Superoxide contributes to vascular dysfunction in mice that express human renin and angiotensinogen

SEAN P. DIDION, MICHAEL J. RYAN, GARY L. BAUMBACH, CURT D. SIGMUND, AND FRANK M. FARACI

Departments of Internal Medicine, Pathology, Physiology, and Pharmacology, Cardiovascular Center, University of Iowa College of Medicine, Iowa City, Iowa 52242

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Didion, Sean P., Michael J. Ryan, Gary L. Baumbach, Curt D. Sigmund, and Frank M. Faraci. Superoxide contributes to vascular dysfunction in mice that express human renin and angiotensinogen. Am J Physiol Heart Circ Physiol 283: H1569–H1576, 2002. First published June 20, 2002; 10.1152/ajpheart.00079.2002.—This study examined vascular function and the role of superoxide in mice that chronically express human renin (R+) and human angiotensinogen (A+). Responses of aortas from R+/A+ mice and from their normotensive littermates (RA− mice) were examined in vitro. Endothelium-dependent relaxation to acetylcholine was impaired in vessels from R+/A+ mice (e.g., maximal relaxation to 100 μM acetylcholine was 45 ± 5% and 65 ± 3% in R+/A+ and RA− mice, respectively; P < 0.05). Relaxation was also impaired to the endothelium-independent dilators authentic nitric oxide and nitroprusside in vessels from R+/A+ mice. Maximal vasorelaxation to acetylcholine, isoprenaline, non-nitric oxide dilator papaverine was similar in R+/A+ and RA− mice. Incubation of vessels from R+/A+ mice with Tiron (1 mM), a superoxide scavenger, improved relaxation to acetylcholine, nitric oxide, and nitroprusside. In contrast, incubation with diethylthiocarbamate (1 mM), an inhibitor of copper-containing SODs, reduced acetylcholine- and nitroprusside-induced relaxation in vessels from both R+/A+ and RA− mice. Basal superoxide levels, measured with lucigenin-enhanced chemiluminescence (5 μM lucigelin) and hydroethidine-based fluorescent confocal microscopy, were higher in vessels from R+/A+ mice and were Tiron and polyethylene glycol-SOD sensitive. These results suggest that increased superoxide contributes to impaired nitric oxide-mediated relaxation in this genetic model of chronic angiotensin II-dependent hypertension.

METHODS

Experimental animals. Double-transgenic (R+/A+) mice were generated by crossbreeding single-transgenic human renin (R+) mice with single-transgenic human angiotensinogen (A+) mice (6, 19). All breeding and genotyping was performed in the transgenic animal facility at the University of Iowa, a virus- and pathogen-free environment. The presence or absence of transgenes in each mouse was assessed by gene- and species-specific polymerase chain reaction of DNA isolated from tail biopsy samples (19).

Angiotensin II affects vascular tone primarily through AT1 receptor-mediated pathways (31). More recently, effects of angiotensin II on vascular tone were demonstrated to include angiotensin II-mediated increases in superoxide (10, 31). For example, angiotensin II was shown to increase basal superoxide production by nearly threefold in cultured smooth muscle (9). Acute infusion of angiotensin II is associated with increases in arterial pressure and increased superoxide levels in vessels (7, 15, 24, 25) that appear to be mediated through activation of AT receptors because these responses can be normalized by losartan administration (24). These findings suggest that acute administration of large pharmacological pressor doses of angiotensin II produces oxidative stress within vessels. However, despite these initial investigations, relatively little is known regarding the effects of chronic and physiological increases in angiotensin II on vascular function and superoxide levels.

Double-transgenic mice that express human renin (R+) and human angiotensinogen (A+) are chronically hypertensive and demonstrate a threefold increase in plasma angiotensin II levels (19). Thus R+/A+ mice provide an excellent model in which to examine mechanisms associated with chronic hypertension induced by the renin-angiotensin system. Because hypertension mediated by angiotensin II involves multiple signaling pathways that display distinctive temporal characteristics (e.g., short-term vs. long-term signaling events; Refs. 26 and 31), the first goal of the present study was to determine whether endothelium-dependent and nitric oxide-mediated relaxation are impaired in R+/A+ mice. Additionally, because angiotensin II can potentially increase superoxide production, the second goal of the present study was to determine whether impaired nitric oxide-dependent relaxation in R+/A+ mice is related to increases in vascular superoxide.

Address for reprint requests and other correspondence: F. M. Faraci, Dept. of Internal Medicine, E315-GH, Univ. of Iowa College of Medicine, Iowa City, IA 52242-1081 (E-mail: frank-faraci@uiowa.edu).

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single-transgenic (R-/A+ and R+/A−) littersmates. Strict species specificity exists between the mouse and human renin-angiotensinogen systems (19). Thus human renin does not proteolytically cleave mouse angiotensinogen, and mouse renin does not cleave human angiotensinogen. We also showed previously (6) that vascular responses to acetylcholine are similar in R-/A−, R-/A+, and R+/A− mice. Therefore, because blood pressure and vascular responses to acetylcholine are not different between R-/A−, R-/A+, and R+/A− mice, and because of the species specificity reaction, responses from these three groups are pooled and are referred to as RA−. In the present study, systolic blood pressure was measured in R+/A+ and RA− mice with an automated tail-cuff device (BP-2000, Visitech Systems, Apex, NC) as described previously (14, 27, 28). All experimental protocols were approved by the University of Iowa Animal Care and Use Committee.

Vascular studies. Mice were euthanized with pentobarbital sodium (75–100 mg/kg ip), and the thoracic aorta was quickly removed and placed in Krebs buffer (pH 7.4) with the following ionic composition (mmol/l): 118.5 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose. Loose connective tissue from the adventitial surface was removed, and the aorta was cut into four rings (each 4 mm in length). Vascular rings were mounted on pairs of triangular hooks and suspended in individual organ chambers containing 20 ml of Krebs buffer maintained at 37°C and bubbled continuously with 95% O2/5% CO2. The rings were connected to force transducers, which measured isometric tension (contraction plateau, concentration-response curves were generated in the absence and presence of extracellular [EC]-SOD and cyclooxygenase and to scavenge superoxide levels were measured). Superoxide levels were measured in R+/A+ and RA− mice with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long-pass filter. Laser settings were identical for acquisition of images, and vessels from R+/A+ mice and RA− mice were processed and imaged in parallel. In some experiments, vessels were preincubated with either vehicle (PBS) or polyethylene glycol-SOD (PEG-SOD; 50 U/ml) for 30 min before application of hydroethidine. Relative increases (based on low-, medium-, and high-intensity fluorescence) in ethidium bromide fluorescence were determined with Scion Image software for the PC (version 4.02). Ethidium bromide fluorescence was normalized to the cross-sectional area of the vessel wall for each section.

Drugs. Acetylcholine, DETC, DPI, indomethacin, papaverine, PEG-SOD, sodium nitroprusside, and Tiron were obtained from Sigma (St. Louis, MO), and all were dissolved in saline with the exception of DPI and indomethacin, which were dissolved in DMSO (final concentration <0.01%) and Na2CO3 (0.1 M), respectively. Hydroethidine was obtained from Molecular Probes (Eugene, OR) and prepared as described previously (20, 21). Authentic nitric oxide was prepared as described previously (5). All other reagents were of standard laboratory grade.

Statistical analysis. Relaxation is expressed as a percent relaxation to PGF2α)-induced contraction. All data are expressed as means ± SE. Comparisons were made with ANOVA for repeated measures with Tukey’s post hoc test (concentration-response curves), one-way ANOVA (superoxide levels), and a Student’s t-test (baseline characteristics). A probability value of <0.05 was considered significant.

RESULTS

Blood pressure and baseline characteristics. Systolic blood pressure was higher (P < 0.05) in R+/A+ mice than in RA− mice (Table 1), consistent with what we previously reported (19). In addition, heart weight-to-body weight ratio (an index of cardiac hypertrophy) was significantly larger (P < 0.05) in R+/A+ mice (Table 1). These differences could not be accounted for by differences in age or body weight, because R+/A+ and RA− mice were of similar age (10 ± 1 and 10 ± 1 mo, respectively; P > 0.05) and body weight (30 ± 1 and 27 ± 1 g, respectively; P > 0.05).

Vascular responses. Relaxation to acetylcholine and authentic nitric oxide was impaired (P < 0.05) in vessels from R+/A+ mice compared with RA− mice (Fig. 1), as was the response to nitroprusside (Fig. 2). Although maximal relaxation was similar, a rightward shift was noted in the concentration-response curve to papaverine in vessels from R+/A+ mice (Fig. 2). Impaired responses to all three endothelium-independent contracting agents were observed in R+/A+ mice compared with RA− mice.

Table 1. Baseline characteristics in RA− and R+/A+ mice

<table>
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<tr>
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<th>n</th>
<th>SBP, mmHg</th>
<th>HW-to-BW Ratio</th>
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<tr>
<td>RA−</td>
<td>12</td>
<td>107 ± 4</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>R+/A+</td>
<td>14</td>
<td>130 ± 5</td>
<td>6.5 ± 0.3*</td>
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</table>

Values are means ± SE for n mice. R+, expressing human renin; A+, expressing human angiotensigen; RA−, pooled responses from R−/A−, R+/A−, and R−/A+; SBP, systolic blood pressure; HW, heart weight; BW, body weight. *P < 0.05 vs. RA−.
agonists suggest impairment of vascular function that extends beyond the endothelium. In R+/A+ mice, contraction of the aorta in response to PGF$_2\alpha$ was also significantly higher ($P < 0.05$) than in RA− mice (Table 2). These findings suggest that relaxation to both endothelium-dependent and -independent stimuli are impaired (whereas contraction is enhanced) in vessels from R+/A+ mice.

We showed previously (4) that inhibition of copper-containing SODs is associated with increases in superoxide and impairment of endothelium-dependent relaxation in cerebral blood vessels from the rabbit. In the present study, inhibition of endogenous copper-containing SODs with DETC (1 mM) resulted in a reduction ($P < 0.05$) in vascular responses to acetylcholine in vessels from both R+/A+ and RA− mice (Fig. 3). Likewise, DETC inhibited ($P < 0.05$; data not shown) relaxation to nitroprusside by ~45% and 35% in vessels from R+/A+ and RA− mice, respectively. In contrast, DETC had no effect ($P > 0.05$) on papaverine-induced relaxation in R+/A+ or RA− mice (data not shown). These data suggest that scavenging of nitric oxide by superoxide (i.e., because of increased superoxide levels) contributes to impaired nitric oxide-dependent relaxation in R+/A+ mice.

We showed previously (6) that, in addition to impaired endothelial responsiveness to acetylcholine, the carotid artery from R+/A+ mice displays a transient contractile response to acetylcholine that is sensitive to indomethacin, suggesting possible upregulation of cyclooxygenase activity in R+/A+ mice. To further examine this possibility, we tested effects of indomethacin (10 $\mu$M) on impaired responses of aortas from R+/A+ mice. Indomethacin had no effect ($P > 0.05$) on acetylcholine- and nitric oxide-induced relaxation or PGF$_2\alpha$-induced contraction (Table 2) in vessels from RA− mice. Tiron also had no effect on papaverine-induced relaxation in vessels from either R+/A+ or RA− mice (data not shown). These data suggest that scavenging of nitric oxide by superoxide (i.e., because of increased superoxide levels) contributes to impaired nitric oxide-dependent relaxation in R+/A+ mice.

To test this concept with a second approach, we treated vessels with Tiron (1 mM), a scavenger of superoxide. Tiron restored responses to acetylcholine (Fig. 4), nitric oxide (Fig. 5), and PGF$_2\alpha$ (Table 2) nearly (~90%) to normal in vessels from R+/A+ mice. In contrast, Tiron had no effect ($P > 0.05$) on acetylcholine (Fig. 4)- and nitric oxide (Fig. 5)-induced relaxation or PGF$_2\alpha$-induced contraction (Table 2) in vessels from RA− mice. Tiron also had no effect on papaverine-induced relaxation in vessels from either R+/A+ or RA− mice (data not shown). These data suggest that scavenging of nitric oxide by superoxide (i.e., because of increased superoxide levels) contributes to impaired nitric oxide-dependent relaxation in R+/A+ mice.

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Table 2. Vasomotor response to PGF2α in aortas from RA− and R+/A+ mice

<table>
<thead>
<tr>
<th>PGF2α, μM</th>
<th>Control</th>
<th>Tiron (1 mM)</th>
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<tr>
<td></td>
<td>RA−</td>
<td>RA−</td>
</tr>
<tr>
<td>10</td>
<td>0.66 ± 0.05</td>
<td>0.58 ± 0.12</td>
</tr>
<tr>
<td>30</td>
<td>0.91 ± 0.05</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>100</td>
<td>0.99 ± 0.05</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>R+/A+</td>
<td>R+/A+</td>
</tr>
<tr>
<td>10</td>
<td>0.83 ± 0.06*</td>
<td>0.67 ± 0.11†</td>
</tr>
<tr>
<td>30</td>
<td>1.00 ± 0.06*</td>
<td>0.91 ± 0.13†</td>
</tr>
<tr>
<td>100</td>
<td>1.22 ± 0.04*</td>
<td>1.06 ± 0.12†</td>
</tr>
</tbody>
</table>

Values (in g) are means ± SE; n = 22 (RA− control), 21 (R+/A+ control), 5 (RA−Tiron), and 6 (R+/A+ Tiron). *P < 0.05 vs. RA−; †P < 0.05 vs. control.

Values in parentheses are SD.

DISCUSSION

There are several major new findings of the present study. First, endothelium-dependent and endothelium-independent relaxation to nitric oxide are impaired in aortas from R+/A+ mice. Additionally, contraction to PGF2α was enhanced in R+/A+ mice. Second, inhibition of copper-containing SODs significantly reduced relaxation to acetylcholine and nitroprusside in R+/A+ and RA− mice, suggesting that acute increases in oxidative stress impair nitric oxide-mediated relaxation in murine blood vessels. Third, impaired nitric oxide-mediated relaxation in R+/A+ mice was significantly improved by Tiron, suggesting that increases in superoxide contribute to vascular dysfunction in R+/A+ mice. Consistent with this idea, basal superoxide levels, as measured with two independent methods, were significantly higher in vessels from R+/A+ mice relative to levels in RA− mice. The results obtained with DPI suggest that increases in superoxide in R+/A+ mice are due to increased activity of NAD(P)H oxidase. Thus superoxide-mediated vascular dysfunction is present in an important model of hypertension in which genes of the renin-angiotensin system are overexpressed chronically. From the present data, it cannot be discerned whether the increase in superoxide and vascular dysfunction is due to the increase in blood pressure per se or mediated directly via effects of angiotensin II within the vessel wall.

R+/A+ mice as a model of angiotensin II-dependent hypertension. R+/A+ mice are hypertensive and display elevated plasma renin activity and plasma angiotensin II levels compared with nontransgenic littermates (19). Although several spontaneously hypertensive mouse and rat strains exist, we suggest that R+/A+ mice have several distinctive advantages over these other commonly used models. First, R+/A+ mice represent a defined (angiotensin II induced) and chronic (caused by...
life-long expression of human renin and human angiotensinogen) model of hypertension. Previous studies that have examined the effect of angiotensin II on vascular function and superoxide levels have primarily used relatively short durations of angiotensin II infusion (≤7 days) with minipumps (7, 15, 24). Second, vascular responses in R+/A+ mice are compared with those from normotensive littermates, which are of a genetically similar (>99.9% homogeneity) background (C57BL/6J). This is of importance, because vascular responses become difficult to interpret when comparisons are made between genetically dissimilar backgrounds (i.e., spontaneously hypertensive vs. Wistar-Kyoto rats) (8, 27, 29). Third, a particular advantage of studying R+/A+ mice pertains to the fact that expression of EC-SOD in mice more closely resembles that observed in humans (12, 18). Most previous studies related to changes in vascular superoxide during acute angiotensin II-induced hypertension have been performed in the rat (7, 15, 24, 25). However, the rat may not be particularly representative for these studies because blood vessels from rats have much less EC-SOD (thought to be a key isoform in blood vessels) than other species, making them potentially more vulnerable to superoxide-mediated vascular damage (12, 18). Thus R+/A+ mice provide an excellent genetic, rather than pharmacological, model by which to study the long-term mechanism(s) associated with hypertension induced by the renin-angiotensin system.

**Impaired vascular function in R+/A+ mice.** Although we have observed impaired endothelium-dependent relaxation in carotid artery of R+/A+ mice (6), the role of superoxide has not been examined in this model previously. Consistent with our previous findings (6), in the present study, endothelium-dependent relaxation in response to acetylcholine was impaired in aortas of R+/A+ mice. Additionally, endothelium-independent relaxation to authentic nitric oxide was significantly reduced in R+/A+ mice. Because exogenous nitric oxide and endogenously produced nitric oxide (released by endothelial nitric oxide synthase) require diffusion through the vessel wall, we anticipated that relaxation to authentic nitric oxide and acetylcholine would be impaired in vessels from R+/A+ mice. It was more difficult to predict whether relaxation to nitroprusside, which appears to require metabolic activation in smooth muscle before it can release nitric oxide (13), would be impaired in vessels from R+/A+ mice. Our observation that relaxation to nitroprusside, in addition to acetylcholine and nitric oxide, was significantly reduced in the aorta from R+/A+ mice suggests that nitric oxide-mediated relaxation is markedly im-

![Figure 4](http://ajpheart.physiology.org/)

**Fig. 4.** Relaxation of aorta in response to acetylcholine in the absence (control; □) and the presence (1 mM; •) of Tiron in RA− mice (n = 8; A) and R+/A+ mice (n = 10; B). Values are means ± SE. *P < 0.05 vs. control.

![Figure 5](http://ajpheart.physiology.org/)

**Fig. 5.** Relaxation of aorta in response to nitric oxide in the absence (control; □) and the presence (1 mM; •) of Tiron in RA− mice (n = 9; A) and R+/A+ mice (n = 8; B). Values are means ± SE. *P < 0.05 vs. control.
paired in R+/A+ mice. Although maximal relaxation to the non-endothelium, non-nitric oxide-dependent dilator papaverine was preserved in R+/A+ mice, a rightward shift was noted at lower concentrations, suggesting that vascular dysfunction is not limited to endothelium but may also involve some general (perhaps nonspecific) impairment of function in vascular muscle.

Because oxidative stress has been associated with impaired endothelium-dependent relaxation, we hypothesized that acute inhibition of SOD (i.e., copper-containing SODs) activity with DETC should reduce nitric oxide-dependent relaxation in RA− mice and might produce additional vascular dysfunction in vessels from R+/A+ mice. DETC has been used previously to induce oxidative stress in blood vessels and has been shown to increase superoxide and impair endothelium-dependent relaxation to acetylcholine in the aorta, carotid artery, and cerebral blood vessels (3, 11, 17, 22, 23). In the present study, DETC significantly reduced acetylcholine- and nitroprusside-induced relaxation in vessels from both R+/A+ and RA− mice. Interestingly, the level of vascular impairment produced with DETC in RA− mice was similar to the vascular responses observed in R+/A+ mice in the absence of DETC. In addition, it was noted that impaired nitric oxide-dependent relaxation in R+/A+ mice could be reduced further with DETC, suggesting that vascular dysfunction in R+/A+ mice (in the absence of DETC) is not mediated solely by the loss of expression or activity of either CuZn-SOD or EC-SOD.

Role of increased superoxide contributing to impaired nitric oxide-dependent relaxation. Because increases in oxidative stress with DETC were associated with a reduction in nitric oxide-dependent relaxation and because angiotensin II can produce oxidative stress, we explored the possibility that increased superoxide contributes to vascular dysfunction in R+/A+ mice. Incubation of vessels with the superoxide scavenger Tiron improved acetylcholine- and nitro oxide- but not papaverine-induced vasorelaxation. Tiron also reduced PGF2α-induced contraction in R+/A+ mice toward that observed in RA− mice. In contrast, Tiron had no effect on relaxation or contraction in vessels from RA− mice, suggesting that the effects of Tiron were selective and that superoxide influences vascular responses only in R+/A+ mice. These data are consistent with results obtained with acute (short term) pharmacological increases in angiotensin II in rats (7, 15, 24, 25) and suggest that enhanced superoxide levels play an important role in vascular dysfunction in this chronic model of angiotensin II-induced hypertension (R+/A+ mice).

To complement our functional data with Tiron, we also measured levels of superoxide in R+/A+ and RA− mice by two independent methods. We found that basal superoxide levels were significantly higher in aortas from R+/A+ mice by lucigenin-enhanced chemiluminescence and by hydroethidine-based confocal microscopy. Both the lucigenin signal and ethidium bromide fluorescence were markedly reduced by superoxide scavengers, suggesting that these signals reflected increases in superoxide. These findings support the conclusion that increases in superoxide contribute to impaired vascular responses either indirectly, via decreases in nitric oxide bioavailability, or alternatively via superoxide-mediated vasoconstriction. The
observation that the lucigenin signal could be markedly reduced by DPI suggests that increases in superoxide in R+/A+ mice reflect enhanced NAD(P)H oxidase activity.

We showed previously (6) that indomethacin abolishes acetylcholine-induced contractions in carotid artery of R+/A+ mice. In the present study of the aorta, enhanced cyclooxygenase activity does not appear to contribute to impaired vascular function because indomethacin did not improve the response of aortas from R+/A+ mice. These apparent differences may relate to two factors. First, we did not observe transient contractions in response to acetylcholine in the aorta as we had in the carotid artery. Second, in the carotid artery, indomethacin was only efficacious in abolishing the transiently induced contractions in response to acetylcholine; however, indomethacin treatment did not restore overall responsiveness of the carotid artery toward normal.

Both lucigenin-enhanced chemiluminescence and hydroethidine-based confocal microscopy have been previously shown to be sensitive methods for detection of superoxide in vascular tissue (3, 4, 16, 20, 21, 30). The use of hydroethidine is advantageous because it provides additional insight regarding localization of superoxide within the vascular wall (20, 21). On the basis of previous studies, we hypothesized that adventitia and possibly endothelium might be major sources of superoxide in aortas of R+/A+ mice. Although ethidium bromide fluorescence was higher in those components of the vessel wall, increases in superoxide were also observed in vascular muscle in R+/A+ mice. This finding is in contrast with previous studies in which acute (6–7 days) infusion of angiotensin II was associated with selective increases in superoxide in endothelium and adventitia only or smooth muscle only (2, 21, 24). These differences may reflect the fact that overexpression of human angiotensinogen and human renin in R+/A+ mice is both systemic and lifelong. Thus there may be spatial and temporal differences in production and/or localization of superoxide in R+/A+ mice. There may also be differences in the mechanisms that lead up to increased superoxide in the different models (acute vs. chronic). The finding that vascular function in R+/A+ mice could be improved but not completely normalized with Tiron suggests that other mechanisms, in addition to superoxide, may be involved in the chronic model.

In summary, the results of the present study strongly support a role for increased superoxide in producing vascular dysfunction in hypertensive mice that express both human renin and human angiotensinogen transgenes. Enhanced superoxide contributed to diminished endothelium-dependent and nitric oxide-mediated relaxation, because a scavenger of superoxide restored responses to acetylcholine and nitric oxide toward normal. Although the present study did not directly attempt to identify the enzymatic source responsible for the increase in vascular superoxide, preliminary studies suggest that an increase in NAD(P)H oxidase may be involved. The findings also suggest that R+/A+ mice will be a useful model to further examine mechanisms of vascular dysfunction during hypertension.
We thank Pamela K. Tompkins for technical assistance with the hydroethidine assay.

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