**NOX1 Deficiency Protects From Aortic Dissection in Response to Angiotensin II**

Gaetan Gavazzi, Christine Deffert, Candice Trocme, Michela Schäppi, François R. Herrmann, Karl-Heinz Krause

**Abstract**—Oxidative stress leads to vascular damage and participates in the pathomechanisms of aortic dissection and aneurysm formation. Here we study aortic dissection in mice deficient in the superoxide-generating reduced nicotinamide-adenine dinucleotide phosphate oxidase NOX1. Seven days of treatment with the hypertensive agent angiotensin II (3 mg/kg per day) led to aortic dissection in 23% of wild-type C57BL/6J mice but in only 4% of NOX1-deficient mice ($P=0.05$). In contrast, treatment of wild-type C57BL/6J mice with the hypertensive agent norepinephrine (12 mg/kg per day), did not lead to aortic dissection or sudden death, suggesting that hypertension is not sufficient to cause aortic dissection. Interestingly, norepinephrine-dependent blood pressure elevations were conserved in NOX1-deficient mice, demonstrating that, different from angiotensin II, it acts through NOX1-independent hypertensive mechanisms. The resistance of NOX1-deficient mice to angiotensin II–induced aortic dissection suggests a role for NOX1-dependent alterations of the vascular wall. We, therefore, studied gene expression and protease/inhibitor equilibrium. cDNA array analysis demonstrated differential effects of angiotensin II on gene expression in wild-type and NOX1-deficient mice. Tissue inhibitor of metalloproteinase 1 was increased both on the mRNA and the protein level in aortas from NOX1-deficient mice. Thus, our results demonstrate that NOX1 is involved in the mechanisms of angiotensin II–dependent aortic dissection. As one underlying mechanism, we have identified NOX1-dependent suppression of tissue inhibitor of metalloproteinase 1 expression, which could lead to tissue damage through an altered protease/inhibitor balance. (Hypertension. 2007;50:189-196.)

**Key Words:** NOX1 ■ nicotinamide-adenine dinucleotide phosphate oxidase ■ blood pressure ■ aortic dissection ■ angiotensin II

Aortic dissections and formation of aneurysms are serious and frequent medical conditions that are the consequence of damage to the vascular wall. Animals model play a key role in understanding their pathomechanisms. Angiotensin II perfusion in mice has become a particularly valuable model for this pathological process. Angiotensin II–induced vascular alterations include the following: (1) sustained blood pressure elevations; (2) increase in the thickness of the aortic media through mechanisms including smooth muscle hyperplasia, smooth muscle proliferation, and accumulation of extracellular matrix (ECM) proteins; (3) markers of inflammation, including oxidative damage to the vascular tissue, infiltration of monocytes/macrophages, and activation of matrix metalloproteinases; and (4) formation of aortic dissection and aneurysms. Angiotensin II–dependent signal transduction is complex and involves ≥2 different receptors and the activation of multiple intracellular signaling pathways. There is now increasing evidence that reactive oxygen species (ROS) are an important part of angiotensin II–dependent signal transduction involved in the pathogenesis of aortic aneurysms.

Investigations on the source of ROS generation in the vascular system suggest an important role for the NOX family of ROS-generating reduced nicotinamide-adenine dinucleotide phosphate oxidases. More specifically, a role for NOX enzymes in aortic dissection and aneurysm formation is suggested by an increased expression of the p22phox NOX subunit in human aneurysmal aortas and by the decreased aortic aneurysm formation in p47phox-deficient mice. However, it is not clear which NOX enzyme is relevant for aortic dissection and aneurysm formation. In mice, 6 NOX family members are known: NOX1 to 4 and DUOX1 and 2. In the vascular system, NOX1 is expressed in smooth muscle; NOX2 in macrophages, adventitial fibroblasts, and to some extent also in endothelium; and NOX4 in endothelium and smooth muscle. The role of NOX1 in angiotensin II–induced hypertension has...
been demonstrated recently. Here we demonstrate that NOX1 is critically involved in angiotensin II–induced aortic dissection.

Methods
All of the reagents were from standard suppliers and of highest available grade.

Animals
Generation of NOX1-deficient mice has been described previously in detail. Animals were back crossed with C57BL/6J mice, and results shown in this study were obtained with F6-generation animals. Wild-type controls were either littermates or wild-type C57BL/6J mice. For our experiments, only males from 12 to 30 weeks of age (weight: 25 to 35 g) were used.

All of the mice were housed in a quiet room at 25°C with a 12-hour light/dark cycle and free access to food and water. This study and the animal procedures described below were approved by the Ethics Committee of Animal Care in Geneva and the Cantonal Veterinary Office (No. 31.1.1033/3168/3).

Definition of Sudden Death
Mice that were found dead without any preceding signs of suffering were defined as sudden death. Signs of suffering in mice were actively searched for by the following criteria: weight, activity, and fur appearance (during the course of the study, no sign of suffering in any of the mice was detected).

Implantation of Minipumps
For implantation of subcutaneous minipump (Azelt model 1007D), mice were anesthetized with inhaled isoflurane (5% at induction followed by an 2%). Minipumps were implanted through an incision in the midscapular region under sterile conditions. Angiotensin II (Sigma) was dissolved in 0.154 mol/L of NaCl for infusion at 3 mg/kg per day. Norepinephrine (Sigma) was dissolved in 0.154 mol/L of NaCl for infusion at 12 mg/kg per day. Sham-treated animals underwent an identical surgical procedure, except that in the control mice the minipump was replaced with an empty shell of the same size.

Tail Cuff Blood Pressure Measurements
Tail cuff blood pressure (TCBP) was measured in awake mice by tail cuff plethysmography (RTBP 200, Kent Scientific, ADI Instrument). Baseline measurements were performed on ≥3 different days before the implantation of minipumps. Animals were considered well trained if TCBP variability was ≤40±20 mm Hg. All of the reported TCBP values represent an average of 10 to 20 repeated measurements per time point and animal.

Histochemistry
For histology, specimens were fixed in formal, embedded in paraffin, and subsequently sectioned at 5 µm. Three different parts of each aorta were analyzed with optic microscopy after hematoxylin/eosin staining, performed according to standard procedures. Aortic dissection was defined histologically as a splitting of the middle layer (media) from the outer layer of the aorta and/or blood accumulation within the aortic wall. The analysis was performed blinded by an experienced staff member of our histology core facility.

Oligonucleotide Microarray
The total aorta of 3 wild-type mice and 3 NOX1-deficient mice were collected after a 7-day angiotensin II infusion. The total RNA was extracted from 10 to 15 mg of aorta, using the RNeasy Protect mini kit (Qiagen). A total of 0.2 µg of RNA was converted into double-stranded cDNA using a cDNA synthesis kit (SuperScript Choice, Invitrogen) with a special oligo(dT)24 primer containing a T7 RNA promoter site added to the poly-T tract. Biotinylated cRNAs were generated from cDNAs using the GeneChip IVT labeling kit (Affymetrix) and subsequently purified with the RNeasy kit (Qiagen).

All of the amplification started with 100 ng of total RNA for each sample. Two rounds of amplifications were performed for each replicate to synthesize biotinylated cRNA according to the Affymetrix protocol. A total of 17.5 µg of biotinylated cRNA was hybridized to mouse Affymetrix 430 2.0 chips, containing probe features for 45101 transcripts. The chips were washed and scanned and the fluorescence signals analyzed with Affymetrix software GCOS. To identify differentially expressed transcripts, pairwise comparison analyses were carried out with Affymetrix software. Each of the NOX1-deficient aorta samples (knockout) was compared with each of the wild-type aorta, resulting in 9 pairwise comparisons (knockout×wild-type).

Real-Time PCR
Thoracic aorta samples were placed in RNA later (Qiagen). After homogenization with polytron PT 1200E (Kinematica AG) in RNA, RNA was then extracted by the RNeasy Protect mini kit (Qiagen) and reverse transcribed using the superscript reverse transcriptase (Superscript Choice, Invitrogen). A total of 200 ng of sample was used as a template for the real-time PCR. Based on preliminary experiments, 2 constitutively expressed reference genes were selected for normalization of candidate gene expression levels. These genes were EEF1A1 (eukaryotic elongation factor 1A1) and TBP (Tata box binding protein). For PCR primers see the data supplement available online at http://hyper.ahajournals.org.

Metalloproteinases and Tissue Inhibitor of Metalloproteinase 1 Measurement
The explants of whole thoracic aortas from wild-type and NOX1-deficient mice were first homogenized with polytron PT 1200E (Kinematica AG). Gelatinase matrix metalloproteinase (MMP)-9 were evaluated by zymography as described previously. Band intensity was quantified by scanning densitometry (CD 60, Desaga, Sarstedt Group), and gelatinase levels were calculated by reference to the scanning values obtained from known amounts of purified MMP-9. This technique allows us to determine levels of latent and activated MMP-9 that migrate at 92 and 82 kDa, respectively, on the gel and degrade gelatin identically after electrophoresis. Specific activity was expressed in picomoles of gelatinase per microgram of protein.

Tissue inhibitor of metalloproteinase 1 (TIMP-1) levels were determined by a commercial ELISA assay (R&D Systems), of which sensitivity is 2.1 pg/mL according to the manufacturer. Results were expressed in picomoles of TIMP-1 per microgram of protein to calculate the molar MMP-9/TIMP-1 ratio.

Statistical Analysis
Differences in blood pressure values were assessed using ANOVA with a repeated-measures design. For the comparison of quantitative PCR, nonparametric tests were used because of the smaller sample size. Fisher’s exact test was used to compare the percentage of aortic dissections. All of the data were reported as mean±SD. Null hypotheses were rejected when P<0.05. Analyses were performed with the Stata 9.2, statistical package (Stata Corporation).

Results
Susceptibility of NOX1-Deficient Mice to Angiotensin II–Induced Aortic Dissection
We have previously described decreased blood pressure elevations of NOX1-deficient mice in response to angiotensin II. During these initial studies, we have observed in wild-type mice cases of sudden death (see Methods section for definition of sudden death). Because angiotensin II may lead to aortic dissection and aneurysms in C57BL/6 mice, we decided to study to what extent NOX1 might be involved in
the development of this pathology. Over a 7-day period of angiotensin II perfusion, 2 (ie, ≈7%) of the wild-type mice, but none of the NOX1-deficient mice, succumbed to sudden death. In surviving mice, systematic analysis of hematoxylin/eosin-stained aortic sections revealed aortic dissections or aneurysm with thrombi in 6 (of 26) wild-type C57BL/6 mice. In contrast, we found aortic dissection in only 1 (of 25) NOX1-deficient mice (Figure 1A). The difference in the occurrence of aortic dissections in wild-type versus NOX1-deficient mice was statistically significant (Figure 1B; \( P = 0.05 \)). Thus, NOX1-deficient mice are protected from angiotensin II–dependent aortic dissection, and we conclude that NOX1 is involved in the underlying pathomechanism.

**Norepinephrine-Induced Blood Pressure Elevations in NOX1-Deficient Mice**

Angiotensin II–induced blood pressure elevations were blunted in NOX1-deficient mice.\(^{23} \) Therefore, prevention of dissection may be simply because of decreased blood pressure. To study this question, we used norepinephrine, which acts through hypertensive mechanisms distinct from angiotensin II.\(^{31} \) Norepinephrine infusion (12 mg/kg per day) over a 7-day period induced comparable blood pressure elevations in wild-type and NOX1-deficient mice on days 1, 3, and 6 (Figure 2A). Note that the blood pressure increases in response to the selected norepinephrine concentrations were equal or even slightly higher than those observed in wild-type mice with angiotensin II.\(^{23} \) Thus, we conclude that norepinephrine causes substantial blood pressure elevations in the absence of NOX1 and that NOX1 activation is, therefore, not involved in hypertensive signaling by norepinephrine.

**Norepinephrine-Induced Blood Pressure Elevations and Aortic Dissection**

No sudden death was observed after treatment with norepinephrine. Histological sections of explanted aortas after norepinephrine treatment did not reveal aortic dissections in either wild-type or NOX1-deficient mice (data not shown). Figure 2B compares the results obtained with angiotensin II and norepinephrine. There was a significant reduction in angiotensin II–induced blood pressure elevations and aortic dissection in NOX1-deficient mice. Norepinephrine-induced blood pressure elevations were conserved in NOX1-deficient mice, and despite high blood pressure elevations, there were no aortic dissections in norepinephrine-treated mice. This strongly suggests that blood pressure elevations are not sufficient for the induction of aortic dissection and that angiotensin II damages the vascular wall through additional mechanisms, including activation of NOX1.

**NOX1 and Angiotensin II–Dependent Regulation of Gene Expression**

NOX1 might be involved in the mediation of angiotensin II effects through ROS-dependent regulation of gene expression. We, therefore, performed oligonucleotide array analysis in aortic samples from angiotensin II–treated wild-type and NOX1-deficient mice. As shown in the Table, numerous genes were differentially expressed in NOX1-deficient mice. Based on paired analysis (7 of 9 concordant pairs) and a 2-fold increase as cutoff, a total of 185 genes were upregulated, and >576 genes were downregulated in NOX1-deficient mice, as compared with wild-type mice. The 50 genes with the most important changes are shown in the Table. In terms of function gene analysis (GeneSpring, former Silicon Genetics, now Agilent Technologies), these 50 genes repartitioned as follows: cell structure, 15; metabolism, 11; cell DNA function, 6; cell organism defenses, 9; and unclassified, 9. Thus, we conclude that NOX1 is involved in the regulation of gene expression in response to angiotensin II. For this article, we have followed up by real-time PCR...
several genes that appeared of importance for mechanisms of aortic dissection (Figure 3).

The ECM plays an important role in the vascular health and disease. The oligonucleotide array suggested that thrombospondin 4 was increased in angiotensin II–treated NOX1-deficient mice, as compared with wild-type mice. Real-time PCR demonstrated that angiotensin II did not lead to increased thrombospondin 4 levels in wild-type mice. In contrast, angiotensin II caused a massive increase (>10-fold) in thrombospondin 4 mRNA levels in the NOX1-deficient mice (Figure 3A). Thus, NOX1-derived ROS appear to suppress the angiotensin II induction of thrombospondin 4 expression. Relatively little is known about thrombospondin 4. Thrombospondin 4 polymorphisms are associated with cardiovascular disease in epidemiological studies, and there are some indications that it might act as an “adaptor protein in ECM assembly.” More studies on this interesting protein will be needed to investigate whether it might indeed be involved in the pathophysiology of aortic dissection.

In previous studies, we have observed that NOX1 deficiency diminishes the angiotensin II–induced accumulation of ECM proteins in the aortic media. Collagen I and III are the main components of the aortic ECM, and a protein named CTHRC1 (collagen triple helix repeat containing 1) is thought to inhibit collagen expression. The results of the oligonucleotide array suggested that NOX1 deficiency might decrease procollagen type III mRNA levels and increase CTHRC1. We, therefore, measured mRNA levels of procollagen III of CTHRC1 by real-time PCR (Figure 3B and 3C). Thus, there were no statistically significant differences. However, because of the small sample size, we cannot exclude a moderate effect of NOX1 on the expression of procollagen type III and CTHRC1.

NOX1 and MMPs

The balance of protease and antiprotease activity in the aortic tissue is thought to play an important role in pathogenesis of aortic dissection and aneurysm. More specifically, the MMPs MMP-2 and MMP-9 are thought to favor the pathogenesis, whereas the specific inhibitor TIMP-1 is thought to be preventive.

The results of the oligonucleotide array suggested a possible increase in mRNA levels of TIMP-1, whereas there were no apparent differences in MMP levels. These results were confirmed by real-time PCR: NOX1 deficiency had no effect on MMP-2 and MMP-9 mRNA levels (Figure 3D and 3E). Angiotensin II increased TIMP-1 mRNA levels ~6-fold in wild-type mice but ~10-fold in NOX1-deficient mice. This suggests that NOX1 attenuates angiotensin II–induced TIMP-1 gene expression (Figure 3F).

These results raise the possibility that NOX1 alters the protease/antiprotease equilibrium. Indeed, an increase of the MMP-9/TIMP-1 ratio has been associated with aortoaneurysm and dissection. We, therefore, measured MMP9 gelatinolytic activity, as well as MMP-9 and TIMP-1 protein levels, in aortic tissue homogenates (Figure 4A through 4C). The levels of latent and activated MMP-9 were slightly increased after angiotensin II infusion, but there was no apparent difference between wild-type and NOX1-deficient mice (Figure 4A and 4B). In contrast, there was a marked effect of NOX1 deficiency on angiotensin II–induced changes in TIMP-1 protein levels. Angiotensin II did not lead to a statistically significant increase in TIMP-1 protein levels in wild-type mice but to a massive increase of ~70-fold ($P=0.01$) in NOX1-deficient mice (Figure 4C). Taken together, these results demonstrate that NOX1 is an inhibitor of angiotensin II–induced TIMP-1 expression and thereby alters the proteolytic balance in the vascular wall.

Discussion

The finding that NOX1 is of crucial importance for the formation of aortic dissection is novel and unexpected. Mechanisms that lead to angiotensin II–induced aortic dissection include monocyte/macrophage infiltration, activation of proteases (in particular, MMPs), and fibrotic response (reviewed in Reference 4). Because several of these processes involved oxidative stress, there is a mechanistic basis for a potential involvement of NOX enzymes in the pathogenesis.
The 50 Most Important Changes of Gene Expression in Aortic Samples of NOX1-Deficient Mice Versus Wild-Type Mice (n=3)

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<th>Affimetrix Probe Set Number</th>
<th>Gene Name</th>
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Positive numbers in the Fold Change column mean an increased expression and negative numbers a decreased expression in NOX-deficient mice.
of aortic dissection. However, our observation that NOX1 is a key player is unexpected. Indeed, the marked macrophage infiltration in early aortic dissection and aneurysm argues for a role of the phagocyte nicotinamide-adenine dinucleotide phosphate oxidase NOX2. More stringently, deficiency in the phagocyte nicotinamide-adenine dinucleotide phosphate oxidase subunit p47phox was shown to prevent angiotensin II–dependent aneurysm formation. Two explanations can account for the apparently diverging results: concomitant activation of NOX1 and NOX2 is necessary for development of aortic dissection and aneurysms, and p47phox might be used by NOX1 as an organizer subunit in the vascular wall. Studies with NOX2-deficient mice will be necessary to distinguish between the 2 possibilities.

Because NOX1-deficient mice have a decreased blood pressure response to angiotensin II, it was conceivable that the prevention of aortic dissection in NOX1-deficient mice was simply explained by the decreased blood pressure. We, therefore, used norepinephrine, a hypertensive agent thought to act through different pathways than angiotensin II. Interestingly, norepinephrine caused a full blood pressure increase in NOX1-deficient mice. Yet, no sudden death or aortic dissections were observed in either wild-type or NOX1-deficient mice treated with norepinephrine. This

Figure 3. Angiotensin II–induced gene expression in wild-type and NOX1-deficient mice. Mice were treated with angiotensin II (ang II) as described in Figure 1 and euthanized at day 7. mRNA levels of MMPs 2 and 9, TIMP-1, thrombospondin-4, procollagen III, and collagen triple helix type 1 (CTHRC1) were determined by real-time PCR; values shown are normalized with respect to 2 reference genes (RG). n=8 (A); n=4 (B through F).

Figure 4. Effect of NOX1 deficiency on MMP-9 and TIMP-1 levels. Mice were treated with angiotensin II (ang II) as described in Figure 1 and euthanized at day 7. Thoracic aortas were explanted and homogenized. Representative zymogram of MMP-9 in sham and angiotensin II–infused wild-type and NOX1-deficient mice (A), levels of latent MMP-9 (B), levels of activated MMP-9 (C), and TIMP-1 (D) were measured as described in the Methods section.
clearly demonstrates that blood pressure elevations are not sufficient to induced aortic dissection in wild-type mice and that NOX1 activation is an important factor in this process. Our observation that blood pressure increase by itself is not sufficient for aortic dissection, and aneurysm formation is in line with observations by other groups.20,40

Through which mechanisms does NOX1-derived ROS participate in the formation of aortic dissection and aneurysm? Our results demonstrate an exquisite sensitivity of TIMP-1 mRNA and protein levels to NOX1. This observation is corroborated by the recent demonstration that ROS induce downregulation of the TIMP-1 protein.41 In our study, there is an obvious difference between the strong effects of NOX1 deficiency on the TIMP-1 protein level (Figure 4C) and the more moderate effects on TIMP-1 mRNA levels (Figure 3F). Because the relationship between mRNA levels and protein levels is not linear, we cannot exclude that NOX1 exerts its effect solely through transcriptional regulation. However, it is tempting to speculate that, in addition, there is a direct effect of NOX1-derived ROS on TIMP-1 protein synthesis or stability.

TIMP-1 inhibits MMPs, in particular, the proform of MMP-9.38,42 There are several lines of arguments suggesting that TIMP-1 plays an important role in the development of aortic dissection and aneurysms. TIMP-1 is secreted by vascular smooth muscle cells.38 TIMP1-deficient mice have an increased risk of aneurysm formation in different mouse models of aneurysm,43–45 and TIMP-1 polymorphisms are linked to human risk for aortic aneurysm.46 Thus, together with the published information, our results provide a strong argument in favor of TIMP-1 as an important downstream mediator of NOX1-derived oxidative stress in the pathogenesis of aortic dissection and aneurysm.

NOX1-dependent downregulation of TIMP-1 in wild-type mice is expected to enhance the proteolytic activity in the vascular wall. However, we have previously documented an increased accumulation of collagen-type ECM in angiotensin II–stimulated wild-type mice, as compared with NOX1-deficient animals.73 At first glance, this is counterintuitive, because an increase in MMP activity should lead to an increased degradation of collagen. So, how can this be conceptualized and how is the high protease/antiprotease ratio in wild-type mice linked to aortic dissection? We consider several not virtually exclusive possibilities: (1) MMPs might preferentially degrade elastin, a key matrix protein for the aortic stability, and collagen accumulation might only be a secondary compensatory phenomenon;13–38; (2) MMPs promote basement membrane degradation, phenotypic modulation, migration, and proliferation of vascular smooth muscle cells and might thereby destabilize the aortic wall;47, and (3) the proteolytic activity of MMPs might also be involved in the activation of signaling molecules, such as transforming growth factor-β.48

Is there a direct effect of NOX1-derived ROS on MMPs in addition to the indirect effect via TIMP-1 described in this study? Angiotensin II clearly activates MMPs.13,14,20 Several in vitro studies indeed point toward a role of oxidative stress in this process.49,50 However, the in vivo evidence for a role of oxidative stress in angiotensin II–dependent MMP activation is relatively weak. Vitamin E had a protective effect on aneurysm formation but did not prevent activation of MMPs.13,14,20 p47phox deletion diminished, to some extent, MMP-2 (but not MMP-9) activity in the vascular wall.20 Note, however, that these effects were relatively small as compared with the effects on TIMP-1 described in this study. Indeed, angiotensin II appears to double the activity of MMP-2 and MMP-9 in the vascular wall,13,14,20 whereas p47phox deletion diminishes MMP-2 activation by ~20%.20 For comparison, NOX1 deletion leads to a 6-fold increase in TIMP-1 protein levels.

Perspectives

Our study documents a new role of NOX1 in vascular pathology. The link between NOX1 activation and TIMP-1 suppression also provides new evidence for the protective role of TIMP-1. The essential role of NOX1 in aneurysm formation opens interesting therapeutic avenues. NOX1 might become a prime drug target for prevention and early treatment of aortic aneurysms. Indeed, NOX1 inhibition would target both high blood pressure23,24 and blood pressure–independent modifications within the vascular wall. In addition, as opposed to NOX2, NOX1 inhibition is not expected to lead to immunodeficiency. Thus, a long-term treatment with NOX1 inhibitors, which would be needed to prevent progression of aneurysms, is a realistic perspective.

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Disclosures
None.

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