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S Rajagopalan, S Kurz, T Münzel, M Tarpey, B A Freeman, K K Griendling, D G Harrison

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Research Article

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Angiotensin II–mediated Hypertension in the Rat Increases Vascular Superoxide Production via Membrane NADH/NADPH Oxidase Activation

Contribution to Alterations of Vasomotor Tone

Sanjay Rajagopalan,* Sabine Kurz,*‖ Thomas Münzel,‖ Margaret Tarpey,§ Bruce A. Freeman,§ Kathy K. Griendling,* and David G. Harrison*‡

**Department of Medicine, Division of Cardiology, Emory University School of Medicine, Atlanta, Georgia 30322; ‡Atlanta Veterans Administration Medical Center, Atlanta, Georgia 30033; §Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, Alabama 35233; and the ‖Albert Ludwig University of Freiburg, Freiburg, Germany 79106*

Abstract

We tested the hypothesis that angiotensin II–induced hypertension is associated with an increase in vascular $\cdot O_2^-$ production, and characterized the oxidase involved in this process. Infusion of angiotensin II (0.7 mg/kg per d) increased systolic blood pressure and doubled vascular $\cdot O_2^-$ production (assessed by lucigenin chemiluminescence), predominantly from the vascular media. NE infusion (2.75 mg/kg per d) produced a similar degree of hypertension, but did not increase vascular $\cdot O_2^-$ production. Studies using various enzyme inhibitors and vascular homogenates suggested that the predominant source of $\cdot O_2^-$ activated by angiotensin II infusion is an NADH/NADPH-dependent, membrane-bound oxidase. Angiotensin II–, but not NE-, induced hypertension was associated with impaired relaxations to acetylcholine, the calcium ionophore A23187, and nitroglycerin. These relaxations were variably corrected by treatment of vessels with liposome-encapsulated superoxide dismutase. When Losartan was administered concomitantly with angiotensin II, vascular $\cdot O_2^-$ production and relaxations were normalized, demonstrating a role for the angiotensin type-1 receptor in these processes. We conclude that forms of hypertension associated with elevated circulating levels of angiotensin II may have unique vascular effects not shared by other forms of hypertension because they increase vascular smooth muscle $\cdot O_2^-$ production via NADH/NADPH oxidase activation. (*J. Clin. Invest.* 1996. 97:1916–1923.) Key words: hypertension • superoxide • nitric oxide • angiotensin II • endothelium

Introduction

Angiotensin II exerts numerous effects on the cardiovascular system. These include vasoconstriction, induction of vascular smooth muscle cell growth (1, 2), stimulation of protooncogene expression (3–5), modulation of myocardial hypertrophy and fibrosis (6–9), and modulation of ventricular remodeling after myocardial infarction (for review see reference 10). A variety of pathologic states, including certain forms of hypertension, congestive heart failure, and nitrate tolerance are associated with elevated plasma renin activity and circulating levels of angiotensin II. Additionally, renin and angiotensin II produced locally in the vessel wall may have important autocrine and paracrine effects, even in the setting of normal or low circulating angiotensin II levels (10).

Traditionally, it has been thought that the predominant effect of angiotensin II on vascular tone was achieved via its direct vasoconstrictor effect, mediated via interactions with the vascular smooth muscle angiotensin type-1 (AT_1)[1] receptor. Endothelial cells also possess angiotensin II receptors, and can release both nitric oxide (11) and vasoconstrictor prostanoids (under certain pathophysiological conditions) in response to the octapeptide (12).

Recently, a novel signaling mechanism for angiotensin II has been described, which may have important implications for both its physiological and pathophysiological effects. In cultured vascular smooth muscle cells, 3–4 h treatment with angiotensin II increases production of $\cdot O_2^-$ anions via membrane-bound NADH- and NADPH-driven oxidases (13). The pathways leading to this activation remain undefined, but likely involve stimulation of phospholipase A_2 and the release of aracidonic acid.

These previous studies were performed in cultured vascular smooth muscle cells after several passages. Such cells undergo transformation to a synthetic phenotype and exhibit signaling responses to angiotensin II which may differ from responses of the vascular smooth muscle in vivo. Because of these issues, it was not clear that a similar effect could be produced by modest levels of angiotensin II in vivo. In the present experiments, we examined the effect of angiotensin II–induced hypertension on vascular $\cdot O_2^-$ production and attempted to characterize the oxidase activated. Studies were also performed to determine if another form of pharmacologically induced hypertension, that due to the infusion of NE, had similar effects on vascular $\cdot O_2^-$ production. Finally, we sought to determine if this alteration in vascular $\cdot O_2^-$ production had any impact on endothelial regulation of vasomotion.

Methods

Animal preparation. Male Sprague-Dawley rats (wt 250–300 g; Harlan Sprague Dawley Inc., Indianapolis, IN) were anesthetized with in-

Address correspondence to David G. Harrison, Division of Cardiology, Emory University School of Medicine, 1639 Pierce Drive, Woodruff Memorial Building, Atlanta, GA 30322. Phone: 404-727-3710; FAX: 404-727-3330.

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1. *Abbreviations used in this paper:* AT_1, angiotensin type-1; L-NMMA, N^G-monomethyl-L-arginine.

traperitoneal ketamine 80 mg/kg and xylazine 10 mg/kg. Using sterile techniques, an incision was made in the midscapular region and osmotic minipumps (Alzet model 2002; Alza Corp., Palo Alto, CA) containing angiotensin II (infusion rate 0.7 mg/kg per d) were implanted. Sham-operated animals underwent an identical surgical procedure, except that either no pump, or an empty osmotic pump, was implanted.

Systolic blood pressures were measured by tail cuff plethysmography immediately before surgery, and in most animals immediately before death. In some animals (n = 12), daily systolic pressures were obtained to examine the time course of blood pressure rise in response to the angiotensin II infusion.

On the fifth day of angiotensin II infusion, the animals were given a lethal injection of Na pentobarbital. After the pentobarbital injection, but before death, heparin (2,500 U) was given via intracardiac injection. The aortas were harvested and used in subsequent studies.

In some studies, the selective AT_1 receptor antagonist Losartan (Dupont-Merck, Wilmington, DE) (25 mg/kg per d) was administered in the drinking water for 2 d before and during angiotensin II infusions.

To examine vascular production of $\cdot O_2^-$ in another model of hypertension, a similar protocol was followed in additional studies, except that NE (2.8 mg/kg per d) was infused. For these studies, NE was dissolved in 0.2 mM sodium metabisulfite and pH adjusted to 5.0 to ensure stability.

In a final series of rats, we examined the effect of low dose angiotensin II infusion (0.3 mg/kg per d).

Vessel preparation. The aorta was placed in chilled, modified Krebs/Hepes buffer (buffer A composition [mM]: NaCl, 99.01; KCl, 4.69; $CaCl_2$, 1.87; $MgSO_4$, 1.20; K_2HPO_4, 1.03; $NaHCO_3$, 25.0; Na-Hepes, 20.0, and glucose, 11.1, pH 7.4), cleaned of excessive adventitial tissue, and cut into 5-mm ring segments. In some vessels, the endothelium was removed by inserting the closed tips of a metal hemostat into the ring segment and rolling it gently on moistened filter paper.

Estimation of vascular $\cdot O_2^-$ production. $\cdot O_2^-$ anion production was measured using lucigenin chemiluminescence. The details of this assay have been published previously (14–16). Briefly, after preparation, the vessels were placed in a modified Krebs/Hepes buffer and allowed to equilibrate for 30 min at 37°C. Scintillation vials containing 2 ml Krebs/Hepes buffer with 250 μM lucigenin were placed into a scintillation counter switched to the out-of-coincidence mode. After dark adaptation, background counts were recorded and a vascular segment was added to the vial. Scintillation counts were then recorded every 2 min for 15 min, and the respective background counts were subtracted. The vessels were then dried by placing them in a 90°C oven for 24 h, for determination of dry weight. Lucigen counts were expressed as counts $\cdot 10^3$ per min per mg dry weight.

In some experiments, either diphenylene iodinium (10 μM), oxypurinol (100 μM), rotenone (100 μM) N^G-monomethyl-L-arginine (L-NMMA) (10 μM), or indomethacin (10 μM) was added to the vessel segments to examine a role for flavin-containing enzymes, xanthine oxidase, mitochondrial respiration, nitric oxide synthase, and cyclooxygenase, respectively.

Examination of sources of $\cdot O_2^-$ in vascular homogenates. Aortic segments (2–3 cm) were placed in chilled buffer A. Periadventitial tissue was carefully removed and the vessels were repeatedly washed to remove adherent blood cells. A 10% vessel homogenate was prepared by homogenizing in a glass-to-glass motorized homogenizer (Contorque power unit; Eberbach Corp, Ann Arbor, MI). The homogenizing buffer (buffer B) was a 50-mM phosphate buffer which contained 0.01 mM EDTA. The homogenate was subjected to low speed centrifugation (1,000 g) for 10 min to remove unbroken cells and debris. 20-μl aliquots of the supernatant were then added to glass scintillation vials containing 250 μm lucigenin in 2 ml buffer B. The chemiluminescence which occurred over the ensuing 5 min in response to the addition of either NADH or NADPH (both 100 μM) was recorded. In preliminary experiments, homogenates alone, without addition of NADH or NADPH, gave only minimal signals. Also, neither NADH nor NADPH evoked lucigenin chemiluminescence in the absence of homogenate. In some experiments, arachidonic acid (100 μM), xanthine (100 nM), succinate (5 mM, with and without 30 μM antimycin A) were added to determine if these could act as substrates for superoxide production. Antimycin A was used to inhibit the complex III of mitochondrial electron transport, while succinate was used as a substrate for this process. The combination of these two would exaggerate any contribution of electron leak from mitochondrial sources. $\cdot O_2^-$ production in response to NADH was also examined after the addition of indomethacin (10 μM), nordihydroguaretic acid (100 μM), or L-NMMA (10 μM). Other experiments were performed in the presence of heparin-binding superoxide dismutase to determine the superoxide dismutase inhibitable fraction. The latter is a recombinant superoxide dismutase with an affinity for glycosaminoglycans on the plasma membrane.

In additional experiments, vessels were initially homogenized in buffer C (50 mM Tris-HCl buffer [pH 7.4] containing the protease inhibitors, 1 mM PMSF, and 1 μg/ml each of antipain, aprotinin, beestatin, leupeptin, soybean trypsin inhibitor, pepstatin A, and 0.1% 2-mercaptoethanol). The supernatant from the low speed centrifugation was subjected to 100,000 g ultracentrifugation for 45 min to separate membrane and cytosolic fractions. 20 μl of either the supernatant or the particulate fraction (which had been resuspended in 250 μl buffer C) were used to examine oxidase activity of these cellular subfractions. The chemiluminescence signals were standardized using a standard curve generated from known quantities of xanthine and xanthine oxidase. Values were standardized to the amount of protein present. Protein content was measured using a commercially available kit (D_C protein assay; Bio Rad Laboratories, Hercules, CA).

Isolated vascular ring experiments. Eight 5-mm ring segments of the thoracic aorta were suspended in individual organ chambers filled with Krebs buffer (25 ml) of the following composition (mM): NaCl, 118.3; KCl, 4.69; $CaCl_2$, 1.87; $MgSO_4$, 1.20; K_2HPO_4, 1.03; $NaHCO_3$, 25.0; and glucose, 11.1, pH 7.40. The solution was aerated continuously with a 95% O_2-, 5% CO_2 mixture and maintained at 37°C. Care was taken not to injure the endothelium during preparation of the rings. Tension was recorded with a linear force transducer. Over a period of 1 h, the resting tension was gradually increased and the ring segment frequently exposed to 80 mM KCl, until the optimal tension for generating force during isometric contraction was reached. In preliminary experiments, this proved to be 2.0 g in all subsets of animals. The vessels were left at this resting tension throughout the remainder of the study. To prevent synthesis of PGs, we performed all experiments in the presence of 10 μM indomethacin. The vessels were then precontracted with L-phenylephrine (0.15 μM). In experiments where vessels from NE-treated animals were studied, PG $F_{2\alpha}$ (3 μM) was employed to obtain preconstricted tone. This vasoconstrictor was used because the previous NE treatment prevented constrictions to reasonable concentrations of phenylephrine. For these studies, separate sham-operated and angiotensin II–treated rats were used. After a stable contraction plateau was reached, the rings were exposed to either acetylcholine (1 nM–3 μM), the calcium ionophore A23187 (1 nM–1 μM), or nitroglycerin (1 nM–1 μM).

Liposomal-encapsulated superoxide dismutase. In previous studies, we have found that administration of conventional Cu-Zn superoxide dismutase only minimally increases vascular superoxide dismutase levels (16, 17). We therefore augmented the vascular levels of superoxide dismutase by using liposomal-encapsulated superoxide dismutase prepared as previously described (16). Aortic rings from control and angiotensin II–treated rats were incubated for 30–45 min at 37°C in a Krebs/Hepes buffer containing 750 U/ml of liposomal-encapsulated superoxide dismutase (final vol 1.0 ml). Thereafter, the aortic rings were removed from the liposomal-encapsulated superoxide dismutase solution, washed, and placed in organ chambers as described above. Liposomes without superoxide dismutase (empty liposomes) were used as controls for these experiments.

Data analysis. Data in the manuscript are expressed as mean±SEM. Comparisons between groups of animals or treatments were made us-

ing one-way ANOVA. When significance was indicated, a Student-Newman-Keuls post hoc analysis was used. To examine interactions between angiotensin II or sham treatment and liposome-encapsulated superoxide dismutase administration, a two-way ANOVA was used, where either sham or angiotensin II treatment was one independent variable and either empty liposomes or liposome-encapsulated superoxide dismutase administration was the other independent variable. Significance was considered present when P was < 0.05.

Results

Effect of angiotensin II and NE on systolic blood pressure. Angiotensin II infusion (0.7 mg/kg per d) caused a progressive increase in systolic blood pressure from 130±2 mmHg to 192±6 mmHg by the fifth day before death. NE infusion caused a similar increase in blood pressure from 130±3 to 189±7 mmHg (Fig. 1). Losartan prevented the rise in blood pressure in response to angiotensin II infusion (systolic pressure 125±4 mmHg before death).

Vascular $\cdot O_2^-$ production. Superoxide production by vascular segments from sham-operated animals averaged 5.3±0.3 counts · 10^3 per mg dry wt of vessel per min and was increased twofold in vascular segments from angiotensin II–treated rats ($P < 0.001$, Fig. 2). In contrast, NE infusion had no effect on vascular superoxide production (Fig. 2). Treatment with Losartan decreased $\cdot O_2^-$ production in vascular segments from angiotensin II–infused rats to a value below that observed in vessels from sham-operated rats ($P < 0.001$). Removal of the endothelium decreased lucigenin chemiluminescence in vessel segments from both sham and angiotensin II–treated rats to a small but equal extent (Table I). Incubation with 10 μM diphenylene iodinium for 10 min markedly attenuated the lucigenin signal in both shams and angiotensin II–treated segments (Table I). In contrast, neither oxypurinol, rotenone, L-NMMA, nor indomethacin affected the lucigenin signal in intact vascular segments (Table I).

Superoxide production by vascular homogenates. Superoxide production in response to the addition of a variety of substrates was examined in vascular homogenates. In homogenates of vessels from sham-operated animals, the $\cdot O_2^-$ generated in response to NADH was ~ 1.6-fold higher than that generated by NADPH (12.1±1 vs 7.78±1 nmol·min^{-1}·mg protein^{-1}, respectively, $P < 0.05$, Fig 3). In homogenates from angiotensin II–treated animals, the effect of both NADH and NADPH on $\cdot O_2^-$ generation was approximately doubled (Fig. 3). Heparin-binding superoxide dismutase (30 U/ml), a recombinant form of superoxide dismutase, which contains a glycosaminoglycan binding region, was highly effective in reducing the chemiluminescence signal. It decreased lucigenin chemiluminescence from 12.1±0.77 to 2.4±0.2 and 23.97±2.13 to 3.8±0.5 nmol· min^{-1}·mg protein^{-1} in response to NADH, and from 7.78±0.95 to 1.4±0.2 and 14.67±1.03 to 2.2±0.8 nmol· min^{-1}·mg protein^{-1} in response to NADPH, in sham- and angiotensin-infused animals, respectively.

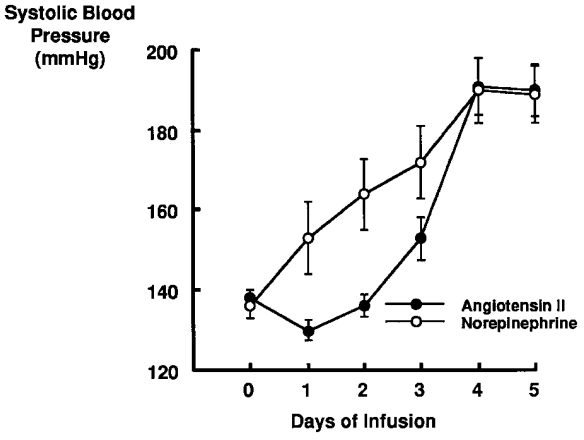

Figure 1. Effect of infusion of either angiotensin II or NE on systolic blood pressure. Both drugs were infused subcutaneously by means of an osmotic minipump implanted between the scapulae. Angiotensin II was infused at a rate of 0.7 mg/kg per d, while NE was infused at 2.8 mg/kg per d.

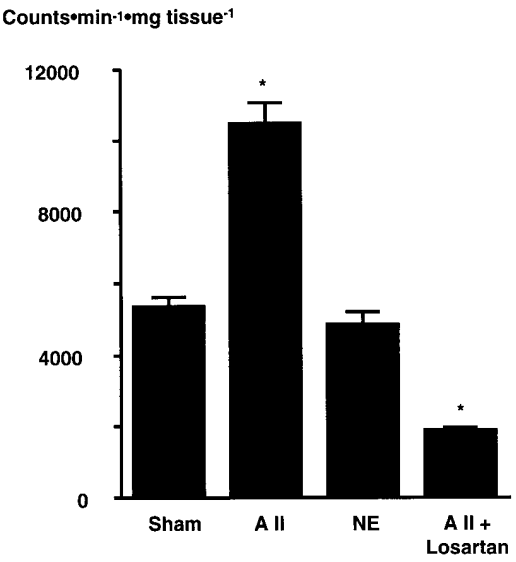

Figure 2. Vascular $\cdot O_2^-$ production assessed by lucigenin chemiluminescence. Aortic segments from sham-operated, angiotensin II–infused (AII), and NE-infused (NE) animals were studied. The effect of concomitant treatment with Losartan (25 mg/kg per d) in angiotensin II–treated rats (AII + Losartan) was also examined. Data are expressed per dry weight of vessel. *$P < 0.001$ *vs sham*.

Table I. Superoxide Production in Intact Aortic Ring Segments, in Response to Various Interventions

Interventions	n	Sham operated	Angiotensin II infused
Endothelium (+)	11	5.3±0.3	10.5±0.6*
Endothelium (−)	5	4.0±0.3‡	8.4±0.8*‡
Diphenylenieiodinium (100 μM)	6	1.7±0.2§	1.8±0.2§
Oxypurinol (100 μM)	5	5.5±0.5	11.6±0.2
L-NMMA (10 μM)	3	5.0±0.6	9.7±0.5
Indomethacin (10 μM)	2	4.8±0.1	12.2±0.2
Rotenone (100 μM)	3	6.1±0.7	9.5±0.3

All values are means±SEM. Superoxide is expressed as counts·10^3/mg of dry wt of vessel/min. §$P < 0.001$ vs vessels without the interventions; ‡$P < 0.05$ vs nondenuded vessels; *$P < 0.001$ vs sham (statistical analysis only performed on endothelium (+), endothelium (−), and diphenyleniodinium groups).

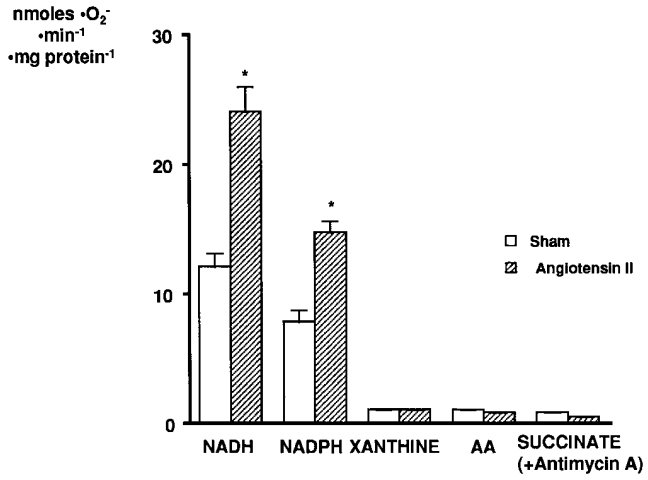

Figure 3. Production of $\cdot O_2^-$ by vascular homogenates in response to several potential substrates. Either NADH, NADPH, xanthine, arachidonic acid, or succinate in the presence of antimycin A were added to tissue homogenates from sham-operated and angiotensin II–infused animals. Only NADH and, to a lesser extent, NADPH significantly induced superoxide production. $*P < 0.001$ *vs sham.*

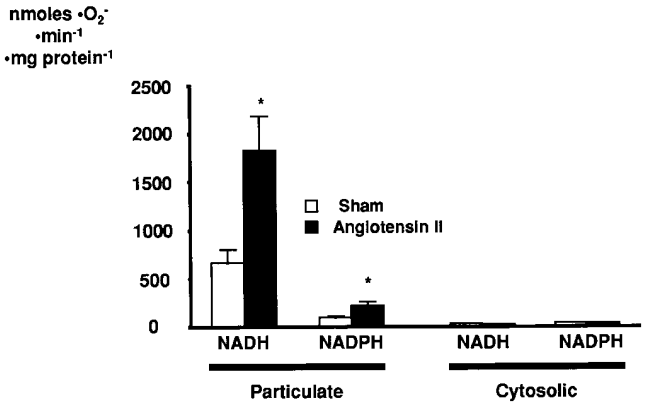

Figure 4. Subcellular location of NADH- and NADPH-oxidase activity in vascular homogenates from sham-operated and angiotensin II–infused rats. Homogenates were subjected to 100,000 *g* ultracentrifugation for 45 min. Superoxide production in response to NADH and NADPH in both the supernatant and resuspended particulate fractions was examined. Virtually all activity was localized to the particulate fraction.$*P < 0.01$ *vs sham.*

In contrast to NADH and NADPH, arachidonic acid, xanthine, and succinate (in the presence of antimycin A) produced only small increases in chemiluminescence by vascular homogenates (Fig. 3).

Membrane-bound vs cytosolic oxidase activity. NADH-driven $\cdot O_2^-$ production averaged 708±172 vs 1,832±457 nmol· min^{-1}·mg $protein^{-1}$ in the particulate fraction of vessel homogenates of sham- and angiotensin II–treated animals, respectively ($P < 0.01$). NADPH-driven $\cdot O_2^-$ production was also increased, although to a lesser degree, in the angiotensin II– vs sham-vessel homogenates (245±61 vs 117±5 nmol· min^{-1}·mg $protein^{-1}$, respectively, Fig. 4). Cytosolic activity was minimal (Fig. 4).

Vascular relaxations in angiotensin II–induced hypertension. Acetylcholine produced a 91±3% relaxation of aortic segments from sham-operated controls. This effect of acetylcholine was markedly reduced in vessels from angiotensin II–treated animals (Fig. 5 and Table II). Treatment with Losartan prevented the impairment of relaxations to acetylcholine in angiotensin II–treated animals (Fig. 5, Table II). Relaxations to nitroglycerin were blunted by approximately one-half log in the angiotensin II–treated vessels compared to control vessels (Table III).

To determine if the alteration in endothelium-dependent vascular relaxation was due to an effect of hypertension or the effect of angiotensin II in stimulating $\cdot O_2^-$ production, we also examined vascular responses in NE-treated rats. In contrast to the effect of angiotensin II–induced hypertension, NE-induced hypertension had no effect on relaxations to either acetylcholine, the calcium ionophore A23187, or nitroglycerin (Fig. 6 and Table III).

Effect of liposome-encapsulated superoxide dismutase on endothelium-dependent vascular relaxation. Liposome-encapsulated superoxide dismutase markedly increased peak relaxations to acetylcholine and the calcium ionophore A23187 in vessels from angiotensin II–treated animals (Fig. 7, Table IV). Relaxations to nitroglycerin were substantially blunted in vessels from angiotensin II–treated animals, and were normalized by treatment with liposome-encapsulated superoxide dismutase (Fig. 7, Table IV). In control vessels, liposome-encapsulated superoxide dismutase produced a small leftward shift in the dose-response curve to acetylcholine (Fig. 7, Table IV). Two-way ANOVA revealed an interaction between liposome-encapsulated superoxide dismutase and angiotensin II vs sham treatment, such that the effect of the superoxide dismutase was greatest in the angiotensin II–treated animals.

Effect of low dose angiotensin II on vascular oxidase activity. To further dissociate the pressure-dependent and independent effects of angiotensin II, we studied an additional 10 rats. Five were treated with low dose angiotensin II (0.3 mg/kg per d) and five served as sham-operated controls. 5 d after an-

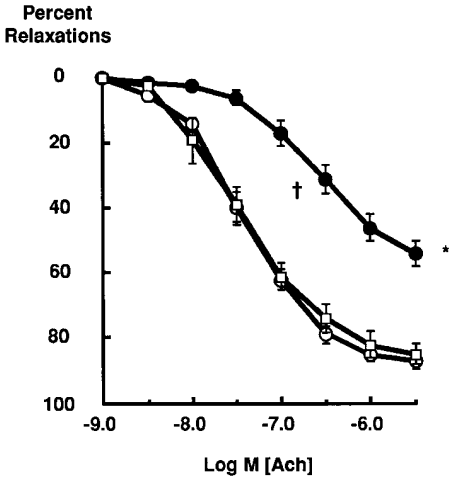

Figure 5. Vascular relaxations to acetylcholine in sham-operated and angiotensin II–infused rats and effect of Losartan. Vessels were studied as ring segments in organ chambers. Relaxations were studied after the vessels had been preconstricted with phenylephrine (0.1 μM). $*P < 0.05$ vs sham for percent maximal relaxation; $^{\dagger}P < 0.05$ vs sham for ED_{50}. ○, Control; ●, angiotensin II; □, angiotensin II–Losartan.

Table II. Responses of Isolated Vessels to Acetylcholine: Effects of Angiotensin II Infusion and Losartan Treatment

Intervention	Ach ED_{50}	Ach Peak Relaxation
Sham	7.39±0.06	91±3
Ang II	6.50±0.09*	55±7*
Ang II + Losartan	7.53±0.15	89±4

Data are mean±SEM. ED_{50}s are -Log [M]. Relaxations are peak responses as a percentage of preconstricted tension. Ach, acetylcholine. *$P < 0.05$ vs sham.

giotensin II infusion or sham surgery, systolic blood pressure averaged 138±9 mmHg in the angiotensin II–treated animals and 128±7 mmHg in the sham-treated animals. NADH oxidase activity was 20±1 nmol·min^{-1}·mg protein^{-1} in the angiotensin II–treated animals, as opposed to 11.9±0.4 nmol·min^{-1}·mg protein^{-1} in the sham-operated animals. NADPH oxidase activity was similar between the animals receiving low dose angiotensin II and sham-operated animals (8.4±0.4 vs 8.1±1.8 nmol·min^{-1}·mg protein^{-1}).

Discussion

In these studies, we found that angiotensin II–induced hypertension is associated with increased vascular $\cdot O_2^-$ production and impaired vascular relaxations to acetylcholine, the calcium ionophore A23187, and nitroglycerin. In studies of vascular homogenates, the predominant source of this increased $\cdot O_2^-$ production seemed to be membrane-bound vascular NADH and NADPH oxidases. The alteration of vascular relaxations to endogenous and exogenous nitric oxide was likely, at least in part, due to the increase in vascular $\cdot O_2^-$ production, as it was partially corrected by augmenting vascular superoxide levels with liposome-entrapped superoxide dismutase. In contrast to the effect of angiotensin II infusion, NE infusion, which produced a similar degree of hypertension, did not increase vascular $\cdot O_2^-$ production and did not alter endothelium-dependent vascular relaxation.

During the past 2 yr, it has become evident that the NADH/NADPH oxidases represent the most important source of $\cdot O_2^-$ in both endothelial cells and vascular smooth muscle (18–21). Recent observations by Griendling et al. reveal that angiotensin II activates an NADH/NADPH oxidase in cultured vascular smooth muscle cells in a dose- and time-dependent fashion (13). The present studies add to these recent observations by demonstrating that angiotensin II can exert this effect in vivo, and that this increase in $\cdot O_2^-$ production may contribute to alterations in endothelium-dependent vascular relaxation and responses to exogenous nitrovasodilators in the intact vessel.

The oxidase activated by angiotensin II in vivo was membrane bound and was activated by NADH to a greater extent than NADPH. These features are virtually identical to those previously observed in cultured vascular smooth muscle cells (13). In intact vascular segments, neither oxypurinol, rotenone, indomethacin, L-NMMA, nor nordihydroguaretic acid affected vascular superoxide production. Likewise, in studies of vascular homogenates, xanthine, arachidonic acid, and succinate had only minimal effects on the chemiluminescence signal. These findings excluded an important role for xanthine oxidase, mitochondrial electron transport, cyclooxygenase, NO synthase, and lipooxygenase as sources of $\cdot O_2^-$.

The NADH/NADPH oxidases of vascular tissues have important differences from those of neutrophils. Vascular oxidases produce continuous low levels (nmol·min^{-1}mg protein^{-1}) of superoxide in contrast to the "burst"-like high activity (μmol·min^{-1}·mg protein^{-1}) of neutrophils. The substrate utilization for the neutrophil favors NADPH, while that of the vascular cell favors NADH (this study and references 13, 19, 21, 22). The vascular oxidase, in spite of all the apparent differences from the neutrophil/macrophage NADPH oxidase, may use some of the same components as the neutrophil oxidase. In particular, the vascular smooth muscle–derived NADH oxidase contains a spectrally detectable cytochrome b_{558}, similar to the electron transport component of the neutrophil NADPH oxidase. Recently, we have cloned an abundantly expressed p22 phox protein from vascular smooth muscle which has a > 90% homology to the neutrophil p22 phox (the small subunit of cytochrome b_{558}) (17). Identification of the remaining oxidase components remains to be made.

Removal of the endothelium reduced vascular superoxide production to an equal extent in vessels from sham-operated and angiotensin II–treated animals. This finding suggests that the majority of the increase in vascular $\cdot O_2^-$ production in angiotensin II–treated rats was from vascular smooth muscle. It is possible that other cell types such as monocyte/macrophages or neutrophils contributed to the increase in vascular superoxide production during angiotensin II infusion, but we believe this is unlikely. Previous studies have shown that the degree of inflammatory cell infiltration at these earlier time points of angiotensin II infusion is negligible (23). Further, the preferred substrate for oxidases in macrophages and neutrophils is NADPH rather than NADH.

The mechanisms and signaling processes whereby angiotensin II might increase vascular $\cdot O_2^-$ production via NADH/NADPH oxidases remain poorly defined. It is of interest that these are not shared by another vasoconstrictor, NE. In intact

Table III. Effects of Infused Angiotensin II and NE on ED_{50} and Maximal Relaxation to Acetylcholine, Nitroglycerin, and A23187

Intervention	Ach ED_{50}	Ach Peak relaxation	Ntg ED_{50}	Ntg/Peak relaxation	A23187 ED_{50}	A23187 Peak relaxation
Sham	7.60±0.04	91±2	8.04±0.09	96±4	7.45±0.07	98±3
Ang II	6.78±0.17*	51±8*	7.56±0.27*	92±5	7.10±0.20*	61±9*
Norepinephrine	7.67±0.07	94±3	7.90±0.09	96±2	7.33±0.04	96±2

Data are mean±SEM. ED_{50}s are -Log [M]. Relaxations are peak responses as a percentage of preconstricted tension. Ach, acetylcholine; Ntg, nitroglycerin. *0.05 vs sham.

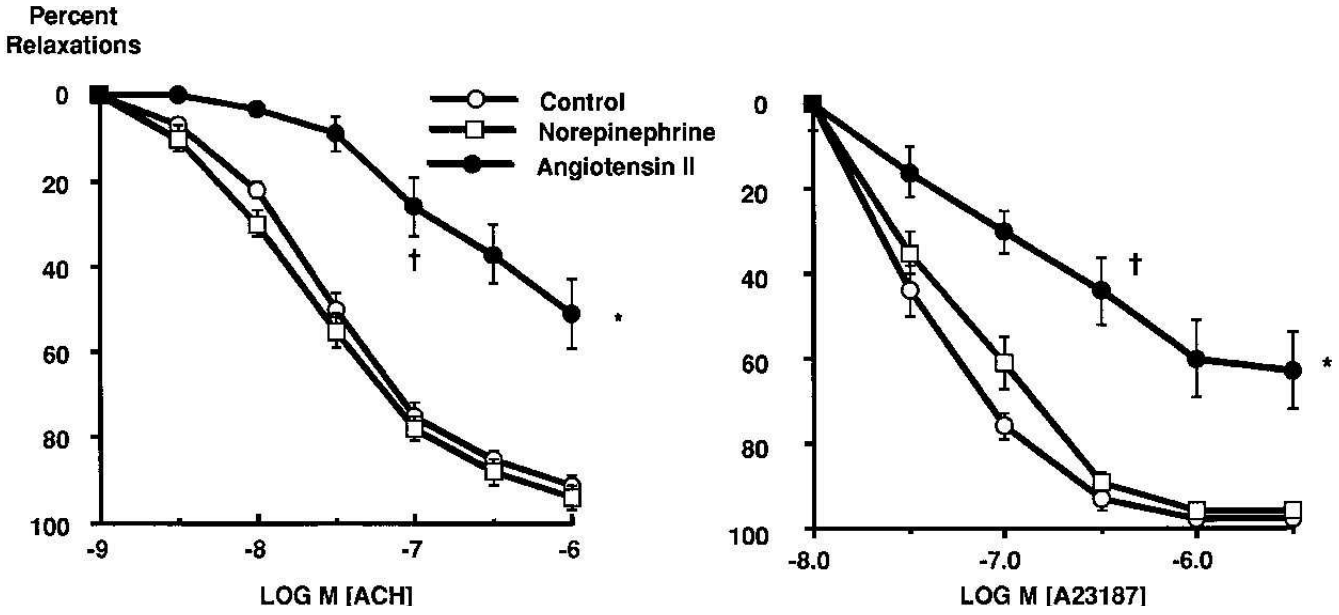

Figure 6. Endothelium-dependent vascular relaxations to acetylcholine and the calcium ionophore A23187 of aortic segments from sham-operated, angiotensin II–infused, and NE-infused rats. Vessels were studied as ring segments in organ chambers, and relaxations to each agent were studied after the vessels had been preconstricted with $PGF_{2\alpha}$ (3 μM). $^{*}P < 0.05$ vs sham for percent maximal relaxation; $^{\dagger}P < 0.05$ vs sham for ED_{50}.

resistance vessels, membrane-associated diacylglycerol kinase activity is increased by NE, but not by angiotensin II (24). This would result in a greater proportion of diacylglycerol formed on NE stimulation, being reincorporated into phospholipids. In contrast, in the absence of such activation, the diacylglycerol formed upon angiotensin II stimulation would more likely lead to the formation of arachidonic acid, through the diacylglycerol lipase pathway. Arachidonic acid has been shown to activate NADH/NADPH oxidases in both neutrophils and in vascular smooth muscle cells (13, 25, 26). It has also been recently shown that the AT_1 receptor is closely associated with Jak2, and that the activity of Jak2 is increased upon angiotensin II stimulation of the receptor (27). It is unknown if similar signaling processes are shared by NE or if Jak2 activation is involved in stimulation of oxidase activity. This issue is further compounded by the finding that arachidonic acid had no effect on oxidase activity of vascular homogenates (unlike what is observed for homogenates of cultured vascular smooth muscle cells). It is conceivable that smooth muscle cells in vivo, exposed to a variety of external stimuli, have near-maximally stimulated oxidase activity, and that the addition of exogenous arachidonic acid to homogenates of the tissue will have little further effect. Signaling mechanisms and metabolic pathways for arachidonic acid may also vary between cells in culture and in vivo.

Several pathophysiological conditions have been associated with increases in vascular $\cdot O_2^-$ production, including hypercholesterolemia (14), diabetes (28, 29), ischemia followed

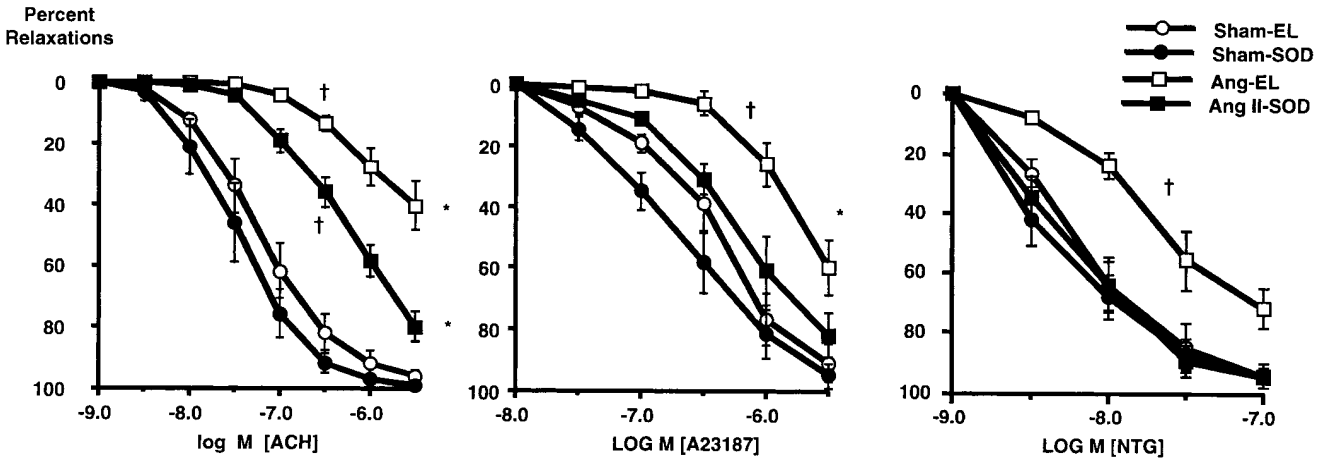

Figure 7. Effect of liposome-encapsulated superoxide dismutase on relaxations to acetylcholine, the calcium ionophore A23187, and nitroglycerin in vessels from sham-operated and angiotensin II–infused rats. Vessels from both groups were exposed for 30–45 min to either empty liposomes (*EL*) or liposomal-encapsulated superoxide dismutase (*SOD*, 750 U/ml). Vascular relaxations were examined after contraction with phenylephrine. $^{*}P < 0.05$ vs sham for percent maximal relaxation; $^{\dagger}P < 0.05$ vs sham for ED_{50}.

Table IV. Effects of Liposomal-encapsulated Superoxide Dismutase on Vascular Responses

Intervention	Ach ED_{50}	Ach/Peak relaxation	Ntg ED_{50}	Ntg Peak relaxation	A23187 ED_{50}	A23187 Peak relaxation
Sham + Empty	7.07±0.16	94±3	8.19±0.09	95±4	6.42±0.11	93±4
Sham + L-SOD	7.61±0.16*	100±0.3	8.34±0.12	93±5	6.64±0.13	94±3
Ang II + Empty	6.25±0.06‡	40±11*‡	7.62±0.08*‡	82±6	5.99±0.05‡	60±9*‡
Ang II + L-SOD	6.44±0.12‡	80±3	8.25±0.13	96±2	6.35±0.11	82±7

Data are mean±SEM. ED_{50}s are -Log [M]. Empty, empty liposomes; L-SOD, liposomal encapsulated superoxide dismutase; Ang II, angiotensin II. *0.05 L-SOD vs empty liposome; ‡0.05 Ang II vs sham.

by reperfusion (30), and nitrate tolerance (16). In several of these, this increase in vascular $\cdot O_2^-$ has been suggested to alter endothelium-dependent vascular relaxation by promoting inactivation of nitric oxide. Nitric oxide has been suggested to have antiatherogenic properties, in terms of inhibiting platelet aggregation (31), adhesion molecule expression (32), and smooth muscle proliferation (33). The reaction rate between $\cdot O_2^-$ and nitric oxide is extremely rapid (6.7×10^9 mol/sec), and actually exceeds the reaction rate between superoxide and superoxide dismutase (34). For this reason, the balance between $\cdot O_2^-$, nitric oxide, and superoxide dismutase in the vessel wall is tenuous, and relatively minor changes in the levels of any of these factors may substantially alter regulation of vascular tone. Thus, conditions in which steady state $\cdot O_2^-$ levels are increased two- to threefold, are associated with rather dramatic impairments in endothelium-dependent vascular relaxation.

Our findings may provide some insight into why forms of hypertension associated with elevated plasma renin activity (and presumably elevated effects of angiotensin II) are associated with increased cardiovascular event rates (35). It is of interest that hypertension induced by NE infusion was not associated with an increase in vascular $\cdot O_2^-$ production, and did not alter endothelial regulation of vasomotion. Likewise, infusion of lower doses of angiotensin II, which had minimal effects on blood pressure, doubled NADH-oxidase activity. These findings are compatible with the concept that hypertension per se is not a stimulus for increased $\cdot O_2^-$ production, and that conditions in which circulating or local levels of angiotensin II are elevated may have unique effects on the vessel wall, independent of elevating blood pressure. Further, hypertension not associated with increases in angiotensin II, or activation of vascular oxidases, may be less prone to produce vascular disease.

The effect of hypertension on endothelium-dependent vascular relaxation has been somewhat controversial (for review see reference 36). Further, the cause of altered endothelial regulation of vasomotion in various forms of hypertension may vary. Based on our current findings, it is interesting to speculate that animal models or human subjects with hypertension associated with elevated levels of angiotensin II, might exhibit greater alterations of endothelium-dependent vascular relaxation than do hypertensive conditions associated with low renin/angiotensin II states. Future studies in which endothelium-dependent vascular relaxation is examined in humans should take into account the renin/angiotensin II profiles of the subjects examined. Finally, these studies may provide insights into why treatment with angiotensin II–converting enzyme inhibitors or angiotensin II–receptor antagonists may have beneficial effects not encountered with other drugs (37–40).

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