Role of superoxide anion in regulating pressor and vascular hypertrophic response to angiotensin II

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Wang, Hui Di, Douglas G. Johns, Shanqin Xu, and Richard A. Cohen. Role of superoxide anion in regulating pressor and vascular hypertrophic response to angiotensin II. Am J Physiol Heart Circ Physiol 282: H1697–H1702, 2002.—Our purpose was to address the role of NADPH oxidase-derived superoxide anion in the vascular response to ANG II. Blood pressure, aortic superoxide anion, 3-nitrotyrosine, and medial cross-sectional area were compared in wild-type mice and in mice that overexpress human superoxide dismutase (hSOD). The pressor response to ANG II was significantly less in hSOD mice. Superoxide anion levels were increased twofold in ANG II-treated wild-type mice but not in hSOD mice. 3-Nitrotyrosine increased in aortic endothelium and adventitia in wild-type but not hSOD mice. In contrast, aortic medial cross-sectional area increased 50% with ANG II in hSOD mice, comparable to wild-type mice. The lower pressor response to ANG II in the mice expressing hSOD is consistent with a pressor role of superoxide anion in wild-type mice, most likely because it reacts with nitric oxide. Despite preventing the increase in superoxide anion and 3-nitrotyrosine, the aortic hypertrophic response to ANG II in vivo was unaffected by hSOD.

3-nitrotyrosine; hypertrophy; hypertension

ANG II is a potent vasoconstrictor, mitogen, and hypertrophic agent that plays an important role in the pathogenesis of hypertension and other cardiovascular diseases. Vascular hypertrophy and inflammation are common features of these disorders. Despite the efficacy of angiotensin-converting enzyme inhibitors and ANG II receptor antagonists in the treatment of hypertension, the mechanisms by which ANG II exerts its effects on the vasculature are incompletely understood.

Accumulating evidence suggests that vascular oxidative stress may have a role in mediating effects of ANG II (9, 25, 36–38). In rats, infusion of ANG II, but not norepinephrine, increased superoxide anion production and the activity of a neutrophil-like NADPH oxidase in the aorta (25). Treatment of ANG II-infused hypertensive rats with superoxide dismutase (SOD) decreased blood pressure (19). Griendling and co-work-

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midscapular region under sterile conditions, and osmotic minipumps (Alzet model 1007D; Alza, Palo Alto, CA) containing 0.15 mol/l NaCl and 1 mmol/l acetic acid were implanted. The delivery rate was 3.2 mg·kg⁻¹·day⁻¹ for 6 days. Sham-treated animals underwent an identical surgical procedure, except that an osmotic minipump containing 0.15 mol/l NaCl and 1 mmol/l acetic acid was implanted. Systolic blood pressure was determined before and at the end of the drug infusion by tail cuff plethysmography. Ten to twenty repeated values were averaged at each determination. This noninvasive method of measuring blood pressure has been validated in mice and correlates well with intra-arterial measurements made in normotensive and hypertensive mice (14). These procedures were approved by the Boston University Medical Center and University of Saskatchewan Institutional Animal Care and Use Committees.

Detection of superoxide anion by lucigenin chemiluminescence. The details of this assay to measure basal levels of superoxide anion in mouse aorta have been published previously (38). Briefly, after the aorta was isolated and cleaned of fat and loose connective tissue, it was incubated in physiological saline in PBS (pH 7.4) containing 5% O₂-5% CO₂. The aorta was then transferred into test tubes containing 1 ml of HEPES-buffered physiological solution (pH 7.4) containing lucigenin (5 μmol/l). This lower concentration of lucigenin was demonstrated not to be involved in redox cycling and to specifically indicate superoxide anion levels in intact vascular tissue (20, 27, 30). This remains the only published method capable of detecting basal superoxide anion production from a single mouse aorta without requiring the addition of NAD(P)H. The luminometer was set to report arbitrary units of emitted light. After a 15-min equilibration, repeated measurements were integrated every 30 s and an average value was reported over a 5-min period. Tiron (10 mmol/l), a cell-permeant, nonenzymatic scavenger of superoxide anion, was then added to quench all superoxide anion-dependent chemiluminescence, and chemiluminescence was integrated over the last 90 s of an additional 5-min period. Superoxide anion is reported as milliunits per minute per milligram of aortic wet weight.

Tissue preparation for histology. The aorta was cleaned of adherent fat, placed in 4% formalin overnight, and then processed and embedded in paraffin. Sections (5 μm) were obtained from the descending thoracic aorta, 3 mm distal to the left subclavian artery.

Localization of 3-nitrotyrosine immunohistochemistry. After removal of paraffin and rehydration, slides were treated with 10 mM citric acid (pH = 6). Tissue sections were microwave heated to recover antigenicity (3 × 2 min at 700 W). Nonspecific binding was blocked with 10% normal goat serum in PBS (pH = 7.4) for 30 min before incubation with either polyclonal anti-nitrotyrosine antibody (1 μg/ml; Upstate Biotechnology, Lake Placid, NY) or PBS with 1% BSA overnight at 4°C. Tissue sections were then incubated for 30 min at room temperature with a biotinylated anti-rabbit IgG (1:800) secondary antibody and the Vectastain ABC kit (Vector). Vector red alkaline phosphatase substrate (Vector) was used to visualize positive immunoreactivity for 3-nitrotyrosine. Specificity of the antibodies was confirmed by preincubation of antibody with free 3-nitrotyrosine (10 mmol/l). Semiquantitative analysis of tissue immunoreactivity for nitrotyrosine was done by three observers, blinded both to the sample identification and experimental protocol, using an arbitrary grading system from 1 to 4 to estimate the degree of positive staining.

Measurement of aorta medial area. Two cross sections, each spaced 50–70 μm apart, were stained with hematoxylin and eosin and photographed at a magnification of ×100. The images from these microscopic sections were displayed on a computer with Photoshop software. The aortic media was then outlined on the image, and the medial area was measured with NIH Image software. The data from each of the two sections from each animal were averaged.

Data analysis. Data are expressed as means ± SE. Statistical comparisons were made by one- or two-way ANOVA. Significance was accepted when P was <0.05.

RESULTS

Baseline blood pressure and pressor responses to ANG II. In 12- to 13-wk-old mice, baseline systolic blood pressure was similar in hSOD mice and wild-type mice (Table 1).

In mice expressing hSOD the systolic blood pressure reached after infusion of ANG II was significantly less than in wild-type mice (Table 1). The change in blood pressure from baseline was also significantly smaller in the hSOD mice than in wild-type mice.

Superoxide anion levels in mouse aorta in response to ANG II. ANG II infusion increased both total and Tiron-quantifiable aortic chemiluminescence approximately twofold in wild-type mice. In mice expressing hSOD ANG II did not significantly increase either parameter of aortic superoxide anion production (Table 2).

Tiron-quantifiable aortic chemiluminescence was significantly less in both sham-infused and ANG II-infused hSOD-expressing mice (Table 2).

Localization of 3-nitrotyrosine by immunohistochemistry. 3-Nitrotyrosine protein moieties were assessed as a marker of oxidative stress. In wild-type mice infused with ANG II, immunohistochemistry performed with a polyclonal antibody raised against 3-nitrotyrosine localized the greatest amounts of 3-nitrotyrosine to the adventitia and the endothelium (Fig. 1). Lesser amounts of staining were observed in the media. Immunoreactivity was not observed when the anti-3-nitrotyrosine antibody was preincubated with 3-nitrotyrosine (10 mmol/l) or when the primary antibody was omitted, indicating that the staining was specific. Semiquantitative analysis of the 3-nitrotyrosine staining showed that staining in ANG II-infused wild-type mice was statistically and visibly increased approximately twofold compared with sham-treated mice (Fig. 2). Staining of the aorta of sham-treated mice expressing hSOD was significantly lower than in wild-type mice.

<table>
<thead>
<tr>
<th>Table 1. Systolic blood pressure in experimental mice</th>
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<tr>
<td>Experimental Design</td>
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<tr>
<td>Before ANG II infusion</td>
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<td>After ANG II infusion</td>
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<td>Change in pressure due to ANG II infusion</td>
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Data (in mmHg) are means ± SE tail systolic pressures in wild-type mice and mice expressing human superoxide dismutase (hSOD). *The change in pressure caused by ANG II infusion in hSOD mice was significantly lower than in wild-type mice.
mice (P < 0.01), and ANG II caused no significant increase in nitrotyrosine staining (P > 0.2).

Aortic hypertrophic responses to ANG II infusion. The medial cross-sectional areas of sham-treated wild-type and hSOD-expressing mice were not significantly different (Fig. 3, P > 0.5). ANG II infusion significantly increased aortic medial area in wild-type mice ~1.6-fold (P < 0.02). In mice expressing hSOD, aortic medial area was also significantly increased to an extent similar to that in wild-type mice (P < 0.02). Examples of the medial hypertrophy that occurred in response to ANG II in wild-type and hSOD transgenic mice aortas can be seen in Fig. 1.

DISCUSSION

Recent studies have suggested that hypertension is associated with oxidative stress. This includes ANG II-dependent hypertensive models (5, 19, 23, 36, 38), spontaneously hypertensive rats (28, 29, 33), and Dahl salt-sensitive rats (2, 32). The contribution of oxidant stress to hypertension is supported by the ability of antioxidants to reduce blood pressure in those models (19, 22, 28, 29). In addition, Vaziri et al. (35) reported that oxidative stress induced by glutathione depletion caused severe hypertension in normal rats. However, because of the abundant concomitant biochemical and hemodynamic disorders that can contribute to hypertension, as well as potential multiple nonspecific effects of antioxidants, it is difficult to associate hypertension to a direct effect of oxidative stress per se.

The present study was performed in hopes that a more specific definition of oxidative stress would result in greater understanding of its role in the vascular response to hypertension. The hypothesis that NADPH

Table 2. Basal superoxide anion levels in mouse aortic rings

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<th>Wild-Type Mice</th>
<th>hSOD Mice</th>
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<tr>
<td></td>
<td>Sham ANG II</td>
<td>Sham ANG II</td>
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<tr>
<td>Lucigenin</td>
<td>6.0 ± 1.2</td>
<td>3.4 ± 1.0†</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>(P &lt; 0.04)</td>
<td>(P &gt; 0.1)</td>
<td></td>
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<tr>
<td>Lucigenin + Tiron</td>
<td>2.9 ± 1.2</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 6)</td>
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<tr>
<td>(P &gt; 0.5)</td>
<td>(P &lt; 0.02)</td>
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<tr>
<td>Tiron quenchable</td>
<td>3.2 ± 0.9</td>
<td>0.9 ± 0.6†</td>
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<tr>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 6)</td>
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<tr>
<td>(P &lt; 0.02)</td>
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Values (in mU·mg⁻¹·min⁻¹) are means ± SE chemiluminescence of the aorta for n mice. Comparison of superoxide anion levels in aorta of sham-treated and ANG II-infused wild-type mice and mice expressing hSOD is shown. Infusion of ANG II for 6 days increased aortic superoxide anion levels twofold in the aorta of wild-type mice but did not do so in mice expressing hSOD. Chemiluminescence in sham-treated or ANG II-infused hSOD mice was significantly less than that in wild-type mice. *P < 0.05 vs. sham-treated group; †P < 0.05 vs. wild-type mice.
Peroxynitrite can nitrate tyrosine constituents of proteins (7). Although other factors such as myeloperoxidase can tyrosine nitrate proteins (31), only the formation of peroxynitrite involves superoxide anion.

oxidase-derived superoxide anion plays a critical role in ANG II-induced hypertension has been suggested by the following findings: 1) infusion of ANG II increases blood pressure and NADPH oxidase derived superoxide anion levels in rat, rabbit (23, 25, 36), and mouse (38) aortic segments; 2) treatment of ANG II-infused hypertensive rats with liposomal SOD or tempol decreases blood pressure (19, 29); and 3) ANG II increases blood pressure to a lower value in gp91phox-deficient mice (38) and, in this study, in mice overexpressing hSOD compared with wild-type mice.

The mechanisms by which increased superoxide anion levels regulate blood pressure in response to ANG II are not known. One factor is the inactivation of the vasodilator properties of nitric oxide by superoxide anion derived from NADPH oxidase (37) that results in vasoconstriction (36) and contributes to blood pressure regulation. This suggestion is compatible with the higher blood pressure seen with inhibitors of, or in genetic deficiency of, nitric oxide synthase (10, 13, 16, 26). Indeed, Kato et al. (16) found that treatment of rats with a nitric oxide synthase inhibitor caused similar pressor and aortic hypertrophic responses as infusion with ANG II, and treatment with both together had no additive effect. In addition, the combination of superoxide anion and nitric oxide produces peroxynitrite, which is known to inactivate prostacyclin synthase, decreasing the production of the prostanooid vasodilator (41). Also, oxidation of arachidonic acid may produce F_2-isoprostanes, which could mediate further vasoconstriction (21).

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The fact that mice overexpressing hSOD in this study and those lacking gp91phox (38) do not increase aortic superoxide anion levels or nitrotyrosine strongly suggests that peroxynitrite is the cause of vascular tyrosine nitration in response to ANG II. Accumulation of aortic nitrotyrosine from peroxynitrite is likely due to an enzyme process, suggested by the fact that sham-treated hSOD transgenic mice had significantly less staining compared with wild-type mice. The pattern of the most intense nitrotyrosine staining in endothelium and adventitia in both normal and ANG II-infused wild-type aorta is similar to that found in the rat and rabbit aorta with vital staining for superoxide anion with nitroblue tetrazolium (37) or with immunohistochemistry for multiple subunits of NADPH oxidase (23, 24, 36–38), indicating that these aortic regions are most involved by the generation and reaction of reactive oxygen and reactive nitrogen species by NADPH oxidase in vivo. Although the functional effects of tyrosine nitration are not addressed in this study, it has been shown that this chemical modification is likely to be important in the dysfunction of many vascular proteins such as SOD (39) and prostacyclin synthase (41).

The smooth muscle hypertrophic response to ANG II is likely mediated by NADPH oxidase-derived reactive oxygen species. Ushio-Fukai et al. (34) showed that antisense to p22phox decreased [3H]leucine incorporation as a measure of hypertrophy in cultured rat aortic smooth muscle cells. Furthermore, we found (38) that gp91phox-deficient mice failed to increase aortic superoxide anion or to develop vascular hypertrophy in vivo in response to ANG II infusion despite a pressor response that otherwise would be expected to cause hypertrophy. Interestingly, although there was a reduced pressor response to ANG II, as well as lower levels of superoxide anion and nitrotyrosine accumulation, the aorta of hSOD transgenic mice developed hypertrophy to an extent similar to that in wild-type mice in response to ANG II. Together with our finding of an
absent hypertrophic response in gp91phox-deficient mice, this finding might be explained by the dismutation of superoxide anion to hydrogen peroxide and its catabolites in mice expressing hSOD. Supporting this speculation, hydrogen peroxide produced by NADPH oxidase was found to mediate hypertrophy of smooth muscle cells in culture in response to ANG II (40). Of course, changes in other mediators including decreased nitric oxide or increased ONOO− or vasoconstrictor eicosanoids could also contribute to regulation of smooth muscle growth during ANG II infusion. As mentioned earlier, a decrease in nitric oxide bioactivity contributes to medial hypertrophy in response to ANG II (16). However, it is doubtful that nitric oxide can explain the hypertrophy that persists in the hSOD-expressing mouse aorta because more, rather than less, bioactive nitric oxide would be expected in mice with hSOD. An increase in nitric oxide bioactivity or decreased vasoconstrictor eicosanoids might contribute to the decreased pressor response to ANG II in mice expressing hSOD. Also, the fact that a similar hypertrophic response occurred in hSOD transgenic mice at a lower blood pressure is consistent with the blood pressure-independent nature of medial hypertrophy mediated by ANG II (12, 38).

A corollary of our observations is that the increased accumulation of nitrotyrosine concentrated in endothelium and adventitia in aortas of mice infused with ANG II, which is attenuated in hSOD transgenic mice, is apparently not essential for medial hypertrophy. Supporting this finding, we recently reported (15) that the aorta of mice deficient in inducible nitric oxide synthase also does not show increased nitrotyrosine when infused with ANG II but is hypertrophied to a similar extent as in wild type. This also suggests that inducible nitric oxide synthase and NADPH oxidase participate in formation of peroxynitrite and the protein tyrosine nitration that occurs in response to ANG II.

In conclusion, our data support the hypothesis that NADPH oxidase-derived superoxide anion and its derivative peroxynitrite play a role in regulating blood pressure in ANG II-dependent hypertension, possibly because of the inactivation of nitric oxide. In contrast, the aortic hypertrophic response to ANG II in vivo does not appear to be directly due to superoxide anion, peroxynitrite, or altered function of tyrosine-nitrated proteins but is likely mediated by other reactive oxygen species derived from NADPH oxidase.

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