gp91phox-Containing NADPH Oxidase Mediates Endothelial Dysfunction in Renovascular Hypertension

O. Jung, MD; J.G. Schreiber; H. Geiger, MD; T. Pedrazzini, MD; R. Busse, MD; R.P. Brandes, MD

Background—Isoforms of the NADPH oxidase contribute to vascular superoxide anion (O$_2^-$) formation and limit NO bioavailability. We hypothesized that the endothelial gp91phox-containing NADPH oxidase is predominant in generating the O$_2^-$ to scavenge endothelial NO and thus is responsible for the development of endothelial dysfunction.

Methods and Results—Endothelial dysfunction was studied in aortic rings from wild-type (WT) and gp91phox-knockout (gp91phox$^{-/-}$) mice with and without renovascular hypertension induced by renal artery clipping (2K1C). Hypertension induced by 2K1C was more severe in WT than in gp91phox$^{-/-}$ mice (158±2 versus 149±2 mm Hg; P<0.05).

Endothelium-dependent relaxation to acetylcholine (ACh) was attenuated in rings from clipped WT but not from clipped gp91phox$^{-/-}$ mice. The reactive oxygen species (ROS) scavenger Tiron, PEG-superoxide dismutase, and the NADPH oxidase inhibitory peptide gp91ds-tat enhanced ACh-induced relaxation in aortae of clipped WT mice. Inhibition of protein kinase C, Rac, and the epidermal growth factor receptor kinase, elements involved in the activation of the NADPH oxidase, restored normal endothelium-dependent relaxation in vessels from clipped WT mice but had no effect on relaxations in those from gp91phox$^{-/-}$ mice. Relaxations to exogenous NO were attenuated in vessels from clipped WT but not clipped gp91phox$^{-/-}$ mice. After removal of the endothelium or treatment with PEG-superoxide dismutase, NO-induced relaxations were identical in vessels from clipped and sham-operated WT and gp91phox mice.

Conclusions—These data indicate that the formation of O$_2^-$ by the endothelial gp91phox-containing NADPH oxidase accounts for the reduced NO bioavailability in the 2K1C model and contributes to the development of renovascular hypertrophy and endothelial dysfunction. (Circulation. 2004;109:1795-1801.)

Key Words: stress, oxidative ■ angiotensin ■ endothelium ■ hypertension

Endothelial dysfunction, a situation of attenuated nitric oxide (NO) availability, is associated with an enhanced cardiovascular mortality. The amount of bioactive NO, which is the predominant antiantiatherosclerotic principle in the vascular wall, is determined by the activity of the endothelial NO synthase (eNOS) and by the amount of NO-scavenging superoxide anions (O$_2^-$). In situations of an activated renin-angiotensin system or increased wall stretch, vascular isoforms of the NADPH oxidase contribute to O$_2^-$ formation. To date, NADPH oxidases have been demonstrated to be functional in all vascular layers, including the endothelium, the smooth muscle layer, and the adventitia. In each of these layers, several isoforms of the leukocyte enzyme are expressed. In endothelial cells and in the adventitia, the Nox homologue gp91phox, also called Nox2, is expressed. Nox4 is reported to be expressed through the vessel wall, whereas Nox1 expression is restricted to the smooth muscle layer.

Nox1- and gp91phox-mediated O$_2^-$ production is increased by angiotensin II and renovascular hypertension in humans is associated with endothelial dysfunction and increased vascular O$_2^-$ formation. It is unknown, however, which isoform of the leukocyte NADPH oxidase and which vascular layer yields O$_2^-$ formation in renovascular hypertension and thus is responsible for the development of endothelial dysfunction.

We hypothesized that the endothelial gp91phox-containing NADPH oxidase is the predominant enzyme involved in the generation of O$_2^-$, which leads to endothelial dysfunction in renovascular hypertension. To test this hypothesis, we performed experiments in wild-type (WT) and gp91phox$^{-/-}$ mice with and without renovascular hypertension induced by the 2-kidney, 1-clip model.

Methods

Study Design and Animal Procedures

Male gp91phox$^{-/-}$ mice (background C57 black 6) were obtained from Jackson Laboratories, Bar Harbor, Maine. Matched C57 black 6 control mice were obtained from Charles River Breeding Laboratories, Sulzfeld, Germany. At the age of 8 weeks, animals were
subjected to sham operation or clip application as described elsewhere.\textsuperscript{22} A single injection of ampicillin (50 mg/kg SC) was applied to prevent wound infection after the operation. Blood pressure was measured 14 and 28 days later by the tail-cuff technique (Visitech Systems), and organ chamber experiments were performed at day 29.

Successful induction of renovascular hypertension, defined by a blood pressure $>120$ mm Hg 2 weeks after clip application was observed in $\approx70\%$ of the animals. Animals that did not meet this criterion were excluded from the study. Experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23) and were approved by the local government (II/25.3-19c20/15-F61/16).


table

<table>
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<tr>
<th>Gene</th>
<th>Wild-Type Sham (n=8)</th>
<th>Wild-Type Clip (n=8)</th>
<th>gp91\textsuperscript{\textdagger/- Sham (n=8)</th>
<th>gp91\textsuperscript{\textdagger/- Clip (n=8)</th>
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<td>5'-GGAGCCCTGTTGATCCTGG-3'</td>
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<td>p22phox</td>
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<td>5'-GTTGGTATAGGTGGTGTATG-3'</td>
<td>1.00±0.10</td>
<td>1.09±0.13</td>
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</table>

Values are expressed relative to the copy numbers determined in sham-operated wild-type mice.

**Organ Chamber Experiments**

Organ chamber experiments were performed as described\textsuperscript{4} in aortic rings. The concentration of phenylephrine was adjusted to obtain an identical preconstriction level of 80% of the contraction elicited by KCl (80 mmol/L). No statistical difference was observed in the preconstriction level and force development of the different study groups (data not shown).

**Organ Culture**

Aortic segments were obtained under sterile conditions from WT and gp91phox\textsuperscript{\textdagger/- mice, cleaned, and incubated for 24 hours at 5% CO\textsubscript{2} and 37°C in MCD1 131 culture medium (Gibco) containing 0.1% BSA, penicillin, and streptomycin in the presence of different concentrations of [\textit{val}-angiotensin II. After this period, rings were subjected to organ chamber experiments.

**Vascular Radical Generation**

Measurements were performed by use of a lucigenin (5 μmol/L)-enhanced chemiluminescence assay in intact mouse aortic rings as described previously.\textsuperscript{4}

**Angiotensin II Plasma Levels**

Angiotensin II levels from ETDA plasma were determined with a commercially available radioimmunoassay (IBL).

**Immunoblotting**

Western blot analysis from Triton X-100 (1%)-soluble aortic protein was performed as described previously\textsuperscript{4} with the following antibodies: mouse anti-eNOS (BD Transduction), mouse anti-Rac-1 (BD Transduction), sheep anti-CuZnSOD (Calbiochem), and rabbit anti-catalase (Calbiochem).

**Real-Time PCR**

Primers against NADPH oxidase subunits were designed using the sequence information of the NCBI database (Table 1). Total RNA was isolated from aortic rings with the "Absolutely RNA RT PCR Miniprep kit" (Stratagene Europe), and the extracted total RNA (between 250 and 350 ng) was used for reverse transcription (Superscript RT II, Invitrogen) with pdN\textsubscript{5} primers (Amersham/Pharmacia) in a total volume of 50 μL. These reactions were diluted, and 10 μL of the dilutions was used for amplification in the Mx4000 (Stratagene) with the oligonucleotides described, Platinum Taq Polymerase (Invitrogen), and SYBR green I according to the manufacturer’s instructions. The polymerase chain reaction (PCR) conditions were as follows: initial denaturation, 95°C, 7 minutes; 40 to 45 cycles of denaturation (95°C, 30 seconds), annealing (55 to 67°C, 30 to 60 seconds), and elongation (72°C, 30 to 60 seconds).

**Statistics**

All values are mean±SEM. Maximal relaxations were calculated from individual dose-response curves. Statistical analysis was carried out by ANOVA for repeated measurements followed by Fisher’s least significant difference test or paired t test, if appropriate. Values of $P<0.05$ were considered statistically significant.

**Results**

Four weeks after clip application, cardiac hypertrophy, shrinkage of the clipped kidney, and hypertrophy of the contralateral side was observed in WT and gp91phox\textsuperscript{\textdagger/- mice. Angiotensin II plasma levels were similarly increased after renal artery clipping in both mouse strains (Table 2).

**Renal Artery Clipping Does Not Induce Endothelial Dysfunction and Results in Less Hypertension in gp91phox\textsuperscript{\textdagger/- Mice**

Endothelium-dependent relaxation to acetylcholine (ACh) was significantly attenuated 4 weeks after renal artery clipping in aortic rings of WT mice. In vivo treatment with PEG-SOD or ex vivo application of the antioxidant Tiron (1 mmol/L) or the SOD mimetic manganese TBAP (10 μmol/L, data not shown) significantly improved relaxation (Figure 1, A and B), demonstrating that scavenging of NO by O$_2^-$ underlies endothelial dysfunction in the present study. Endothelium-dependent relaxation was slightly better in aortic rings from sham-operated gp91phox\textsuperscript{\textdagger/- than in rings from WT mice. In contrast to WT animals, clip application had no effect on endothelium-dependent relaxation in gp91phox\textsuperscript{\textdagger/- mice (Figure 1C).
Blood pressure in sham-operated animals was identical between WT and gp91phox\(^{-/-}\) mice. Clipping increased the blood pressure in both strains. The hypertensive effect of the procedure was significantly more pronounced in WT than gp91phox\(^{-/-}\) animals (Figure 1D).

### Inhibitors of NADPH Oxidase Activation Selectively Restore Endothelial Function in Aortic Rings of WT Mice

To demonstrate that the attenuated endothelium-dependent relaxation in aortic rings from clipped WT mice is a consequence of NADPH oxidase activation, inhibitors of different pathways involved in oxidase activation\(^{14}\) were tested. Inhibition of protein kinase C, Rac, and the epidermal growth factor receptor kinase all restored normal endothelium-dependent relaxation in rings from clipped WT mice, whereas none of the inhibitor had any effect on relaxation in rings from clipped or sham-operated gp91phox\(^{-/-}\) mice (Figure 2). Compared with the peptide control, the NADPH oxidase-specific but isoform-unselective peptide inhibitor gp91ds-tat improved ACh-induced relaxation in vessels from clipped but not sham-operated animals (Figure 3A).

<table>
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<th>Characteristics of the Animals</th>
<th>WT</th>
<th>gp91phox(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=8)</td>
<td>Clip (n=7)</td>
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<tr>
<td>Cardiac weight index, mg heart wt/g body wt</td>
<td>4.39±0.07</td>
<td>4.80±0.16*</td>
</tr>
<tr>
<td>Left/right kidney ratio</td>
<td>1.00±0.04</td>
<td>0.52±0.10*</td>
</tr>
<tr>
<td>Angiotensin II plasma levels, nmol/L</td>
<td>20.9±0.9</td>
<td>35.4±4.5*</td>
</tr>
</tbody>
</table>

\(P<0.05\) sham vs clipped group of identical strain.

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**Table 2.** Characteristics of the Animals

**Figure 1.** Effect of renal artery clip application on endothelium-dependent relaxation and blood pressure in WT and gp91phox\(^{-/-}\) mice. Effect of antioxidant Tiron (A, 1 mmol/L) and in vivo PEG-SOD (B) on clip-induced attenuation of endothelium-dependent relaxation in WT mice: concentration-response curves to ACh were obtained in phenylephrine-preconstricted mouse aortic rings. \(P<0.05\) clipped vs sham-operated group and Tiron or PEG-SOD present vs absent. C, Endothelium-dependent relaxation in mouse aortic rings from clipped and sham-operated WT mice and gp91phox\(^{-/-}\) mice, \(n=7\) to \(8\) in each group. \(P<0.05\) clipped vs sham-operated WT animals. D, Blood pressure measured by tail-cuff technique 4 weeks after sham or clip operation in WT and gp91phox\(^{-/-}\) (gp91\(^{-/-}\)) mice. \(P<0.05\) clipped WT vs clipped gp91phox\(^{-/-}\) mice. \(n=8\).

**Figure 2.** Effect of inhibitors of NADPH oxidase activation on endothelium-dependent relaxation. Endothelium-dependent relaxation to ACh in aortic rings from clipped and sham-operated WT (left) mice and gp91phox\(^{-/-}\) (right) mice was studied in absence and presence of substances interfering with activation of NADPH oxidases. A, Preincubation with protein kinase C inhibitor Ro31-8220 (1 \(\mu\)mol/L, 30 minutes); B, preincubation with Clostridium difficile lethal toxin B (TcdB, 60 minutes, 40 ng/mL); C, preincubation with epidermal growth factor receptor kinase inhibitor AG1478 (1 \(\mu\)mol/L). \(n=6\) in each group. \(P<0.05\) clipped vs sham-operated WT animals in absence of inhibitors.
A trend toward an increased O$_2^-$ formation was observed in rings from gp91phox$^{-/-}$ mice, but this value did not reach statistical significance (Figure 3B). The contribution of the endothelium to vascular radical formation was studied for agonist-induced O$_2^-$ production. Stimulation with phorbol myristate acetate (100 nmol/L) resulted in a pronounced increase in O$_2^-$ formation in aortic segments from WT animals only (data not shown), as reported previously. Removal of the endothelium attenuated the phorbol myristate acetate–induced O$_2^-$ formation by 89% and 92%, respectively, in aortic segments from sham- and clip-operated WT mice.

Accordingly, in contrast to the observations made in WT animals, the antioxidant Tiron had absolutely no effect on endothelium-dependent relaxation of clipped gp91phox$^{-/-}$ mice, suggesting that in these animals, NO bioavailability is not limited by O$_2^-$ (Figure 3C).

**Vascular Gene Expression Is Similar Between gp91phox$^{-/-}$ and WT Mice**

No difference in the expression of eNOS, catalase, CuZn-SOD, extracellular SOD, and Rac was observed between gp91phox$^{-/-}$ and WT animals from the sham-operated and clipped groups (Figure 4). Expression of NADPH oxidase subunits was studied by real-time PCR. Because the gp91phox$^{-/-}$ mice were generated by the insertion of a neomycin cassette into exon 3, gp91phox mRNA is detectable in gp91phox$^{-/-}$ mice, although no intact protein is translated. mRNA levels for p22phox, Nox1, gp91phox, and Nox-4 were not different between sham-operated and clipped mice (Table 1).

**Endothelial NADPH Oxidase Limits NO Bioavailability**

gp91phox is expressed predominantly in the endothelium and the adventitia. To study which of these 2 layers limits NO bioavailability, responses to exogenously applied NO released by Deta-NONOate were studied in endothelium-intact and denuded vessels. NO-induced relaxations were attenuated in vessels from clipped WT mice compared with sham-operated animals. Clipping had no effect on relaxation in vessels from gp91phox$^{-/-}$ mice. Removal of the endothelium by the detergent CHAPS (5 mg/mL dissolved in glucose solution 50 g/L, exposure for 45 seconds) improved NO-dependent relaxation. After endothelial denudation, NO-mediated relaxation was identical in all 4 groups. Accordingly, relaxations of endothelium-intact rings to sodium nitroprusside, an NO donor that releases NO directly in the smooth muscle by metabolism through an NADH oxidoreductase, were identical in all groups (Figure 5, A through C). In vivo treatment with PEG-SOD (Figure 5D) or ex vivo treatment with MnTBAP (data not shown) restored complete relaxation to Deta-NONOate in vessels from clipped WT mice, demonstrating that scavenging of NO underlies the attenuated responses after clipping.

**High Concentration of Angiotensin II Attenuated Endothelium-Dependent Relaxations in gp91phox$^{-/-}$ Mice**

Experiments using angiotensin II infusion clearly demonstrated that the smooth muscle layer is an important source of...
O$_2^-$ in response to angiotensin II. We therefore hypothesized that a different reactivity to angiotensin II between the endothelium and the smooth muscle layer might underlie our observation of a predominant endothelial role of NO scavenging in the present study. To test this hypothesis, aortic rings from WT and gp91phox$^{-/-}$ mice were incubated in organ culture at different concentrations of angiotensin II.

Incubation for 24 hours with 100 nmol/L angiotensin II attenuated responses in vessels from WT and gp91phox$^{-/-}$ mice. In contrast, 30 nmol/L of angiotensin II had no effect on relaxation in gp91phox$^{-/-}$ mice but was equally effective in impairing relaxation as 100 nmol/L angiotensin II in rings from WT mice (Figure 6).

**Discussion**

In the present study, we identified the mechanism underlying attenuated endothelium-dependent relaxation in renovascular hypertension in a mouse model. Genetic deletion of the gp91phox subunit of the NADPH oxidase prevented the endothelium-induced endothelial dysfunction and to some extent attenuated renovascular hypertension. In WT mice, renovascular hypertension was associated with increased NO scavenging of exogenously applied NO, an effect sensitive to removal of the endothelium or deletion of gp91phox. These data indicate that O$_2^-$ formed by an endothelial gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension by scavenging endothelium-derived NO.

Renovascular hypertension arises from an increased generation of angiotensin II as a consequence of increased renal renin release. Via several different effector pathways, angiotensin II increases blood pressure, including aldosterone-mediated sodium and water retention and an increase in sympathetic drive. Angiotensin II also leads to the induction and activation of NADPH oxidases in all vascular layers. This

Figure 4. Aortic protein expression in clip- and sham-operated animals. Protein expression of endothelial NO synthase, catalase, extracellular SOD, CuZnSOD, and Rac was determined from aortic lysates of sham- and clip-operated WT and gp91phox$^{-/-}$ mice. Numbers below blots indicate results of densitometry. In n=8 animals per group, no significant differences in protein expression were observed.

Figure 5. Role of endothelium in clip-induced attenuation of NO-induced relaxation. Concentration-response curves to NO donor Deta-NONOate in endothelium-intact (A) and endothelium-denuded (B) rings and to sodium nitroprusside (SNP) in endothelium-intact (C) aortic rings from sham- and clip-operated WT and gp91phox$^{-/-}$ mice. *P<0.05 WT vs gp91phox$^{-/-}$, P<0.05 sham-operated vs clipped WT mice. n=8. D, Relaxations to DetaNONOate in endothelium-intact aortic rings from sham- and clip-operated WT mice 12 hours after injection of saline or PEG-SOD.

Figure 6. Dose effect of ex vivo application of angiotensin on endothelium-dependent relaxation. Aortic rings from WT (A) and gp91phox$^{-/-}$ (B) mice were incubated in organ culture for 24 hours with 0, 30, and 100 nmol/L [5val]-angiotensin II (Ang II). Subsequently, endothelium-dependent relaxation to ACh was studied. n=8, *P<0.05 control (CTL) vs Ang II 30 nmol/L and 100 nmol/L in aortic segments from WT mice. *P<0.05 CTL and Ang II 30 nmol/L vs Ang II 100 nmol/L in gp91phox$^{-/-}$ mice.
process results in scavenging of endothelium-derived NO and subsequent attenuation of endothelium-dependent relaxation as well as vascular remodeling and hypertrophy. Despite similar angiotensin II levels in hypertensive WT and gp91phox−/− mice, renovascular hypertension was significantly less pronounced in gp91phox−/− mice, illustrating the importance of gp91phox-derived O$_2^−$ for blood pressure control. The observed differences, however, were small and emphasize the role of the other pro-hypertensive pathways for renovascular hypertension mentioned above.

The lack of gp91phox also prevented clip-induced endothelial dysfunction, and this was an unexpected observation. Given that all vascular cells have the capacity to generate O$_2^−$ in response to angiotensin II via different NADPH oxidase isoforms, why should gp91phox be so important? One possible explanation could be derived from the ex vivo incubation with angiotensin II. It was observed that higher concentrations of angiotensin II, whereas at higher concentrations of angiotensin II, whereas at higher concentrations, the action of the endothelial AT$_2$ receptor blocks radical formation$^{10,16}$; the latter mechanism was not observed in smooth muscle cells.$^{17}$ Alternatively, the close spatial relationship of endothelial NO formation and endothelial gp91phox−mediated NO scavenging might be of greater relevance for NO bioavailability than scavenging of NO in the smooth muscle cell layer. A third important point relates to a potential uncoupling of endothelial NO synthase in the present model. Renal artery clipping appeared to be less effective in attenuating the relaxation to exogenously applied NO compared with the effects of clipping on responses to ACh, the stimulus of endothelial NO release. This may suggest that O$_2^−$ formed by the gp91phox-containing endothelial NADPH oxidase in renovascular hypertension not only scavenges NO but also impairs NO synthase–dependent NO formation, a mechanism recently described as NO synthase uncoupling.$^{18–20}$ Indeed, activation of NADPH oxidases appears to be a prerequisite for such an uncoupling.$^{20}$

In the present study, angiotensin II levels were elevated but mRNA expression of the NADPH oxidase remained unchanged. This suggests that in the 2-kidney, 1-clip model, activation rather than induction of NADPH oxidases underlies endothelial dysfunction. Indeed, 3 completely unrelated inhibitors that have previously been shown to block NADPH oxidase activation in cultured cells$^{14}$ restored normal endothelium-dependent relaxation in clipped WT animals without affecting responses in gp91phox−/− mice. In line with this, it has been suggested that protein kinase C activity, which is involved in NADPH oxidase activation, is increased constitutively in renovascular hypertension.$^{21}$ Moreover, increased wall stretch, angiotensin II, and hypertension per se are known to increase protein kinase C activity.$^{22–25}$

Without a doubt, the impact of renovascular hypertension on endothelium-dependent relaxation was small in the present study compared with previous observations in humans$^{31}$ and rats.$^{21}$ However, it is well known that mice are less susceptible to endothelial dysfunction than, for example, rats. The aorta of mice contains large amounts of extracellular SOD, attenuating the impact of oxidative stress on NO bioavailability.$^{26}$ A fact that might also explain the relatively mild increase in vascular O$_2^−$ formation after clipping of WT animals. Moreover, even a dysfunctional NO synthase in mice can induce some endothelium-dependent relaxation via the formation of dilating H$_2$O$_2$. Finally, the small caliber of the mouse aorta has a more favorable intima-to-media ratio and thus a higher NO bioavailability at the effector enzyme (soluble guanylyl cyclase) in the smooth muscle cells. Even in humans, endothelial dysfunction is most evident in larger vessels.

In conclusion, the present study demonstrates that endothelial dysfunction in renovascular hypertension of mice results from activation of the endothelial gp91phox-containing NADPH oxidase.

Acknowledgments

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References


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