Effects of Angiotensin II Infusion on the Expression and Function of NAD(P)H Oxidase and Components of Nitric Oxide/cGMP Signaling

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Abstract—Angiotensin II infusion causes endothelial dysfunction by increasing NAD(P)H oxidase–mediated vascular superoxide production. However, it remains to be elucidated how in vivo angiotensin II treatment may alter the expression of the gp91phox isoforms and the endothelial nitric oxide synthase (NOS III) and subsequent signaling events and whether, in addition to the NAD(P)H oxidase, NOS III contributes to vascular superoxide formation. We therefore studied the influence of in vivo angiotensin II treatment (7 days) in rats on endothelial function and on the expression of the NAD(P)H oxidase subunits p22phox, nox1, nox4, and gp91phox and NOS III. Further analysis included the expression of NO-downstream targets, the soluble guanylyl cyclase (sGC), the cGMP-dependent protein kinase I (cGK-I), and the expression and phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at Ser239 (P-VASP). Angiotensin II caused endothelial dysfunction and increased vascular superoxide. Likewise, we found an increase in vascular protein kinase C (PKC) activity, in the expression of nox1 (6- to 7-fold), gp91phox (3-fold), p22phox (3-fold), NOS III mRNA, and protein. NOS-inhibition with N\textsuperscript{G}-nitro-L-arginine decreased superoxide in vessels from angiotensin II–treated animals, compatible with NOS-uncoupling. Vascular NO assessed with electron paramagnetic resonance was markedly reduced. Likewise, a decrease in sGC-expression and P-VASP levels was found. In vivo PKC-inhibition with chelerythrine reduced angiotensin II–induced superoxide production and markedly inhibited upregulation of NAD(P)H oxidase subunits. We therefore conclude that angiotensin II–induced increases in the activity and the expression of NAD(P)H oxidase are at least in part PKC-dependent. NADPH oxidase–induced superoxide production may trigger NOS III uncoupling, leading to impaired NO/cGMP signaling and to endothelial dysfunction in this animal model. The full text of this article is available at http://www.circresaha.org.

Key Words: angiotensin II ■ nitric oxide synthase uncoupling ■ nox expression ■ cGMP-dependent protein kinase ■ vasodilator-stimulated phosphoprotein

Endothelial dysfunction in response to long-term angiotensin II treatment has been shown to be secondary to increased superoxide production within the endothelium, the media, and/or adventitial layer.\(^1\)\(^\text{-}\)\(^\text{3}\) Subsequent studies identified the NAD(P)H oxidase as a predominant superoxide source.\(^2\)\(^\text{2}\)\(^\text{-}\)\(^\text{4}\)\(^\text{-}\)\(^\text{6}\) The vascular NAD(P)H oxidase shares several characteristics with the multicomponent enzyme complex described in neutrophils.\(^7\) The endothelial as well as the adventitial NAD(P)H oxidase express the flavocytochrome b\(_{558}\) subunits gp91\(_{\text{phox}}\) and p22\(_{\text{phox}}\), as well as the cytosolic factors p47\(_{\text{phox}}\) and p67\(_{\text{phox}}\) and the small molecular weight G protein rac-1.\(^8\)\(^\text{-}\)\(^\text{9}\) In contrast, smooth muscle cells lack gp91\(_{\text{phox}}\) and recent studies identified the existence of several gp91\(_{\text{phox}}\) homologues such as nox1 and nox4.\(^1\)\(^\text{0}\) In vitro studies revealed that nox1 expression is increased in response to angiotensin II in a protein kinase C (PKC)–dependent fashion.\(^1\)\(^\text{0}\) Antisense nox1 mRNA completely inhibited angiotensin II–induced superoxide production, supporting a role of nox1 in redox signaling in vascular smooth muscle cells.\(^1\)\(^\text{0}\) No information is available concerning the regulation of nox isoforms in response to in vivo angiotensin II treatment, and it is unknown whether changes in the expression of nox isoforms are paralleled by changes in vascular PKC activity.

Another mechanism for impairing endothelial function in vascular disease is the uncoupling of NOS III to produce superoxide. Several recent studies of situations of increased...
oxidative stress and endothelial dysfunction such as atherosclerosis\textsuperscript{11} and diabetes\textsuperscript{12} demonstrated an upregulated but dysfunctional, uncoupled NOS III. It has become clear from studies on both NOS I and NOS III that in the absence of the substrate $\omega$-arginine or the cofactor tetrahydrobiopterin, heme reduction in the enzyme uncouples NOS, thereby producing superoxide rather than NO,\textsuperscript{13,14} which may contribute to oxidative stress and endothelial dysfunction. The relative contributions of an uncoupled NOS III and NAD(P)H oxidases to the generation of reactive oxygen species in the setting of high angiotensin II levels are unknown.

Increased production of reactive oxygen species by NAD(P)H oxidase activation and NOS III uncoupling can result in a reduction of vascular NO bioavailability, leading to a subsequent decrease in the activity of the cGMP-dependent kinase I (cGK-I) in vascular tissue.\textsuperscript{11} It remains to be established whether a similar phenomenon can be demonstrated in vessels from angiotensin II–treated animals. Thus, in this study, we examined the effect of angiotensin II infusion on expression and activity of the novel NAD(P)H oxidase proteins nox1 and nox4 and on NOS III uncoupling. We also assessed the functional consequences by measuring vascular NO production and expression of the soluble guanylyl cyclase and cGMP-dependent protein kinase (cGK-I). The role of PKC in the regulation of NADPH oxidase subunit expression and vascular superoxide production was assessed by in vivo treatment with the PKC-inhibitor chelerythrine. We found that both nox1 expression in smooth muscle cells and NOS III uncoupling in endothelial cells potentially contribute to the endothelial dysfunction that accompanies angiotensin II–induced hypertension, and that these phenomena are at least in part mediated by PKC.

Materials and Methods
The present study was conducted in accordance with the guidelines for animal experimentation at the University Hospital Eppendorf, Hamburg, Germany.

Animal Model, In Vivo Angiotensin II Infusion
Wistar rats (210 to 250 g; Charles River, Sulzfeld, Germany) were treated with a subcutaneous osmotic minipump (Alza Corp) filled with either angiotensin II or NaCl (sham-treated rats) for 7 days. The angiotensin II infusion rate averaged 1 mg/kg/d, as described.\textsuperscript{16} To address the role of PKC and NOS III in superoxide production, some rats were treated concomitantly with the PKC-inhibitor chelerythrine for 7 days (Alzet pumps, 5 mg/kg/d).\textsuperscript{15} In separate experiments, systolic blood pressure was measured by tail cuff plethysmography immediately before surgery, and in most animals, immediately before euthanasia.

Organ Chamber Experiments
Vasodilator responses to nitroglycerin (NTG) and the endothelium-dependent vasodilator acetylcholine (ACH) were tested in organ chambers, as previously described.\textsuperscript{16}

Determination of Vascular PKC Activity
Aortas were dissected free from tissue surrounding the adventitia and homogenized in a glass homogenizer on ice. Homogenates were sonicated 3 times for 10 seconds and centrifuged for 10 minutes at 2000g. The protein concentration of the supernatant was determined using the Bio-Rad Protein Assay and samples were stored overnight at 4°C. PKC activity was measured using an ELISA kit from Calbiochem. Briefly, 6 $\mu$g of protein was mixed with a reaction buffer containing (in mmol/L) 25 Tris-HCl, pH 7.0, 3 MgCl$_2$, 0.2 ATP, 2 CaCl$_2$, and 50 $\mu$g/mL phosphatidylserine. For the negative control, CaCl$_2$ was omitted from the buffer and 20 mmol/L EGTA was added in place of phosphatidylserine. To inhibit protein kinase A activity, 80 mmol/L protein kinase A inhibitor 14 to 22 amide was added to the buffer. Samples were transferred into a 96-well plate coated with pseudosubstrate. PKC present in the sample catalyzed the phosphorylation of pseudosubstrate on serine. A biotinylated antibody was added, which bound to the phosphorylated pseudosubstrate. This was followed by a peroxidase-catalyzed color reaction of which the OD was measured at 492 nm.

RNase Protection Analysis for the Expression of NOS III in Vascular Tissue
A cDNA fragment of rat NOS III was generated with reverse transcriptase–polymerase chain reaction (RT-PCR) as described,\textsuperscript{12} using 2 $\mu$g of total RNA from rat aorta. Oligonucleotide primers were 5'-GACATTGAGACAAAGGGCTGC-3' (sense) and 5'-CGCTTGTGCACTCCTGG-5' (antisense). RNase protection assays were performed with a mixture of RNase A and RNase T1 using 40 $\mu$g of total RNA from rat aorta as described.\textsuperscript{17} The protected RNA fragment of NOS III was 425 nt.

Spin Trapping of Basal NO Using Colloid Fe(DETC)$_2$
Segments from rat aorta (10 mm) were incubated (37°C for 30 minutes) in 24-well plates in 0.4 mL Krebs solution in the presence of 200 mmol/L colloid Fe(DETC)$_2$, as described recently.\textsuperscript{17} Electron paramagnetic resonance (EPR) studies were performed on a table-top x-band spectrometer Miniscope (Magnettech). Recordings were made at 77 K, using a Dewar flask (Wilmad). Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 60 seconds of sweep time, and the number of scans 10.

Estimation of Vascular Superoxide Production

Lucigenin-Enhanced Chemiluminescence
Vascular superoxide was estimated using lucigenin-chemiluminescence as previously described.\textsuperscript{18} To address the influence of endothelial (NOS III–derived) NO and NOS-mediated superoxide production as well as PKC on vascular lucigenin-enhanced chemiluminescence, vessels were preincubated with NO$\_2$-nitro-$\omega$-arginine (L-NNA, 1 mmol/L) and chelerythrine, respectively, for 30 minutes as described.\textsuperscript{19}

Oxidative Fluorescent Microtopography
The oxidative fluorescent dye dihydroethidium was used to evaluate the in situ concentration of superoxide, as described recently.\textsuperscript{10} To address the role of PKC and NOS III in superoxide production, some vessels were preincubated with chelerythrine or L-NNA (1 mmol/L) for 30 minutes, as described.\textsuperscript{19}

Measurement of NADH- and NADPH-Dependent Oxidase(s) Activity
Rat aortas were homogenized and plasma membrane fractions were isolated as recently described.\textsuperscript{21} Membranes (1 $\mu$g protein) were resuspended in 50 $\mu$L phosphate buffer (0.15 mol/L, pH 7.4) prepared from supra-pure grade NaCl, KH$_2$PO$_4$, K$_2$HPO$_4$, and supplemented with 50 mmol/L desferoxamine and 100 mmol/L DTPA. Membrane suspensions were incubated in the presence of 1 mmol/L CP-H (a superoxide-sensitive probe, Alexis) at 37°C for 30 minutes in the absence or presence of 400 mmol/L NADH or NADPH. The samples were then stored on ice and measured within 30 minutes. Spectra of EPR were recorded at room temperature using a x-band spectrometer (Magnettech). EPR parameters were 10 mW of microwave power, 0.3 mT of amplitude modulation, 100 kHz of modulation frequency, 60 seconds of sweep time, 336 mT of center field, 5 mT of sweep width, and the number of scans 2. The increases of the
EPR signals on stimulation with NADH or NADPH over background signals were superoxide dismutase (SOD)–inhibitable and reflected superoxide production by NAD(P)H oxidase(s). The background EPR signals (samples without NADH or NADPH) were electronically subtracted.

Quantitative Polymerase Chain Reaction for the Determination of nox1, nox4, and p22phox
Vascular cDNA was amplifed using the LightCycler (Roche) real-time thermocycler. Copy numbers were obtained from standard curves generated with genuine rat nox1, nox4, and p22phox templates, as described recently.10

Semi-quantitative Polymerase Chain Reaction for the Determination of gp91phox
Total RNA from rat aortic sections was isolated by guanidinium thiocyanate/phenol chloroform extraction (RNAzolTM, WAK-Chemie, D-61348 Bad Homburg).22 The gp91phox mRNA expression was determined by standard-calibrated, competitive RT-PCR as described.12

Western Blot Analyses for NOS III and sGC Expression
Rat aortic tissue was homogenized and subjected to SDS-PAGE and subsequently blotted to nitrocellulose membranes (BioRad). The blots were developed with a mouse monoclonal antibody to human sGC β1 subunits was performed as described previously.12

Detection of cGK-I and VASP Expression and VASP Phosphorylated at Ser239
Expression levels of cGK-I and VASP as well as the extent of VASP phosphorylated at serine 239 (P-VASP were determined by immunoblotting techniques as described recently.11,20

Statistical Analysis
Results are expressed as mean±SEM. The ED50 value for each experiment was obtained by log10-transform. To compare O2− production, P-VASP and VASP, sGC, gp91phox, nox isoforms, and cGK-I expression in normal and angiotensin II–treated animals, 1- or 2-way ANOVA was employed. Comparisons of vascular responses were performed using multivariate analysis of variance. A Scheffe’s post hoc test was used to examine differences between groups when significance was indicated. A value of P<0.05 was considered significant.

Results
Effects of In Vivo Angiotensin II Treatment on Blood Pressure, Vasorelaxant Responses, and Vascular PKC-Activity
As previously reported, infusion of angiotensin II caused a progressive increase in blood pressure from 164±4 to 219±5 mm Hg (Figure 1A). At day 7, hypertension was associated with marked endothelial dysfunction, a significant shift to the right of the NTG concentration-response curve (Table 1) and an increase in vascular PKC-activity (Figure 1B).

Effects of In Vivo Angiotensin II Treatment on the Expression of nox Homologues and p22phox Oxidase Activity
The expression of p22phox, nox1, and nox4 was assessed by quantitative PCR. As shown in Figure 2, angiotensin II infusion for 7 days increased nox1 expression ≈7-fold and p22phox expression 3-fold and slightly increased nox4 expression (Figure 2). gp91phox mRNA expression was increased to 330±31% of control. In vivo PKC inhibition did not modify NAD(P)H oxidase subunit expression of sham-treated rats, but markedly inhibited upregulation of nox1 and p22phox (Figure 2). A partial inhibition of gp91phox upregulation was also observed (data not shown).

NAD(P)H oxidase activity was assessed in membrane fractions by EPR after stimulation with either NADPH or NADH. The NAD(P)H-induced EPR-signals were about 2-fold higher as compared with the NADH-induced signals. Treatment with angiotensin II doubled superoxide production in response to both substrates (Figure 3). These data are in good agreement with our previous findings.

Effects of In Vivo Angiotensin II Treatment on NOS III Expression and Vascular NO Production
Angiotensin II infusion for 7 days significantly increased NOS III mRNA to 247±21% of control as determined by RNase protection analyses (Figure 4A). Likewise, NOS III protein expression was upregulated to 220±19% (Figure 4B). These findings clearly demonstrate that angiotensin II–induced endothelial dysfunction is accompanied by an upregulation rather than a downregulation of NOS III.

Vascular NO production as assayed with an intracellular Fe(DETC), was found to be markedly reduced in angiotensin II–infused animals as compared with controls (Figure 5).

Effects of Angiotensin II Treatment on Vascular Superoxide Production
In rats infused with angiotensin II, the vascular superoxide levels were about 3-fold higher as compared with vessels

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<th>Potency (−log M ED50)</th>
<th>Efficacy (% max. rel.)</th>
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<tr>
<td>ACh</td>
<td>NTG</td>
</tr>
<tr>
<td>Control (n=11)</td>
<td>7.69±0.09</td>
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<tr>
<td>Angiotensin II (n=20)</td>
<td>6.75±0.08*</td>
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Data are presented as mean±SEM; *P<0.05 vs control.

ED50 indicates concentrations that cause half maximal relaxation (max. rel.) of the vessel; NTG, nitroglycerin; and ACh, acetylcholine.

![Figure 1](image_url) Effects of angiotensin II infusion on systolic blood pressure and vascular protein kinase C (PKC) activity. *P<0.05 vs control.
treated with vehicle only (Table 2). Incubation of control vessels with the NOS inhibitor L-NNA increased lucigenin signals, indicating a significant attenuation of the baseline chemiluminescence signal by endothelium-derived NO, as described before. In vessels from angiotensin II–treated rats, superoxide levels were significantly decreased in response to L-NNA, indicating a significant contribution of NOS III to superoxide production in vessels from angiotensin II–infused animals. Likewise, we found a marked inhibition of both superoxide steady-state levels and superoxide production in response to angiotensin II after in vitro incubation and after in vitro treatment with the PKC inhibitor chelerythrine, consistent with a contribution of NAD(P)H oxidase enzymes, whose activity and expression are regulated by PKC.

Incubation of vessel sections from control and angiotensin II–infused animals with dihydroethidium revealed a marked increase in ethidium fluorescence in angiotensin II–infused animals in the endothelium, the media, and the adventitia, all to a similar extent (Figure 6). Pretreatment with chelerythrine in vitro did not modify vascular superoxide levels in controls vessels, but markedly decreased them in vessels from angiotensin II–treated animals. The decrease in superoxide was seen throughout the vessel wall. Likewise, in vivo PKC-inhibition with chelerythrine decreased superoxide mainly in the smooth muscle cell layer.

Incubation of control vessels with L-NNA increased superoxide signals in the endothelium consistent with our lucigenin measurements. In contrast, in vessels from angiotensin II–treated animals, L-NNA decreased ethidium fluorescence mainly in the endothelial layer, compatible with NOS III uncoupling (data not shown).

Figure 2. Effect of angiotensin II infusion (A II) with and without PKC-inhibition with chelerythrine on the expression of the NAD(P)H oxidase subunits p22phox, nox1, and nox4. Expression levels were assayed by real-time PCR (nox1, nox4, and p22phox). The upregulation of nox1 and p22phox were markedly inhibited by in vivo chelerythrine treatment. Data are the mean of 4 to 6 aortas; *P < 0.05 vs control.

Figure 3. Effects of NADH and NADPH on superoxide production in membrane fractions of vascular tissue from control animals (C) and animals treated with angiotensin II (A II). Data are mean±SEM from 4 independent experiments.

Figure 4. Effect of angiotensin II infusion on NOS III mRNA and protein expression in rat aorta. A, RNase protection analyses were performed with aortas from control rats (C) and angiotensin II–treated animals (A II) using antisense RNA probes to rat NOS III. Top, An autoradiograph of a representative gel. Bottom, The result of densitometric analyses of 6 different gels (*P < 0.01 vs control). B, Western blots were performed with aortas from control rats (C) and angiotensin II–treated animals (A II) using a monoclonal antibody to NOS III. Top, An original Western blot with samples from 2 different aortas. Bottom, Densitometric quantification of 8 blots for both groups (*P < 0.05).
Effect of In Vivo Angiotensin II Treatment on sGC, cGK-I, and VASP Expression and P-VASP Levels

Infusion of angiotensin II resulted in a significant decrease in the expression of the sGC subunit $\beta_1$ (Figure 7). A comparable decrease was observed for the sGC subunit $\alpha_1$ (from 100±10% to 23±4%, $P<0.05$), although the expression of cGK-I was not changed at all (Figure 8). In contrast, P-VASP was markedly decreased in vessels from angiotensin II–infused animals, indicating a decrease in cGMP-dependent protein kinase activity (Figure 8). Total VASP expression was not modified by angiotensin II infusion (data not shown). These data indicate that in addition to inactivation of NO by superoxide, angiotensin II may contribute to vascular dysfunction by downregulating sGC expression, thereby reducing cGMP-mediated effects such as cGK-I activity.

Discussion

The present data indicate that angiotensin II infusion increases vascular superoxide production and increases the expression of the NADPH oxidase subunits p22phox, nox1, and gp91phox, at least in part in a PKC-dependent fashion. Angiotensin II also leads to NOS III uncoupling, which may further enhance oxidative stress in vascular tissue, thereby decreasing NO bioavailability. In spite of an increase in NOS III expression, reduced vascular NO production, as well as the downregulation of the NO target enzyme sGC, inhibits the activity of cGK-I. The combination of these events can contribute to endothelial dysfunction and hypertension in this animal model.

**TABLE 2. Effects of Inhibition of NOS III by L-NNA and Inhibition of Protein Kinase C by Chelerythrine In Vitro and In Vivo on Vascular Superoxide Production in Control and Angiotensin II–Treated Rats as Determined With Lucigenin (5 μmol/L)**

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<th>Control (counts/mg/min)</th>
<th>Angiotensin II (counts/mg/min)</th>
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<tr>
<td>E+</td>
<td>1332±87</td>
<td>3307±191*</td>
</tr>
<tr>
<td>+L-NNA</td>
<td>2334±306*</td>
<td>1866±103†</td>
</tr>
<tr>
<td>+Chelerythrine (in vitro)</td>
<td>1246±224</td>
<td>1983±189†</td>
</tr>
<tr>
<td>+Chelerythrine (in vivo)</td>
<td>—</td>
<td>2644±350†</td>
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Data are mean±SEM (4 to 13 experiments). E+ indicates endothelium present. *$P<0.05$ vs control; †$P<0.05$ vs angiotensin II–treated.

Effects of Angiotensin II Infusion on Endothelial Function, nox Isoform Expression, and Vascular Superoxide Production

Previously, we and others have shown that treatment of rats with angiotensin II leads to a marked attenuation of vascular responses to endothelium-dependent vasodilators. Impaired endothelial function was associated with increased NAD(P)H oxidase activity and increased expression of the NADPH oxidase subunit p22phox. Importantly, relaxation was partly restored by in vitro and in vivo administration of liposomal-entrapped SOD or recombinant heparin-bound SOD. These observations suggest that NO degradation, due to increased vascular superoxide, significantly contributes to endothelial dysfunction and hypertension in this model. The vascular NAD(P)H oxidase shares several characteristics with the multicomponent enzyme complex described in neutrophils. The endothelial as well as the adventitial NAD(P)H oxidase express the flavocytochrome b558 subunits gp91phox and p22phox as well as the cytosolic factors p47phox and p67phox. In contrast, smooth muscle cells lack gp91phox, and recent studies identified the existence of gp91phox homologues such as nox1 and nox4. Because gp91phox and nox harbor the electron transfer functions of the enzyme and thus serve as the catalytic moiety, regulation of these subunits is of utmost importance to the ultimate function of the enzyme. Our current data show that both the endothelial/adventitial homologue gp91phox and the smooth muscle homologue nox1 are upregulated by angiotensin II, consistent with the concept that all layers of the vessel wall contribute to increased vascular superoxide production (Figure 5, Table 2).

Interestingly, recent in vitro studies revealed that angiotensin II downregulates nox4 while markedly upregulating the nox1 isofrom. Upregulation of nox1 was also observed in response to the phorbol ester phorbol myristate acetate (a direct activator of PKC), and the angiotensin II–induced upregulation of nox1 was inhibited by specific inhibitors of PKC, suggesting a crucial role for PKC in the upregulation of the nox1 message. Antisense nox1 mRNA completely inhibited angiotensin II–induced superoxide production, supporting a role for nox1 in redox signaling in vascular smooth muscle cells. The results obtained from our in vivo experiments support this concept. Using a nonradioactive PKC assay, we found a 2-fold increase in whole vessel homogenates from angiotensin II–treated animals compared with controls. Although nox4 expression was increased only slightly by angiotensin II treatment, a dramatic increase in the expression of nox1 (≈7 fold) and p22phox (3-fold) was
observed (Figure 2). Likewise a significant increase in the expression of gp91phox (3-fold) was found. The angiotensin II–induced upregulation of nox1 was strikingly reduced by in vivo treatment with chelerythrine, suggesting that as shown in vitro, the expression of nox1 in smooth muscle cells is regulated in a PKC-dependent manner.10 An interesting new aspect of the present studies is that upregulation of p22phox was attenuated by in vivo PKC inhibition as well, suggesting that PKC may serve as a general regulator of oxidase function by regulating both expression (present data) and activity.7

PKC-activation and increased expression of nox1 and in vascular tissue was associated with a significant increase in the activity of the NAD(P)H oxidase. As quantified by EPR method, the SOD-inhibitable NADPH- and NADH-stimulated superoxide production was significantly increased in membrane fractions of vessels from angiotensin II–infused animals as compared with controls. Furthermore, in vitro as

![Figure 6](image_url)

**Figure 6.** Effect of angiotensin II infusion on vascular superoxide production in rat aorta as detected with dihydroethidium. Fluorescent photomicrographs of confocal microscopic sections of aortas from rats receiving sham treatment (left top panel) or angiotensin II (left bottom panel), with or without incubation with the protein kinase C inhibitor chelerythrine (10⁻⁵ mol/L, middle panels) and with in vivo PKC-inhibition with chelerythrine (5 mg/kg/d for 7 days; right panels) are shown. Vessels were labeled with the dye dihydroethidium, which produces a red fluorescence when oxidized to ethidium by superoxide. Angiotensin II infusion increased superoxide production in the endothelium, the media, and adventitia. Incubation of vessels from angiotensin II–treated animals with chelerythrine markedly reduced superoxide. E indicates endothelium; M, media; and A, adventitia. Data are representative of 4 experiments.

![Figure 7](image_url)

**Figure 7.** Effects of angiotensin II infusion on the expression of the soluble guanylyl cyclase (sGC) subunit β₁ as assessed by Western blot. In aortas from angiotensin II–treated animals (A II), the expression of the sGC subunit β₁ was markedly reduced as compared with controls (C). Top, An original blot. Bottom, Results of the densitometric analyses. Data are the mean±SEM of 4 independent experiments.

![Figure 8](image_url)

**Figure 8.** Effects of angiotensin II infusion on the expression of the cGMP-dependent protein kinase (cGK-I), the vasodilator-stimulated phosphoprotein (VASP), and on the phosphorylation of the VASP at Ser239 (P-VASP; 16C2) in rat aortas. Top, An original blot of 3 controls (C) and 3 angiotensin II (A II)–treated aortas. Bottom, Densitometric quantification. Equal protein loading was verified by α-actinin immunostaining. VASP expression levels, determined by the use of phosphorylation independent antibodies, were not affected (data not shown). Bars represent the mean±SEM of 6 independent experiments; *P<0.05 vs control.
well as in vivo treatment with chelerythrine significantly inhibited dihydroethidium fluorescence, suggesting that activity of superoxide-producing enzymes strongly depends on vascular PKC activity. Taken together, these data suggest that the smooth muscle NAD(P)H oxidase may indeed contribute to increased superoxide production in response to angiotensin II.

**Effects of Angiotensin II Infusion on NOS III Expression and NOS-Mediated Nitric Oxide and Superoxide Production**

We have previously shown that NAD(P)H-derived superoxide may act as a "kindling radical" to cause NOS III uncoupling in a diabetic model. The present data suggest that a similar mechanism may be operative in angiotensin II–induced hypertension. Angiotensin II infusion increases, rather than decreases, the expression of NOS III mRNA and protein (Figure 4). These findings are in line with previous observations in different models of oxidative stress such as atherosclerosis and diabetes mellitus, where NOS III expression was significantly upregulated. Although the precise stimulus for increases in NOS III expression in such conditions remains unclear, recent studies have shown that PKC increases the expression of NOS III at the transcriptional level. In addition, the superoxide dismutase product H$_2$O$_2$ was demonstrated to be a strong regulator of NOS III expression at the transcriptional as well as the posttranscriptional level. Taken together, these findings may explain the upregulation of NOS III observed in this particular model of oxidative stress.

Despite the significant increase in NOS III gene expression, however, EPR measurements revealed that vascular NO production was strikingly reduced (Figure 5). Further experiments indicated that NOS III itself may become uncoupled. Using lucigenin-enhanced chemiluminescence, we found a marked increase in steady-state superoxide levels in vessels from angiotensin II–infused animals as compared with controls. Experiments with the NOS inhibitor L-NNA suggested NOS III as a significant superoxide source. Incubation of control vessels with L-NNA increased lucigenin and coelenterazine-enhanced chemiluminescence signals, compatible with NOS III production. Superoxide levels, increased by incubation of control vessels with L-NNA, decreased, rather than increased, vascular steady-state superoxide levels, compatible with NOS III–mediated superoxide production.

These findings are supported by results obtained with the fluorescent dye dihydroethidium. Angiotensin II infusion caused a significant increase in superoxide, not only in the endothelium and the adventitia, but also in the media. Incubation of control vessels with L-NNA increased dihydroethidium staining in the endothelium of control animals while decreasing it in the endothelium of angiotensin II–infused animals. To quantify NO production in intact vascular tissue, we employed a recently developed EPR-based method using Fe(DETC)$_2$ as a spin trap, which is thought to trap intracellularly-formed NO. Previously, we have shown that moderately elevated increases in extracellularly formed superoxide do not interfere with the NO-EPR signal. Based on these findings, we speculate that the NO signals obtained by Fe(DETC)$_2$ may be sensitive mainly to intracellular formed superoxide (eg, by NOS III itself), and that the markedly depressed vascular NO signal is therefore, at least in part, secondary to an uncoupled NOS III. These findings strongly suggest that, similar to conditions found in hyperlipidemia and diabetes mellitus, NO uncoupling contributes to endothelial dysfunction and increased superoxide production in this animal model of angiotensin II–induced oxidative stress.

**Angiotensin II Treatment Decreases Activity/Expression of the sGC and cGMP Signaling Without Affecting cGK-I and VASP Expression**

Reductions of intracellular NO bioavailability by NAD(P)H oxidase activation and/or NO uncoupling would be expected to lead to a marked inhibition of the activity of cGK-I. It is now well established that the NO-cGMP pathway is a key regulator of vascular tone and that cGK-I mediates many of these effects. Studies with cGK-I–deficient human cells and mice demonstrated that cGK-I ablation disrupts the NO/cGMP pathway in vascular cells and tissues. Gene-targeted loss of murine cGK-I abolished NO/cGMP-dependent relaxation of smooth muscle resulting in severe vascular and intestinal dysfunctions, whereas cAMP-mediated smooth muscle relaxation was not impaired. These recent developments highlight the importance of assessing cGK expression and/or activity in the setting of endothelial dysfunction. In order to assess whether downstream-targets of NO/cGMP were affected under our experimental conditions, expression levels of cGK-I and VASP as well as P-VASP levels were determined. The use of specific antibodies directed against the specific VASP phosphorylation sites allows the analysis of both cAMP and cGMP signaling in vascular cells and tissues, because cAK and cGK preferentially phosphorylate VASP at Ser157 and Ser239, respectively. Recent studies in vascular tissue demonstrated that in situations where endothelial dysfunction is encountered and NO bioavailability decreased, Ser239 P-VASP levels were strikingly reduced.

In the present study, we found that angiotensin II infusion caused endothelial dysfunction in vessels associated with a strong reduction in P-VASP. It is not likely that the observed decrease in P-VASP was due to decreased substrate availability because total VASP expression was not different in vessels from sham-treated and angiotensin II–infused animals (data not shown). Although the decrease in vascular NO by enhanced vascular superoxide production would be expected to decrease P-VASP, other mechanisms such as downregulation of sGC or cGK-I may contribute to this phenomenon. When analyzing the effect of angiotensin II infusion of rats on the expression of sGC, we found a significant decrease in both sGC subunits (α$_1$ and β$_1$). These findings are congruent with recent studies by Ruetten et al, where endothelial dysfunction in another model of hypertension (SHR) was associated with a significant decrease in the expression of both sGC subunits. In contrast, no changes with respect to the expression of the cGK-I were detected.

**Summary**

Although angiotensin II is known to increase vascular superoxide production and to cause endothelial dysfunction, it affects the function of many proteins in the vessel wall. In the present study, we
provide insight into the mechanisms linking these 2 events. We found that angiotensin II increases the expression of both the endothelial and smooth muscle gp91phox homologues in a PKC-dependent fashion, and we provide evidence that all layers of the vessel wall contribute to superoxide production. The increased oxidative stress uncouples NOS III, leading to further increases in superoxide. The ability of angiotensin II to impair endothelial dysfunction is not, however, limited solely to its effects on the integrity of NO, because it also downregulates the downstream target of NO, sGC. Thus, our data clearly show that angiotensin II coordinates multiple mechanisms contributing to vascular dysfunction in hypertension.

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