Nox4 Is a Protective Reactive Oxygen Species Generating Vascular NADPH Oxidase

Katrin Schröder,* Min Zhang,* Sebastian Benkhoff, Anja Mieth, Rainer Pliquett, Judith Kosowski, Christoph Kruse, Peter Luedike, U. Ruth Michaelis, Norbert Weissmann, Stefanie Dimmeler, Ajay M. Shah, Ralf P. Brandes

Rationale: The function of Nox4, a source of vascular H$_2$O$_2$, is unknown. Other Nox proteins were identified as mediators of endothelial dysfunction.

Objective: We determined the function of Nox4 in situations of increased stress induced by ischemia or angiotensin II with global and tamoxifen-inducible Nox4$^{-/-}$ mice.

Methods and Results: Nox4 was highly expressed in the endothelium and contributed to H$_2$O$_2$ formation. Nox4$^{-/-}$ mice exhibited attenuated angiogenesis (femoral artery ligation) and PEG-catalase treatment in control mice had a similar effect. Tube formation in cultured Nox4$^{-/-}$ lung endothelial cells (LECs) was attenuated and restored by low concentrations of H$_2$O$_2$, whereas PEG-catalase attenuated tube formation in control LECs. Angiotensin II infusion was used as a model of oxidative stress. Compared to wild-type, aortas from inducible Nox4-deficient animals had development of increased inflammation, media hypertrophy, and endothelial dysfunction. Mechanistically, loss of Nox4 resulted in reduction of endothelial nitric oxide synthase expression, nitric oxide production, and heme oxygenase-1 (HO-1) expression, which was associated with apoptosis and inflammatory activation. HO-1 expression is controlled by Nrf-2. Accordingly, Nox4-deficient LECs exhibited reduced Nrf-2 protein level and deletion of Nox4 reduced Nrf-2 reporter gene activity. In vivo treatment with hemin, an inducer of HO-1, blocked the vascular hypertrophy induced by Nox4 deletion in the angiotensin II infusion model and carbon monoxide, the product of HO-1, blocked the Nox4-deletion-induced apoptosis in LECs.

Conclusion: Endogenous Nox4 protects the vasculature during ischemic or inflammatory stress. Different from Nox1 and Nox2, this particular NADPH oxidase therefore may have a protective vascular function. (Circ Res. 2012;110:1217-1225.)

Key Words: angiogenesis ■ carbon monoxide ■ hypertension ■ nitric oxide ■ redox regulation

Reactive oxygen species (ROS) exert a range of different effects in the vascular system. Superoxide anions (O$_2^-$) react with nitric oxide (NO) to form peroxynitrite (ONOO$^-$), a process that limits NO availability, results in NO synthase (NOS) uncoupling, and, through the action of ONOO$^-$, leads to protein and thiol oxidation as well as tyrosine nitration. Hydrogen peroxide (H$_2$O$_2$), the dismutation product of O$_2^-$, also elicits multiple effects, among them smooth muscle cell hypertrophy, activation of metalloproteinases, and, in higher concentrations, NOS inhibition by phosphorylation of tyrosine 657 through the redox-activated tyrosine kinase Pyk2. Interestingly, H$_2$O$_2$ also induces positive endothelial effects because it can activate protein kinase-G Ia by thiol oxidation and subsequent dimerization. Moreover, H$_2$O$_2$ induces as well as activates endothelial NOS (eNOS).

In addition to uncoupled eNOS and mitochondria, the most prominent vascular sources of ROS are NADPH oxidases of the Nox family. In the vascular system Nox1, Nox2, Nox4, and Nox5 are expressed and at least Nox1 and Nox2 have been identified as mediators of endothelial dysfunction.

Recently, Nox4 gained considerable attention because it differs from Nox1 and Nox2 in several aspects; Nox4 mRNA *K.S. and M.Z. contributed equally to this work.

From the Institute for Cardiovascular Physiology (K.S., S.B., A.M., R.P., J.K., C.K., U.R.M., R.P.B.) and Institute of Cardiovascular Regeneration (S.D.), Goethe-University, Frankfurt, Germany; Cardiovascular Division (M.Z., A.M.S.), King’s College London British Heart Foundation Centre of Excellence, London, UK; Division of Cardioangiology (P.L.), Pulmonology and Vascular Medicine, University Hospital, Düsseldorf, Germany; University Lung Centre (N.W.), Justus-Liebig University, Giessen, Germany.

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Correspondence to Katrin Schröder or Ralf P. Brandes, Institut für Kardiovaskuläre Physiologie, Goethe-Universität, Theodor-Stern Kai 7, 60590 Frankfurt, Germany. E-mail schroeder@vrc.uni-frankfurt.de or brandes@vrc.uni-frankfurt.de

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Non-standard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CORM</td>
<td>CO-releasing molecule</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>HO</td>
<td>heme oxygenase</td>
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<tr>
<td>LEC</td>
<td>murine lung endothelial cell</td>
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<tr>
<td>Nrf-2</td>
<td>NF-E2-related factor 2</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>WT</td>
<td>wild-type</td>
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expression is higher than that of the other Nox homologues and different from Nox1 and Nox2, which are induced by angiotensin II in vascular smooth muscle cells. Nox4 expression increases in the course of differentiation and is required for the maintenance of a differentiated phenotype in cultured cells. In particular, during the resolution phase after vascular injury, a transient massive upregulation of Nox4 has been observed. Overexpression studies, mostly in HEK293 cells so far, also suggested that Nox4, different from Nox1 and Nox2, is independent of cytosolic activator subunits and thus is potentially constitutively active, suggesting that Nox4-dependent ROS production is controlled by the abundance of the enzyme. This aspect does not exclude the possibility that other interacting proteins, such as Poldip2 or protein disulfide isomerase, alter the activity of Nox4. In overexpression systems, Nox4 releases predominantly H₂O₂, which seems to be a consequence of a particular property of its E-loop. Increased H₂O₂ formation is also apparent in mice with endothelial-specific Nox4 overexpression. In support of this, Nox4 overexpression in the presence of NO does not lead to ONOO⁻ formation, and this strongly argues against significant O₂⁻ formation by the enzyme. A careful analysis of ROS production of vascular smooth muscle cells also suggested that endogenous Nox4, different from Nox1, produces predominantly H₂O₂ rather than O₂⁻. It should, however, be mentioned that many other publications linked Nox4 to O₂⁻ formation mainly on the basis of an increased fluorescence signaling in the dihydroethidium O₂⁻ assay. Nox4 is able to yield an increased fluorescence signal in this assay, but this is not the consequence of an increased formation of the specific O₂⁻ adduct, oxyethidium, but rather reflects formation of ethidium by a simple oxidation of the redox probe.

In the light of the profoundly different physiological functions of O₂⁻ and H₂O₂, the endogenous vascular functions of Nox4 are still unknown. Recently, it was demonstrated that endothelial-specific overexpression of the enzyme reduces the blood pressure, improves endothelial function, and increases angiogenesis in the ischemic hind limb of mice. The suitability of overexpression data is, however, limited for the demonstration of the function of the endogenous enzyme and, in that respect, little is known about Nox4. Thus, we generated global and inducible knockout mice and characterized their vascular function in response to two stress models: ischemia and angiotensin II.

Methods

Detailed Methods are provided in the Online Supplement. Tamoxifen-inducible Nox4⁺⁻ mice were generated by crossing Nox4⁺⁻/⁺ with ERT2-Cre⁺⁻ transgenic mice. Mouse lung endothelial cells (LECs) were isolated with magnetic beads. Endothelium-dependent relaxation was studied in preconstricted aortic rings. The mRNA expression was determined by quantitative real-time polymerase chain reaction.

Results

Nox4 Is Highly Expressed in Endothelial Cells

Quantitative real-time-polymerase chain reaction identified high basal Nox4 mRNA expression in human umbilical vein endothelial cells as compared to human aortic smooth muscle cells (Online Figure 1). Similarly, Nox4 mRNA expression was significantly higher in cultured murine LECs than in cultured murine aortic smooth muscle cells. On the protein level, Nox4 expression was readily detectable by Western blot in LEC and the intact mouse aorta and carotid artery, whereas when the endothelium of the arteries was removed by detergent treatment, and not only Nox4 mRNA expression declined but also Nox4 protein decreased substantially (Supplemental Figure 1). Thus, in normal murine arteries, Nox4 expression is higher in endothelial than in smooth muscle cells.

Nox4 Contributes to Basal Vascular H₂O₂ Formation

To determine whether endogenous Nox4 contributes to basal vascular H₂O₂ formation, a tamoxifen-inducible Nox4 knockout mouse was generated and a previously characterized global Nox4 knockout mouse was also studied. Nox4 protein was absent in the kidney and lung of global Nox4⁺⁻ mice. Tamoxifen treatment of Nox4⁺⁻/⁺ERT2-Cre⁺⁻ mice led to a rapid loss of Nox4 protein (Figure 1A, B). Importantly, basal H₂O₂ levels in LECs harvested from Nox4⁺⁻/⁻ mice were lower than in wild-type (WT) littermates. Tamoxifen treatment also reduced H₂O₂ formation in Nox4⁺⁻/⁺ERT2-Cre⁺⁻ LECs but not in WT LECs. Transforming growth factor-β1, which induces Nox4 in endothelial cells, increased the H₂O₂ formation in WT cells, and this effect was greatly attenuated in LECs from tamoxifen-treated Nox4⁺⁻/⁺ERT2-Cre⁺⁻ (Figure 1C). To determine the contribution of Nox4 to total vascular H₂O₂ formation, the ferrous oxidation xylenol orange peroxide assay was performed from the intact mouse aorta and demonstrated that Nox4 accounts for approximately 75% of the vascular H₂O₂ formation (Figure 1D). Thus, endogenous Nox4 is a constitutively active and inducible source of H₂O₂ in endothelial cells and the mouse aorta.

Nox4-Derived Endogenous H₂O₂ Promotes Angiogenic Responses

To determine the role of Nox4 in response to ischemia, we subjected knockout mice to femoral artery ligation. Blood flow recovery was significantly attenuated in global Nox4⁺⁻/⁻ mice as well as in inducible Nox4⁺⁻/⁻ mice as compared to WT littermates. This effect was not observed with genetic deletion of Nox1 or Nox2 and the blood flow recovery of Nox1⁺⁻/⁻ mice was even faster than that of WT littermates.
Accordingly, capillary density was significantly lower in recovering muscles of global and inducible Nox4 knockout mice than in WT mice (Supplemental Figure II).

(Figure 2 top). Accordingly, capillary density was significantly lower in recovering muscles of global and inducible Nox4 knockout mice than in WT mice (Supplemental Figure II).

To address whether Nox4-derived H$_2$O$_2$ is involved in angiogenic responses of endothelial cells, we performed matrigel tube formation assays. LEC is from Nox4$^{-/-}$ mice generated 50% less branching points than WT cells. Low

Figure 1. Nox4 contributes to H$_2$O$_2$ formation. A and B, Western blot analysis of Nox4 expression (approximately 65 kDa band) in the tissues and mouse strains indicated. C, Amplex red H$_2$O$_2$ assay in cultured murine lung endothelial cells (LECs ; n=3). D, Ferrous oxidation-xylene orange (FOX) assay for aortic peroxide generation. H$_2$O$_2$ formation was measured as the PEG-catalase-sensitive (200 U/mL) portion of the signal (n=5). WT, wild-type mice; Nox4$^{-/-}$, Nox4$^{flox/flox}$-ERT2-Cre$^{+/-}$; CTL, Nox4$^{flox/flox}$-ERT-Cre$^{0/0}$. Expression of the Cre recombinase was induced in vivo by the treatment with tamoxifen. *P<0.05 vs appropriate control. #P<0.05 with and without transforming growth factor-β1 treatment (10 ng/mL, 16 hours).

Figure 2. Endogenous Nox4 and H$_2$O$_2$ are required for blood flow recovery after femoral artery ligation (A) and endothelial tube formation (B). A, Laser Doppler images and statistics for days 7 and 14 of the blood flow ratio of the ischemic (R) and the control (L) leg in litter mates. For Nox4, global (Nox4$^{-/-}$) as well as inducible knockouts were studied. Nox4$^{flox/flox}$-ERT2-Cre$^{+/-}$ (Cre flox) and Nox4$^{flox/flox}$-ERT2-Cre$^{0/0}$ litter mates (CTL flox) were both pretreated with tamoxifen before operation. Nox4+PEG catalase denotes an experiment in which both groups received intravenous injections of PEG catalase (100,000 U/kg) before and 1 day after surgery. ~PEG-catalase denotes C57BL6/J mice treated with PEG catalase (+PEG catalase) or solvent (wild-type [WT]) *P<0.05. For technical reasons, the laser Doppler measurements on the Nox2 strain were only performed 2 weeks after the operation. B, Matrigel tube formation assay in lung endothelial cells from Nox4$^{+/+}$ (WT) and Nox4$^{-/-}$ mice in the presence or absence of H$_2$O$_2$ and PEG catalase (100 U/mL) n=6 to 7. *P<0.05 WT vs Nox4. ~P<0.05 with vs without H$_2$O$_2$ or PEG catalase.
concentrations of H$_2$O$_2$ (1 and 3 μmol/L) had no effect on WT LECs but normalized branching in Nox4-deficient cells. High concentrations of H$_2$O$_2$ (30 μmol/L) blocked tube formation in both groups (Figure 2 bottom). In keeping with the observation that low concentrations of H$_2$O$_2$ are required for a normal response in this assay, PEG-catalase significantly inhibited basal tube formation in WT LECs with little effect on Nox4-deficient endothelial cells. To determine whether in vivo H$_2$O$_2$ also is required for angiogenic responses, PEG-catalase was injected on the day of and 1 day after femoral artery ligation in WT mice. In vivo PEG-catalase significantly attenuated the blood flow recovery in this model (Figure 2). Thus, angiogenic responses require low concentrations of Nox4-derived H$_2$O$_2$.

Endogenous Nox4 Does Not Affect Angiotensin II–Induced Hypertension

Angiotensin II–induced hypertension is the prototypic situation of increased vascular oxidative stress. We therefore determined the impact of genetic deletion of Nox4 in this situation. Angiotensin II was applied at a low pressor dose (0.7 mg/kg/d). Lack of Nox4 had no effect on basal blood pressure or the angiotensin II–induced hypertensive response, as measured by telemetry in inducible knockout mice or by the tail-cuff technique in global Nox4$^{-/-}$ animals (Supplemental Figure III).

Lack of Nox4 Potentiates the Vascular Effects of Angiotensin II

Because blood pressure is a complex read-out, we also determined vascular nitric oxide availability by bioassay experiments in aortic segments of mice treated with a high proinflammatory concentration of angiotensin II (2.2 mg/kg/d). The acetylcholine-induced endothelium-dependent vasodilation of isolated aortic segments under basal conditions was similar between WT and global as well as inducible Nox4-deficient mice. After in vivo angiotensin II treatment, however, acetylcholine relaxations were impaired in the aorta of Nox4-deficient mice as compared to the aorta of the control animals (Figure 3A, B and Supplemental Figure IVA, B). As a second measure for NO availability, we determined the nitro-L-arginine–induced endothelium-dependent constriction. D and E, Representative aortic sections and medial wall thickness. F and G, Aortic expression of interleukin-6 (IL-6) and IL-1β as determined by quantitative real-time-polymerase chain reaction. n=6. *P<0.05 WT vs identically treated Nox4$^{+/+}$.
netic deletion of Nox1, as reported previously, attenuated angiotensin II–induced vascular hypertrophy (Supplementary Figure V). These observations indicate that loss of Nox4 exacerbates the deleterious effects of angiotensin II in the mouse aorta.

Nox4 Maintains the Activity of Protective Endothelial Mediators

To determine whether the negative effects of Nox4 deletion are a consequence of dysregulation of other Nox homologues or of antioxidant enzymes, quantitative real-time polymerase chain reaction experiments were performed in murine tissue. Deletion of Nox4, however, had no significant effect on the expression of SOD1, SOD2, SOD3, or catalase, or on vascular expression of Nox1 and Nox2 (Supplemental Figure VI). As a possible alternative, Nox4 could control the production of protective endothelial mediators like NO or carbon monoxide (CO). Acute deletion of Nox4 led to a 50% reduction in NO formation by LEC as measured by DAF-2 (Figure 4A). Similarly, plasma nitrite levels 2 weeks after femoral artery ligation were significantly lower in Nox4*/*s compared with control mice (Figure 4B). This effect potentially could be a consequence of a loss of eNOS expression after Nox4 deletion; quantitative real-time-polymerase chain reaction and Western blot from the mouse carotid artery demonstrated that deletion of Nox4 reduced eNOS expression by approximately 50% (Figure 4C, D). Also, heme oxygenase (HO), the enzymatic source of antiinflammatory CO, was modulated by Nox4. Whereas HO-2 is constitutively expressed in endothelial cells, HO-1 expression is redox-sensitive. Nox4 deletion reduced HO-1 but not HO-2 expression of LEC, which was paralleled by an increase in endothelial cell apoptosis as well as endothelial E-selectin expression, a sensitive marker of endothelial activation (Figure 5A, B).

Endogenous Nox4 Controls Basal Endothelial Nrf-2 Activity

HO-1 expression is, among others, controlled by the redox-sensitive transcription factor Nrf-2, which is stabilized in response to oxidative and electrophilic stress. Thus, we hypothesized that endogenous H₂O₂ derived from vascular Nox4, controls Nrf-2. Western blots and reporter gene assays were performed to assess the impact of Nox4 on Nrf-2 in LECs. Before tamoxifen treatment, basal Nrf-2 reporter activity was identical between Nox4flox/flox-ERT2-Cre⁺/⁻ and WT cells. Tamoxifen, probably by a direct antioxidant effect, somewhat decreased the Nrf-2 reporter gene activity in WT cells, whereas tamoxifen-driven deletion of Nox4 resulted in a substantial reduction. After tamoxifen treatment, the Nrf-2 reporter gene activity was significantly lower in Nox4-deficient cells as compared with control cells, suggesting that Nox4 may support Keap oxidation and thus prevents Nrf2 degradation (Figure 5C). Accordingly, Nrf-2 protein expression was significantly lower in Nox4-deficient as compared with WT LECs (Figure 5A), which corresponded to an attenuated vascular expression of HO-1 (Figure 6A).

Increasing the HO-1-CO System Can Compensate the Lack of Nox4

To assess whether Nrf2–driven HO-1 expression contributes to the protective effects of Nox4 in vivo, we treated mice with the HO-1 inducer hemin. Hemin not only increased aortic HO-1 expression (Figure 6A) but also prevented the detrimental effect of Nox4 deletion on angiotensin II–induced vascular hypertrophy (Figure 6B). To determine whether CO, a HO-1 product, could mediate this effect, we focused on endothelial cell apoptosis. Two chemically distinct CO-releasing molecules (CORMs; CORM-A1 and CORM2), but not decomposed CORMs, prevented apoptosis induced by acute deletion of Nox4 in LEC (Figure 6C, D). Thus, downregulation of the Nrf2-HO-1-CO system may contribute...
to the effects occurring in response to the genetic deletion of Nox4.

**Discussion**

This study provides evidence that endogenous Nox4 acts as a vasoprotective protein through mechanisms that include H$_2$O$_2$ generation, maintenance of NO production, and antioxidant HO-1 expression. Genetic deletion of Nox4 therefore attenuated ischemia-induced angiogenesis and potentiated responses to angiotensin II. These observations suggest that endogenous vascular Nox4 may protect vascular function, which is unexpected given the present knowledge of Nox proteins. Numerous studies demonstrated that other Nox proteins, although important for physiological signaling under normal conditions, are mediators of vascular dysfunction, particularly in angiotensin II–induced stress situations. Thus, Nox4 might have an antagonistic function to Nox1 and Nox2 and thus differs from these NADPH oxidases. Nox4 is a special Nox because it has a high constitutive activity and is highly expressed in some cells. Moreover, different from Nox1 and Nox2, Nox4 releases predominantly H$_2$O$_2$, which cannot scavenge NO. Indeed, targeting SOD to the endothelium attenuated the hypertrophic effect of angiotensin II. Hemin could have other off-target effects, but the similar responses observed with CORMs suggest that at least part of its action may be CO-related.

HO-1 expression is controlled by the transcription factor Nrf-2, which accumulates in cells after H$_2$O$_2$-mediated oxidation of its inhibitor Keap. In the absence of H$_2$O$_2$ or other electrophiles, Keap facilitates the degradation of Nrf-2. In line with our observation that basal Nox4 controls Nrf-2, it was observed that myocardial overexpression of Nox4 also increases the level of this transcription factor.

Despite all these possible positive vascular effects, it also should be mentioned that H$_2$O$_2$ is a proliferative stimulus for vascular smooth muscle cells and numerous studies have demonstrated that catalase attenuates the hypertrophic effect of angiotensin II and even linked hypertrophy to NADPH oxidase activation. Thus, how could Nox4-derived H$_2$O$_2$ limit angiotensin II–induced vascular hypertrophy and inflammation? A potential explanation could be that endothelial expression of Nox4 in the vessel is much higher than in the smooth muscle layer. In fact, although it has been demonstrated in cultured smooth muscle cells that Nox4 fulfills many functions in this cell type, until now a careful comparison between endothelial and smooth muscle cell Nox4 expression was not performed. Our data show that in normal vessels Nox4 expression predominates in endothelial cells and is lower in the smooth muscle layer of the vessel. Accordingly, it was previously noted that in the rat carotid artery, smooth muscle Nox4 expression largely increases in the resolution phase after injury and decreases under normal conditions.

**Figure 5. Nox4 maintains endothelial quiescence and Nrf2 activity.** Lung endothelial cells (LECs) from Nox4$^{flox/flox}$-ERT2-CRE$^{19}$ (Nox4$^{+/+}$) and Nox4$^{flox/flox}$-ERT2-Cre$^{20}$ littermates (wild-type [WT]) were studied. A, Western blot analysis for the proteins indicated in LEC 3 days after hydroxytamoxifen treatment. HO-1, heme oxygenase-1; HO-2, heme oxygenase-2. Beta-actin and ERK1/2 were used as loading controls. Numbers below the blots show the result of the relative densitometry. B, Annexin V assay for apoptosis in LECs on days 2 and 3 of hydroxy-tamoxifen incubation (n=3; *P<0.05 WT vs Nox4$^{+/+}$). C, Nrf-2 luciferase reporter gene activity relative to the pGL3P control plasmid in transiently transfected LECs studied in the presence or absence of hydroxytamoxifen (OH-TMX; 10 μmol/L).
murine lung endothelial cells. Cells of Nox4flox/flox-ERT2-Cre0/0 pathway involving NO. Conversely, PEG-catalase was as ineffective. Overexpression of Nox4 increases angiogenesis by a effect of endogenous Nox4 in angiogenesis appears conclusive. CORM2 (200 DCO-releasing molecule (CORM) A1 (200 D) and on the basis of siRNA technology. Not only do cultured cells respond more vigorously to changes than whole organisms but also responses are often complex. For example, as expression of Nox1, Nox2, and p47phox is redox-stimulated, downregulation of Nox4 by siRNA frequently also downregulates these proteins (K. Schroeder, unpublished observation, 2009), whereas this was not the case in the present study. Also, the differentiation state of the cells is of critical importance; whereas transforming growth factor β-1 induces differentiation in endothelial cells, it results in apoptosis of epithelial cells and, interestingly, excessive induction of Nox4 and massive subsequent oxidative stress appears to be involved in this process. The level of Nox4 and subsequently produced H2O2 might be decisive for the effects observed. In the vascular system, too high concentrations of H2O2 are deleterious and this effect also was observed in the present study in the U-shape response of Nox4-deficient endothelial cells to H2O2 in the tube formation assay. These considerations may reconcile the suggested negative role of vascular Nox4 in diabetes or pulmonary hypertension, although these situations have not yet been studied in Nox4 knockout mice. Despite the protective effect on endothelial function in isolated aortic rings, deletion of Nox4 did not alter the hypertensive response to angiotensin II in vivo or the basal blood pressure. These findings can be interpreted in multiple ways. Because the aorta does not contribute to peripheral resistance, it is not known whether our current findings extend into the resistance vessels, where additional vasodilators become important and the ratio of endothelium to smooth muscle is more favorable. It may also support the notion that endothelial dysfunction does not necessarily lead to hypertension. Hypertension is the consequence of a complex dysregulation of many systems not only involving vessels but also involving the heart as well as renal and cerebral regulation loops.

Obviously, the concept of endothelial cells continuously generating significant amounts of ROS (even if it is “only” H2O2) is somewhat counter to the traditional concept of these species as a driving force for disease development. The latter paradigm, however, has been questioned more and more. The cellular redox milieu is rigorously controlled by specialized redox sensors such as the Nrf-2/Keap system, and a notion prevails that the traditional toxic ROS, like hydroxyl radicals, model. The role of Nox1 and Nox2 in angiogenesis is controversial. Nox1 is involved in tumor angiogenesis, whereas in the present study it rather inhibited ischemia-induced angiogenesis. Nox2 is required for vascular endothelial growth factor signaling and, in line with this, attenuated angiogenesis in Nox2-deficient mice was demonstrated. Several more recent studies, similar to the present work, however found no evidence for attenuated angiogenic responses in Nox2-deficient mice. The basis of these discrepant results is currently unclear.

Our present study on a protective function of endogenous Nox4 contrasts with many observations suggesting a deleterious function of Nox4 that link the enzyme to senescence, inflammatory signaling, fibrosis, and even apoptosis. Many of these studies were performed on cultured cells or cell lines and on the basis of siRNA technology. Not only do cultured cells respond more vigorously to changes than whole organisms but also responses are often complex. For example, as expression of Nox1, Nox2, and p47phox is redox-stimulated, downregulation of Nox4 by siRNA frequently also downregulates these proteins (K. Schroeder, unpublished observation, 2009), whereas this was not the case in the present study. Also, the differentiation state of the cells is of critical importance; whereas transforming growth factor β-1 induces differentiation in endothelial cells, it results in apoptosis of epithelial cells and, interestingly, excessive induction of Nox4 and massive subsequent oxidative stress appears to be involved in this process. The level of Nox4 and subsequently produced H2O2 might be decisive for the effects observed. In the vascular system, too high concentrations of H2O2 are deleterious and this effect also was observed in the present study in the U-shape response of Nox4-deficient endothelial cells to H2O2 in the tube formation assay. These considerations may reconcile the suggested negative role of vascular Nox4 in diabetes or pulmonary hypertension, although these situations have not yet been studied in Nox4 knockout mice. Despite the protective effect on endothelial function in isolated aortic rings, deletion of Nox4 did not alter the hypertensive response to angiotensin II in vivo or the basal blood pressure. These findings can be interpreted in multiple ways. Because the aorta does not contribute to peripheral resistance, it is not known whether our current findings extend into the resistance vessels, where additional vasodilators become important and the ratio of endothelium to smooth muscle is more favorable. It may also support the notion that endothelial dysfunction does not necessarily lead to hypertension. Hypertension is the consequence of a complex dysregulation of many systems not only involving vessels but also involving the heart as well as renal and cerebral regulation loops.

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are not subject to regulation in the body simply because of their high reaction speed. Thus, signaling ROS such as H$_2$O$_2$ may have a very different impact on cellular function in particular because the system is much more compartmentalized than initially thought. From a holistic point of view, continuous production of H$_2$O$_2$ maintains a basal level of antioxidant defense and this would desensitize or protect cells against an acute oxidative challenge. Future studies on Nox4 knockout mice therefore will have to prove whether this system is specific for the vasculature or of more general importance. Obviously, expression control of Nox4 will become very important in this aspect. Only moderate levels of the enzyme but not excessive induction will yield the beneficial function. Thus, depending on the disease models used, Nox4 may be protective or harmful. For the vascular system in the settings investigated in this study, however, our data suggest that Nox4 is a beneficial source of ROS.

In conclusion, the present study identifies endogenous Nox4 as a constitutive endothelial generator of H$_2$O$_2$ that positively affects vascular function. Low tonic production of H$_2$O$_2$ is vasoprotective and this could, at least in part, explain the failure of antioxidant therapy to prevent vascular disease in clinical trials. Although endogenous Nox4 appears to have a minor impact on function in healthy vessels, it controls eNOS and HO-1 expression. Thus, in disease situations such as ischemia-induced angiogenesis and angiostatin II–induced superoxide formation and redox-sensitive signaling pathways, Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype.

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Disclosures

None.

References


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Novelty and Significance

What Is Known?

- Nox4 belongs to the family of reactive oxygen species (ROS) generating NADPH oxidases.
- Nox4 is constitutively active and it generates predominantly H2O2.
- Nox1 and Nox2 contribute to physiological signaling of cytokines, hormones, and growth factors, but they also mediate vascular dysfunction in hypertension, hyperlipidemia, and inflammation.

What New Information Does This Article Contribute?

- Nox4 expression is particularly high in endothelial cells.
- By hydrogen peroxide generation, Nox4 maintains endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) expression, which generate protective gaseous transmitters.
- By releasing small amounts of H2O2, Nox4 contributes to ischemia-induced angiogenesis and Nox4 attenuates the hypertensive and hypertonphy effects of angiostatin II.

- Nox4 therefore has functions antagonistic to Nox1 and Nox2.

Traditionally, ROS are thought to contribute to the initiation and progression of vascular disease. Nox (NADPH oxidases) are important sources of ROS. Through the formation of superoxide anions that scavenge protective nitric oxide, Nox1 and Nox2 contribute to vascular dysfunction. Nox4 is highly expressed in the vasculature and it generates mostly H2O2. Using Nox4-knockout mice, we demonstrate that Nox4-derived H2O2 maintains endothelial expression of the protective endothelial enzymes HO-1 and eNOS. We found that loss of Nox4 potentiates the proinflammatory and prohypertrophic effect of angiostatin II and attenuates hypoxic-induced angiogenesis. Thus, Nox4 has functions that are antagonistic to Nox1 and Nox2 and mediate vascular protection.
Nox4 Is a Protective Reactive Oxygen Species Generating Vascular NADPH Oxidase

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Online Figure I: Nox4 expression is higher in endothelial than in smooth muscle cells. (A) qRT-PCR of human aortic smooth muscle cells (HSMC) and human umbilical vein endothelial cells (HUVEC) and (B) murine aortic smooth muscle cells (MSMC) and murine lung endothelial cells (LEC) relative to the house keeping EF2α as determined by ΔACT method. (C/D): Western blot from mouse aorta (C, Nox4 65kDa) and qRT-PCR from mouse carotid artery (D) with (denuded) or without (intact) endothelial removal by CHAPS treatment. n=6, *p<0.05 with vs. without endothelium. eNOS expression (130 kDa) was used as marker to confirm successful endothelial denudation.
Online Figure II: Nox4 contributes to angiogenesis after femoral artery ligation.
Representative image and statistical analysis of capillary density (ratio of endothelial – EC – to muscle cells) of the semimembranosus muscle. Red: PECAM-1, Blue laminin. Effects in global Nox4-/- and Nox4+/+ littermates 14 days after femoral artery ligation and Nox4floxflox-ERT2-CRE+/0 (Nox4+/+) and Nox4floxflox-ERT2-Cre0/0 litter mates (WT). All floxed animals were treated with tamoxifen 10 days prior to the operation. (n≥5, *p<0.05)
Online Figure III: Role of Nox4 in angiotensin II-induced hypertension

(A) Tail cuff measurements: WT and Nox4-/- mice were implanted with osmotic mini pumps delivering angiotensin II (0.7mg/kg/d). Blood pressure was measured daily by tail cuff technique before and after the implantation. n=5-6, *p<0.05 vs. before pump implantation. (B) Telemetry transducers were implanted into Nox4flox/flox-ERT2-CRE+/0 (Nox4+/+) and Nox4flox/flox-ERT2-Cre0/0 littermates (WT). Blood pressure was recorded continuously. At the time point indicated, osmotic mini pumps delivering angiotensin II (0.7mg/kg/d) were implanted and left in place for the remainder of the study. Tamoxifen to induce Cre-recombinase activation was injected i.p. 3 times at the points indicated by arrows. n=5 per group. p=ns.
Online Figure IV: Role of Nox4 in angiotensin II-induced endothelium-dependent relaxation in the aorta of global Nox4-/- mice

WT and Nox4-/- mice were implanted with osmotic mini pumps delivering Angiotensin II (2.2mg/kg/d.). (A&B) Endothelium-dependent relaxation and (C) nitro-L-arginine-induced endothelium-dependent constriction, n≥7, *p<0.05 WT vs. KO, #p<0.05 without vs. with angiotensin II.
**Online Figure V: Role of Nox4 and Nox1 in angiotensin II-induced aortic hypertrophy.**

Normalized thoracic aortic weight in tamoxifen-pretreated Nox4^{flox/flox,ERT2-CRE^{+/-}} (Nox4^{+/-}) and tamoxifen-pretreated Nox4^{flox/flox,ERT2-Cre^{0/0}} littermates (WT) (left) and Nox1^{y/-} and Nox1^{y/+} (WT) litter mates. Subgroups received angiotensin II (AngII) by minipump (2.2 mg/kg/d, 2 weeks). n=6 per group, p<0.05 WT vs. knockout mouse.
Online Figure VI: Expression of antioxidant enzymes (A) and Nox homologues (B) in murine tissue

qRT-PCR was performed from the tissue indicated for the genes shown. Data were normalized to EF2α and are shown as relative difference to the WT control mice. Nox4^{flox/flox-ERT2-Cre}^{+/0} (Nox4^{+/+}) and Nox4^{flox/flox-ERT2-Cre}^{0/0} litter mates (WT) were treated with tamoxifen; tissue was harvested 10 days after treatment. Endothelial removal was performed with a brief exposure to CHAPS. n≥6, *p<0.05 endothelium intact vs. endothelium denuded.
Supplemental Material

Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase
Katrin Schröder, Min Zhang, Sebastian Benkhoff, Anja Mieth, Rainer Pliquett, Judith Kosowski, Christoph Kruse, Peter Luedike, U. Ruth Michaelis, Norbert Weissmann, Stefanie Dimmeler, Ajay M. Shah, Ralf P. Brandes1

Expanded Methods Section

Animals & animal procedures
All experimental procedures were approved by the local governmental authorities (approval number: F28/23) and were performed in accordance with the animal protection guidelines. C57/Bl6J mice were purchased from Charles Rivers (Deisenhofen, Germany). Nox1 and Nox2 are both located on the x-chromosome. As only male animals were used in this study, the knockout mice are designated Nox1y/- and Nox2y/-, respectively. Nox2y/- mice were obtained from Jackson laboratories via Charles Rivers. Nox1y/- mice, kindly provided by Karl-Heinz Krause and previously characterized were used in this study1. Nox1y/- and Nox2y/- mice were bred at the local animal facility. Nox4 -/- mice were generated by targeted deletion of the translation initiation site and of exons 1 and 2 of the Nox4 gene 2 and backcrossed into C57/Bl6J for 5 generations. Breeding was carried out by crossing heterozygous animals so that knockouts and direct wildtype littermates controls (WT) could be used throughout the studies. Tamoxifen-inducible Nox4-/- mice (Nox4flox/flox-ERT2-Cre+/0 mice) were produced by crossing Nox4flox/flox mice (backcrossed more than 10 generations into C57/Bl6J) with Cre-ERT2+/- mice. In these mice, a mutated fusion protein of the human estrogen receptor and the cre recombinase is driven by a CMV-promoter. The constructed is activated only by tamoxifen but not estrogens. Breeding was carried out by crossing Nox4flox/flox mice with Nox4flox/flox-ERT2-Cre+/- animals. Genetic deletion of Nox4 in Nox4flox/flox-ERT2-Cre+/- mice was induced by injection of tamoxifen (40 mg/kg dissolved in autoclaved sunflower oil intraperitoneally for 3 consecutive days).

Cell culture
Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords by dispase digestion or obtained from Lonza (Basel, Switzerland). HUVECs were used between passages 1-3 and cultured in EBM-medium supplemented with 10% FCS, bovine brain supplement and human recombinant EGF.

Lung endothelial cells were isolated from freshly prepared murine lungs as described before. In brief, the tissue was minced and dispase digested at 37°C. After several washing steps, LECs were separated magnetically using CD114 coated Dynabeads (MACS). LECs were used between passages 5-9. For induction of the Cre-promoter in culture, cells were treated with hydroxy-tamoxifen (10µmol/L for three consecutive days.)

Transfections
The Nrf-2-luciferase reporter plasmid was kindly provided by A.v. Knethen (Institute of Biochemistry, Frankfurt am Main, Germany). Transfection of the plasmid was performed using gene eraser according to the manufacturer’s instructions.

Immunoblotting
Western blot analyses were performed with an infrared-based detection system (Odyssey, Licor, Bad Homburg, Germany). The following antibodies were used: E-selectin and Nrf-2 (Santa Cruz), HO-1, HO-2 and Erk (Upstate). For Nox4 a previously reported antibody generated by one of the authors was used.5 The following lysis buffer was used for western blot (pH 7.4, concentrations in mmol/L): Tris-HCl (50), NaCl (150), sodium pyrophosphate (10), sodium fluoride (20), nonidet P40 (1%), sodium desoxycholate (0.5%), proteinase inhibitor mix, phenylmethylsulfonyl fluoride (1), orthovanadate (2), okadaic acid (0.00001). For Nox4 detection, samples were not boiled prior to loading and disulfide bonds were cleaved with TCEP (Thermo Scientific, Nidderau, Germany). In some experiments, the vascular endothelium was removed from the isolated vessel by a brief treatment with the detergent CHAPS (5 mg/mL dissolved in glucose solution 50 g/L, exposure for 45 seconds).

Amplex Red assay for cellular H2O2 production
Cells were grown on 12 well dishes until 90% confluence in the presence of hydroxy-tamoxifen (10 µmol/L). Cells were incubated with or without TGF-β (10ng/mL) overnight. H2O2 production was then measured in phenol-red free medium containing Amplex Red (50 µmol/L, Invitrogen), horse-radish peroxidase (1 U/mL) and no BSA. After 45 min the supernatant was transferred to 96 well plates and H2O2-dependent oxidation of Amplex Red was measured in a microplate fluorimeter (excitation 540 nm, emission 580 nm). Experiments were performed in the presence and absence of catalase (50 U/mL). H2O2 formation was determined as the catalase-sensitive part of the Amplex Red oxidation.
Ferrous oxidation xylenol orange assay for aortic 
H₂O₂ production

Aortic H₂O₂ production was measured with the ferrous oxidation-xylenol orange (Sigma) according to the manufacturers instruction with minor modification as described previously: 6 fresh aorta from mice perfused with PBS to wash out blood was cut into two pieces, weighted and incubated with PBS (0.1ml final vol) at 37°C for 1 h with or without PEG-catalase (200U/ml). Fox reagent was added to final volume of 1ml gently weighted and incubated for 30 min. H₂O₂ was used for calibration curve. The values were normalized by total tissue and expressed as peroxide / g aorta / min as the total or catalase-sensitive portion of the signal.

Luciferase assay

Luciferase activity was assayed with a luciferase assay kit from Promega (Mannheim, Germany) following the manufacturer’s instructions in a Berthold LB9505 luminometer (Bad Wildbad, Germany).

Blood pressure measurements

For radiotelemetric analysis, the left femoral artery of anaesthetized mice was exposed and cannulated with a 0.4 mm catheter connected to a radiotelemetric device (TA11PA-C10, Data Sciences International) anchored subcutaneously. After recovery (7 days), blood pressure and heart rate were monitored continuously for 7 weeks. On day 14 animals received osmotic minipumps (Alzet) delivering angiotensin II (0.7 mg/kg/d) and on day 17, 18 and 19 tamoxifen (40 mg/kg) was injected. Mean value was calculated with the software DQ ART 3.1 Gold (Data Sciences International). Tail cuff measurements were performed with a 4 channel setup (Vistech BP2000) after a 7 day training interval. 20 measures per day were recorded per mouse after acclimatization and the last 10 values were used for the analysis. Measures of 7 days were averaged per mouse.

Vascular hypertrophy and histology

For the induction of medial hypertrophy, osmotic minipumps delivering angiotensin II (2.2 mg/kg/d) were implanted in the back of the mice 10 days after tamoxifen treatment. Animals were sacrificed after two weeks. Aortic sections were cleaned of adventitial tissue, weighed, and cut into segments for vascular function studies or histology. The latter were fixed in PBS-buffered paraformaldehyde (PFA, 4% overnight), desiccated and embedded in paraffin. Serial sections (4um) of the thoracic aorta were cut, rehydrated and stained with the Elastica van Gieson technique. Sections were photographed with a Zeiss Axiolab microscope and media thickness was determined from the average of four points per section and three sections per animal by an observer blinded to the study protocol.

PCR-analysis

Organs were removed, shock frozen in liquid nitrogen and mortared. RNA was isolated using RNA Miniprep Kit (Stratagene, La Jolla, CA, USA). cDNA synthesis was carried out with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers, semiquantitative real-time PCR was performed with ABsolute QPCR SYBR Green Mix and ROX as reference dye (Thermo Scientific) in an Mx4000 cycler (Stratagene) with appropriate primers. Relative expressions of target genes were normalized to eukaryotic translation elongation factor 2 (EF2), analyzed by delta-delta-Ct method and given as percentage compared to control experiments. Primer sequences were as follows: IL-β fw: 5’-GACCTTCCAGGATGAG GACATGAGG-3’, IL-β rev: 5’-GGTG GGG CC TCC CTT CAT TAC-3’ and IL-6 fw: 5’-CCA GTG G CC TTC TGGA TGA CTG ATG-3’, IL-6 rev: 5’-CCT C CG ACT TGT GAA GTG TGA TAG-3’; EF-2 fw: 5’-GAC ATC ACC AAG GGT GTG CAG-3’; EF-2 rev: 5’-GCG TGC AGC ACA CTG GCA TA-3’; Nox1 fw: 5’-GGAG TCTT CCA ACC AACA-3’ Nox2 fw: 5’- AGC TAT GAG GTG GTG ATG TTA GTGG-3’; Nox2 rev: 5’-CAC AAT ATT TGG ACC AGA CAT TGG ACT GAG-3’; Nox4 fw: 5’-TGG TGG GCC TAC TAG GTG TGT TT-3’; Nox4 rev: 5’-AGG GAC CTT CTG TGA TCC TCG-3’; SOD1 fw: 5’-TTCG AGC AGA AGG CAA GGC GGT-3’; SOD1 rev: 5’-TCC GCC GGG CCA CCA TGT TT-3’; SOD2 fw: 5’-CAC CAC GGC GCC TAC GTG AA-3’; SOD2 rev: 5’-AGC CTC CAG CAA CTC TTC TTT GG-3’; SOD3 fw: 5’-CTT GGG AGA GCC TGA CAT GTG CA-3’; SOD3 rev: 5’-ACT TTG GCA TGC GTG TCG CC-3’; Catalase fw: 5’- GCATGAGCCCAGCCTGAC-3’, Catalase rev: 5’-GACATGACCCGGAATGCT-3’, HO-1 fw: 5’-GAC  ATC ACC AAG GGT GTG CAG-3’; EF-2 rev: 5’-AGTCCACAGCCCTGCGCCG-3’. In some experiments, the vascular endothelium was removed from the isolated vessel by a brief treatment with the detergent CHAPS (5 mg/mL dissolved in glucose solution 50 g/L, exposure for 45 seconds).

Vascular function studies

Organ chamber experiments were performed as described7. Relaxations to cumulatively increasing concentrations of acetylcholine (ACh) were recorded in vessels preconstricted to 80% of the maximal KCl (80 mmol/L)-induced contraction using phenylephrine in the presence of diclofenac (10 µmol/L). Relaxations are denoted as percent of the initial constriction obtained by phenylephrine. NO availability was estimated from the constrictor response to the NO synthase inhibitor Nω-nitro-L-arginine (L-NA, 300 µmol/L) in aortic rings preconstricted to 10% of the maximal KCl constriction using phenylephrine.

Apoptosis by FACS analysis

The assay was performed with an Annexin V-7AAD-assay according to the manufacturer’s instructions [BD Biosciences, Heidelberg, Germany]. CORM-A1 was kindly provided by R. Alberto and F. Zobi, Institute of Inorganic Chemistry, University of Zürich.

NO measurement by DAF-2 FACS method

As the chemistry of the DAF-2 assay is complex and background reactions frequently occur, experiments...
were performed with and without an eNOS inhibitor and only the eNOS inhibitor-sensitive portion of the signal was used as a measure for NO production. Cells were detached and preincubated with or without the NOS inhibitor Nitro-L-arginine methylester (300µmol/L) for 15 min. Subsequently, DAF-2-AM (10 µmol/L in DMSO) was added and cells were measured in the FL-1 channel (488 nm). Analysis was carried out in a FACS Calibur machine (BD Biosciences, Heidelberg, Germany).

**Plasma nitrite concentration**

Nitrite and nitrate levels in plasma were measured using HPLC-technique (ENO-20, Eicom) thus combining liquid chromatography with Griess reaction. This enables high sensitivity detection of nitrite and nitrate in biological liquid samples. In brief, plasma samples were precipitated with equal amounts of methanol and mixed using vortex for 10 sec. Samples were centrifuged for 10 min at 10,000xg and the supernatant was collected and analyzed.8

**Tube formation**

For the matrigel assay, LEC were seeded onto matrigel (1.5x10^4 cells/cm²) in EBM medium with or without H₂O₂ or PEG-catalase in the concentrations indicated. Tube formation was assessed after 4 hours and quantified by counting the number of branching points.

**Hindlimb ischemia**

Neovascularization capacity was investigated in a murine model of hindlimb ischemia using 6 to 8 week old mice. The deep femoral artery was ligated with an electric coagulator (ERBOTOM ICC50, ERBE) and subsequently the superficial femoral artery and vein as well as the epigastric arteries were completely excised by electrocoagulation. The overlying skin was closed with 3 surgical staples. Relative blood flow was determined by laser Doppler imaging (Laser Doppler Perfusion Imager System, Wilmington, Germany) at 7 and 14 days post-ligation. The perfusion of the ischemic and non-ischemic limb was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature and to maintain a constant body temperature, mice were exposed to infrared light for 10 minutes before laser Doppler scans. During the scan, mice were lying with their back on a heating pad with their legs stretched and fixed.

To determine capillary density, cross-sections of adductor and semimembranosus muscles embedded in Tissue Tek (Sakura, Heppenheim, Germany) were stained. After fixation in phosphate buffer (100 mmol/L, pH 7.3) containing 4% formalin the tissue was blocked with 3% BSA and permeabilized with 0.5% Triton X-100 followed by incubation with anti-CD31 (BD Pharmingen) and anti-laminin (Abcam, Cambridge, UK) and imaged by confocal microscopy.

**Statistics**

All values are mean ± SEM. Statistical analysis was performed by ANOVA followed by LSD post hoc testing or by T-test if appropriate. Dose response curves were compared by ANOVA for repeated measurements. Densitometry was performed with the odyssey-software. A p-value of less than 0.05 was considered statistically significant.

**Reference List**