTTGAT, CACTGTGATGGCTGAACGAAGA; catalase
GCTGAGAAGCCTAAACAGCAAT, CCCTTCGCAGCCATGTG; SOD1
GGAGCTTATTTTTAATCCTCACTCTCAAG, GGTCTCCAACATGCTCTCTCT; SOD2
CACACATTAACGCGCATCA, GGTGCGGTAGATTGTCCA; SOD3
GGGATGGATCTAGACATTAAAGGA, ACACCTTAGTAAACCCAGAAATCTTTT; M3
muscarinic receptor ATAAGGGCTGACTACTTAATCTTGGATAA,
CGAGGTTGTACTGTACTGTGCAA

**Immunostaining and antibodies**

Immunostaining was performed on 8 µm aortic cryosections. Secondary antibodies were conjugated to Alexafluor 488 or Cy3. Slides were analyzed on a Leica SP5 confocal microscope. The following antibodies were used: polyclonal anti-Nox4 (2); anti-Nox2, anti-eNOS and anti-phospho(Ser1177) eNOS (BD Transduction Laboratories); anti-p22^phox (Santa Cruz), anti-CD31 (Chemicon); anti-VASP and anti-phospho(Ser239) VASP (Cell Signaling); anti-nitrotyrosine and anti-peroxiredoxin 1 (Abcam); anti-peroxiredoxin-SO3 (Abfrontier); and anti-β actin (Sigma). The β-actin level was used as a loading control in densitometric quantification of immunoblots. Levels of phosphorylated vasodilator-stimulated phosphoprotein (VASP) in aorta were measured as a readout of bioavailable NO and protein kinase G activity (3).

**Cultured cells**

Coronary microvascular endothelial cells (CMEC) were isolated and cultured as described (4) and were used at passage 2. Human umbilical vein endothelial cells (HUVEC) were obtained from
Lonza (UK). Adenoviral vectors expressing mouse Nox4 or β-galactosidase were generated using the AdEasy™ Adenoviral Vector System.

**H$_2$O$_2$ measurement**

H$_2$O$_2$ production by CMEC was assayed with a homovanillic acid-based assay which measures H$_2$O$_2$ released from cells (5). CMEC were incubated for 60 min at 37°C with assay mix solution (100 μmol/L homovanillic acid, 4U/mL HRP, 1mmol/L HEPES), the reaction was stopped using 0.1mol/L glycine (pH 10), and fluorescence detected on a fluorimeter (Genosys Pro, Tecan, Männedorf, Switzerland). Inhibition by catalase (325U/mL) was used to confirm specificity for H$_2$O and the results expressed as the catalase-inhibitable signal. A calibration curve was obtained using exogenous H$_2$O$_2$. *In situ* H$_2$O$_2$ production was assessed from the catalase-inhibitable signal in CMEC loaded with 10 µmol/L 5-(and 6)-chloromethyl-2',7'-dichlorodrofluorescein diacetate (DCF) (4).

H$_2$O$_2$ levels in aorta were measured by the ferrous oxidation-xylenol orange (FOX) method (6). This is based on the ability of H$_2$O$_2$ to convert ferrous into ferric ions which then form a purple complex with xylenol orange. Briefly, fresh aortae were rinsed in PBS, divided into two segments and incubated in 0.1ml PBS with or without PEG-Catalase (200U/ml) at 37°C for 1 h. FOX reagent was then added to a final volume of 1ml, gently agitated and incubated for 30 min. The FOX reagent contained 100 µmol/L xylenol orange, 4mmol/L butylated hydroxytoluene, 25mmol/L sulfuric acid and 250 μmol/L ammonium ferrous sulfate dissolved in methanol solution (90% v/v). The samples were gently sonicated (8 W for 10 s) and centrifuged (6000 rpm for 5 min at 4°C). We confirmed that sonication did not affect H$_2$O$_2$ detection by the FOX assay. Supernatants were collected and the absorbance measured at 560 nm. H$_2$O$_2$ was used to generate a calibration curve; the detection limit was ~0.05 nmols. The catalase-inhibitable values were normalized by total tissue weight. The FOX assay can be used to measure both H$_2$O$_2$ and lipid peroxides in biological samples but in our studies the aorta-related increase in peroxide was attributable to H$_2$O$_2$ since it was inhibitable by catalase (6).
Furthermore, the measured signal in aortic tissue was not altered by triphenylphosphine, an agent that degrades organic peroxides (6).

**HPLC-based measurement of DHE oxidation products**

To measure cellular superoxide production, we used HPLC-based detection of the oxidation products of dihydroethidium (DHE, Invitrogen), i.e. 2-hydroxyethidine (EOH) and ethidium (E), as previously described (7,8). HUVEC transfected with Ad.Nox4 or Ad.β-galactosidase were serum starved for 3h in the presence or absence of PEG-catalase (200U/mL), then washed twice with PBS and incubated in PBS/DTPA (0.5 ml) at a final DHE concentration of 100 µmol/L for 30 min. Cells were washed twice with cold PBS, harvested in cold acetonitrile (0.5 ml/well), sonicated (10 s, 1 cycle at 8 W), centrifuged (12,000 g for 10 min at 4°C) and supernatants were dried under vacuum. Pellets were stored at –20°C in the dark until analysis. Samples were resuspended in 80 µl PBS/DTPA and injected (30 µL) into an HPLC system (Shimadzu) equipped with a photodiode array (SPD-M10AVP) and fluorescence (RF-10AXL) detectors. Chromatographic separation was carried out in a C18-Kromosil column (4.6 x 250 mm, 5 µm particle size). DHE was monitored by ultraviolet absorption at 245 nm and EOH and ethidium by fluorescence detection (excitation 510 nm and emission 595 nm) (8). Quantification was performed by comparison of peak signal between the samples and standard solutions under identical chromatographic conditions. DHE-derived products were expressed as ratios of EOH and ethidium generated per DHE consumed (initial DHE concentration minus remaining DHE; EOH/DHE and ethidium/DHE) (8).

**Vascular tone**

Isometric tension was measured in ring segments of descending thoracic aorta (9). Cumulative dose-response curves were obtained for constriction to phenylephrine or prostaglandin F2α. Constriction to L-NMMA was assessed in rings that were lightly constricted to 30% of the phenylephrine maximum. Cumulative dose-response curves for relaxation to acetylcholine, sodium nitroprusside (SNP) or histamine were obtained in rings pre-constricted to 70% of the phenylephrine maximum or to KCL.
Inhibitors were pre-incubated for at least 20 min. Coronary vascular tone was assessed in isolated hearts retrogradely perfused at constant flow, with measurement of coronary perfusion pressure (10).

**Histology**

Tissues were pressure fixed *in vivo* with 0.9% saline perfusion followed by 4% PFA. The proximal portion of the descending aorta was embedded in paraffin. 6µm thick sections were stained with H&E. Wall thickness and cross-sectional area were analyzed using NIH Image software (v1.62), using at least 8 measurements from the internal elastic lamina to the external lamina for wall thickness (11).

**Surgery and blood pressure**

For telemetric studies, animals underwent implantation of transducers (PA-C10, Data Sciences International, St. Paul, MN) via the left carotid artery, with the transmitter body positioned in the right flank, under 2% isoflurane general anesthesia. Telemetry data were collected after a 7d recovery period. Tail-cuff plethysmography was performed after at least 3 training sessions in conscious restrained mice, using a Kent Scientific system (XBP 1000; Torrington, CT).

**In vivo treatments**

To assess the BP response to acute NOS inhibition, anesthetized mice were administered L-NMMA (10mg/kg body weight, as a 10µL bolus over 30s) via the jugular vein. BP was recorded using a 1.4F Scisense (London, Canada) catheter via the left carotid artery. N-acetylcysteine was added to the drinking water (5g/L) while EUK-8 was administered by i.p. injection (25mg/kg/d, 3 injections over 7 days). Ang II was administered over 14d by osmotic minipumps (Model 1002, Alzet, Cupertino, CA).

**Other assays**

SOD activities were assayed in aortic homogenates by zymography on 12% non-denaturing PAGE, as described by Culotta et al (12). Briefly, aortic samples were homogenized in extraction buffer (NaPO₄
10mM; EDTA 5mmol/L; EGTA 5mmol/L; NaCl 50mmol/L; Triton 1%; protease inhibitor cocktail 1% (Sigma); pH7.8). Subsequently, protein in 5% glycerol was separated on a 12% non-denaturing polyacrylamide gel (Invitrogen, UK) in running buffer (Tris base 20mmol/L; glycine 150mmol/L; pH 8.5), then soaked in a dark potassium phosphate (50mmol/L; pH 7.8) solution containing nitroblue tetrazolium (275μg/mL), riboflavin (65μg/mL) and N,N,N9,N9-tetramethyl-ethylenediamine (Temed; 3.2μl/mL). After this, bands were developed by exposing the gel to light whilst washing in dH2O. SOD isoforms were identified by the position on the gel corresponding to their relative molecular weights and subsequently quantified by densitometry.

Total nitrite plus nitrate levels in plasma were measured using a Griess assay kit (Promega), with conversion of nitrate to nitrite by nitrate reductase (13).

Cyclic GMP (cGMP) content in aortic lysates was quantified using a cGMP enzyme-immunoassay (EIA) kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Briefly, frozen aortae were homogenized in 6% TCA and centrifuged at 2,000g. TCA in the supernatant fraction was extracted with diethyl ether and the samples were then lyophilized. The samples were resuspended in assay buffer and subjected to EIA. cGMP levels were expressed as fmol per mg TCA-precipitable protein. Protein was solubilised in SDS sample buffer and protein levels were determined using RC-DC protein assay (BioRad).

Metabolic cage experiments

To assess urinary parameters, mice were studied in metabolic cages (14). 24 hour urine samples as well as serum samples were collected. Urine and serum biochemistry were analyzed using the Advia 2120 system (Siemens Medical Solutions Diagnostics, Newbury, UK).

Statistics

Data are expressed as mean±SEM. Comparisons between Tg and wild-type were made by unpaired Student's t-test for 2 groups, 1-way ANOVA with Bonferroni post-hoc testing for more than 2 groups, or repeated measures ANOVA as appropriate. 2-way ANOVA was used to compare responses to treatments between Tg and wild-type. Concentration-response curves were compared by nonlinear
regression analysis followed by the extra sum-of-squares F test. Statistical analyses were done on
GraphPad Prism (v4.03 for Windows, San Diego, CA). p<0.05 was considered significant.

References


Figure I. Endothelium-targeted overexpression of Nox4 in vivo. (A) Expression of Nox4 transgene and eNOS in tissues of Tg mice. Transgene expression paralleled that of eNOS. (B) Representative Western blots showing protein levels of Nox2 and p22phox in CMEC from Nox4 Tg and wild-type littermate mice. (C) Quantification of immunoblots showing protein levels of Nox2 and p22phox in CMEC (n=3; p=NS).
Figure II. Endothelium-targeted overexpression of Nox4. (A) Aortic sections stained for Nox4 (green) and an endothelial marker, CD31 (red). Yellow color in merged images (bottom panels) denotes co-localization. Scale bars, 50 µm. (B) Mean fluorescence intensity of endothelial Nox4 staining in the immunomicrographs. *, p<0.01.
Figure III. Nox4 staining in WT and Nox4 transgenic aorta. Aortic cryosections (8 µm) were stained with an anti-Nox4 antibody. An HRP-conjugated secondary antibody was used and was visualized by incubation with DAB and counterstaining with hematoxylin.
Figure IV. Expression of Nox subunits, eNOS and antioxidant enzymes in Nox4 Tg and wild-type.
(A) Representative Western blots showing protein levels of p22phox and Nox2 (left) and eNOS and phospho-eNOS (right) in aorta. (B) Quantification of immunoblots for p22phox, Nox2, eNOS and p-eNOS levels in aorta (n=3; p=NS). (C) mRNA levels of SOD1-3 and catalase in Tg and wild-type aorta (n=5; p=NS). (D) Zymographic analysis of SOD activity in aorta (n=4; p=NS)
Figure V. Reduced BP in Nox4 Tg mice. (A) SBP and DBP averaged over 24h. (B) Measure of physical activity averaged over 24h. (C) Mean heart rate. (*, p<0.05; n=5)