

# Paracrine Role of Adventitial Superoxide Anion in Mediating Spontaneous Tone of the Isolated Rat Aorta in Angiotensin II-Induced Hypertension

Hui Di Wang, Susan Hope, Yue Du, Mark T. Quinn, Antonio Cayatte, Patrick J. Pagano, Richard A. Cohen

**Abstract**—The relationship between vascular generation of superoxide anion and spontaneous tone observed in the isolated aorta was studied in hypertensive rats infused with angiotensin II. Aortic rings from hypertensive, but not from sham-operated rats, demonstrated oscillatory spontaneous tone that represented  $52 \pm 5.6\%$  of the maximal contraction to KCl. Spontaneous tone was prevented by calcium-free buffer or by blocking calcium influx through L-type calcium channels with nifedipine. The production of superoxide anion measured by lucigenin chemiluminescence was up to 15-fold higher than in sham-operated rat aorta. The adventitial site of production of superoxide anion was suggested by the fact that lucigenin chemiluminescence was 5.5-fold higher from the adventitia than from the intima. This was confirmed histochemically by demonstrating that the adventitia was the site of reduction of nitroblue tetrazolium as well as immunohistochemical staining of NAD(P)H oxidase subunit proteins. A causal link between superoxide anion production by NAD(P)H oxidase and the spontaneous tone is suggested by the fact that superoxide dismutase or the inhibitor of NAD(P)H oxidase, diphenylene iodonium, decreased both superoxide anion production and spontaneous tone. L-NAME or removal of the endothelium from the aorta had no significant effect on superoxide anion levels or spontaneous tone. However, although superoxide dismutase decreased superoxide anion levels in the presence of L-NAME or in endothelium-denuded rings, it no longer inhibited the tone. This suggests that the effect on tone of superoxide anion originating in the adventitia is mediated by inactivating endothelium-derived nitric oxide, which promotes smooth muscle calcium influx and spontaneous tone. The adventitia is not a passive bystander during the development of hypertension, but rather it may have an important role in the regulation of smooth muscle tone. (*Hypertension*. 1999;33:1225-1232.)

**Key Words:** adventitia ■ superoxide dismutase ■ nitric oxide ■ hypertension ■ NAD(P)H oxidase

Certain types of hypertension are associated with elevated circulating levels of angiotensin II (Ang II), and local production of Ang II in the vessel wall may have autocrine and paracrine effects, even though the circulating peptide level is normal or low.<sup>1</sup> Although Ang II has been thought to mediate its effects on the vasculature by way of its direct contractile effects mediated by AT<sub>1</sub> receptors, it has recently become apparent that vascular superoxide anion production may have a role in this mediation. In rats, hypertension caused by the infusion of Ang II, but not phenylephrine, increased superoxide anion production from aortic segments as well as increased NAD(P)H oxidase activity in aortic homogenates.<sup>2,3</sup> Furthermore, treatment of the Ang II-infused hypertensive rats with superoxide dismutase (SOD) decreased blood pressure, indicating a role for superoxide anion in mediating the hypertension.<sup>4</sup>

It has been reported that both cultured endothelial<sup>5-9</sup> and cultured vascular smooth muscle cells<sup>10,11</sup> are capable of

producing superoxide anion. However, the largest amount of superoxide anion is generated in aorta of normal rabbits<sup>12</sup> and rats<sup>13</sup> by way of a membrane-bound NAD(P)H oxidase which is localized in the adventitia. Furthermore, Ang II increases the production of superoxide anion and increases the activity of NAD(P)H oxidase in cultured aortic adventitial fibroblasts.<sup>14</sup>

Our preliminary studies of the isolated aorta of hypertensive rats infused for 6 days with Ang II showed that the aorta of these rats, but not those from sham-operated normotensive rats, developed oscillatory spontaneous contractile tone. We investigated whether this contractile tone occurred as a result of increased production of superoxide anion and whether the oxidative stress accompanying its production influenced the mechanisms which govern calcium homeostasis in the smooth muscle. Our results demonstrate that the adventitia of the aorta remains the primary site of superoxide anion

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production and that the contractile tone depends on the effect of superoxide anion to inactivate the endogenous vasodilator, nitric oxide.

## Methods

### Animal Model

Male Wistar rats (270 to 300 g) were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine, and an incision was made in the midscapular region under sterile conditions. Osmotic minipumps (Alzet, Alza Corp) containing Ang II dissolved in 0.15 mol/L NaCl and 1 mmol/L acetic acid were implanted. The delivery rate was 0.7 to 1.3 mg · kg<sup>-1</sup> · d<sup>-1</sup> for 6 days. Sham-operated animals underwent an identical surgical procedure, except that an osmotic minipump containing 0.15 mol/L NaCl was implanted. Systolic pressures were determined immediately before surgery and at the end of the infusion period by tail cuff plethysmography. Procedures were according to institutional guidelines.

### Preparation of Rings of Rat Thoracic Aorta

The thoracic aorta was cleaned of adherent fat, and rings 5 mm long were cut and mounted on triangular stirrups for isometric tension recording in organ chambers containing 6 mL of buffer as described.<sup>13</sup> The rings were maintained at 37°C and pH 7.4 with 95% O<sub>2</sub>/5% CO<sub>2</sub> and stretched gradually over 1 hour to optimal tension (50 mN) for smooth muscle contraction. Sodium nitroprusside (10<sup>-7</sup> mol/L) or papaverine (10<sup>-5</sup> mol/L) was introduced for the last 30 minutes to allow adjustment under passive conditions. On the removal of the sodium nitroprusside or papaverine by washing, aortic rings from Ang II-infused hypertensive rats, but not from sham-operated normotensive rats, contracted spontaneously. This is termed spontaneous contractile tone. After being stretched to the resting tension, aortic rings from normotensive and hypertensive rats were at similar points of their length-tension relationship, as indicated by the fact that contractions to KCl (120 mmol/L) were not significantly different after treatment with SOD (see the Table). The role of superoxide anion and nitric oxide were determined by incubating rings with SOD (150 units [U]/mL), diphenylene iodonium (DPI) (10 μmol/L), or L-NAME (300 μmol/L). The role of calcium in mediating the spontaneous tone was examined in rings treated with the L-type channel blocker nifedipine (100 nmol/L) or by replacement of the buffer with one that was identical except that it lacked calcium. Each of these treatments was for ≥90 minutes during the stretching of the ring to its optimal tension. In some studies, nifedipine or calcium-free buffer was added after the spontaneous tone had developed.

At the conclusion of the experiments, the rings were contracted by 120 mmol/L KCl. The spontaneous tone in rings from hypertensive rats was expressed in grams of contraction as well as a percentage of the 120 mmol/L KCl contraction.

To examine the in vitro effect of Ang II on tone and superoxide anion production, aorta of normal rats were contracted with 100 nmol/L of Ang II.

### Detection of Superoxide Anion by Lucigenin Chemiluminescence

The details of this assay have been published previously.<sup>12</sup> Briefly, after completion of the studies in the organ chamber, the rings were rinsed in normal buffer and transferred to test tubes that contained 1 mL of HEPES buffer (pH 7.4) containing lucigenin (250 μmol/L) and maintained at 37°C in the presence or absence of the same treatments as for the tension studies. Because measurements with lucigenin (250 μmol/L) have been questioned because of redox cycling by the indicator and generation of superoxide anion, some studies were done with lucigenin (5 μmol/L) which has been shown not to be responsible for redox cycling.<sup>15</sup> The luminometer was set to report arbitrary units of light emitted and integrated over a 30 second interval; after a 20 minute equilibration, repeated measurements were collected during 5 minutes and averaged. Tiron (10 mmol/L), a cell permeant, nonenzymatic scavenger of superox-

### 120 mmol/L KCl-Induced Contraction and Spontaneous Tone

Groups	120 mmol/L KCl-Induced Contraction, mN
SERIES I:	
Sham: Control	55 ± 2 (6)
+SOD	58 ± 3 (6)
All: Control	73 ± 3 (9)*
+SOD	56 ± 4 (8)‡
+DPI	18 ± 4 (4)‡
+L-NAME	68 ± 4 (6)
+L-NAME+SOD	60 ± 3 (6)†
+Nifedipine	12 ± 2 (6)‡
SERIES II:	
Sham: Control	52 ± 5 (8)
Endothelium-denuded	43 ± 1 (5)
+L-NAME	56 ± 5 (4)
All: Control	61 ± 4 (9)
Endothelium-denuded	49 ± 10 (6)
Endothelium-denuded+SOD	52 ± 16 (5)
SERIES III:	
All: Control	51 ± 6 (6)
+Oxypurinol	49 ± 6 (6)
+Rotenone	45 ± 6 (5)

Data show the maximal contractions in response to KCl (120 mmol/L) obtained at the end of the tension measurement protocol in each of 3 separate series of experiments. Numbers in parentheses indicate the number of animals.

\*P < 0.01, compared with sham control group. †P < 0.05, ‡P < 0.01, compared with Ang II control.

ide anion, was then added to quench the superoxide anion-dependent chemiluminescence; readings from the last 90 seconds of an additional 5-minute period were averaged. To examine the adventitial and intimal production of superoxide anion, the rings were cut longitudinally, flattened, and tied with 3-0 silk suture to a small black plastic plate, with either adventitial or intimal surfaces facing outward.<sup>13</sup> The rings were then transferred to test tubes that contained 5 mL of buffer and were maintained at 37°C and 95% O<sub>2</sub>/5% CO<sub>2</sub> for 1 hour. Rings were then placed into 1 mL of HEPES buffer (pH 7.4) containing lucigenin 5 μmol/L.

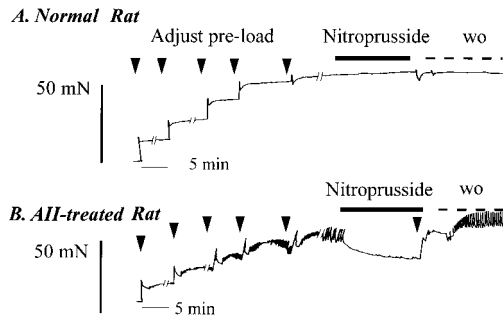
### Localization of Superoxide Anion by Nitroblue Tetrazolium Reduction and Immunohistochemistry

To examine the site of superoxide anion generation in the tissue, aortic rings were incubated with nitroblue tetrazolium to allow superoxide anion generated by the tissue to reduce the nitroblue tetrazolium to blue formazan as described.<sup>13,16</sup>

To localize the source of superoxide production, we analyzed frozen sections of rat aorta by use of immunohistochemistry with monoclonal antibodies that recognize 4 NAD(P)H oxidase proteins known to be essential for NAD(P)H oxidase activity in leukocytes as described.<sup>17-20</sup>

### Drugs

Lucigenin, L-NAME, nitroblue tetrazolium, nifedipine, papaverine, SOD, sodium nitroprusside, and Tiron were purchased from Sigma. Diphenylene iodonium was purchased from Biomol. All drug solutions were made fresh just before each experiment. Drugs were added in aliquots of <1% of the solution volume. DPI and nifedipine were prepared in pure dimethyl sulfoxide. All other drugs were prepared as stock solutions in distilled water.



**Figure 1.** Spontaneous tone in rings from Ang II-infused hypertensive rats. The rings were stretched stepwise over 1 to 1.5 hours to optimal tension (50 mN) for smooth muscle contraction. Nitroprusside ( $10^{-7}$  mol/L) was introduced for the last 30 minutes to allow final adjustment of resting tension under truly resting conditions. On washout of nitroprusside, the rings from Ang II-infused hypertensive (B), but not from sham-operated normotensive rats (A), contracted spontaneously.

### Data Analysis

Data are expressed as mean  $\pm$  SEM. Statistical comparisons were made by Student *t* test. Significance was accepted when *P* was  $<0.05$ .

## Results

### Effect of Ang II on Systolic Blood Pressure

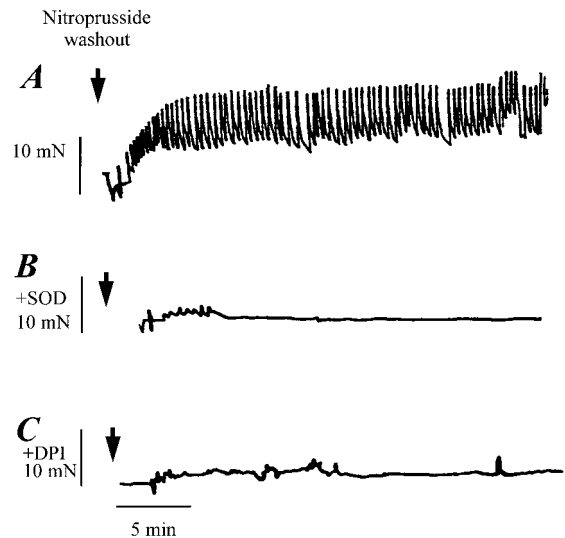
By the seventh day, Ang II infusion increased systolic blood pressure ( $188 \pm 4$  mm Hg,  $n=12$ ) with a range of 170 to 206 mm Hg. This blood pressure was significantly higher than in sham-operated rats ( $128 \pm 4$  mm Hg,  $n=7$ ,  $P<0.01$ ) with a range of 113 to 162 mm Hg.

### Spontaneous Tone in Rings From Ang II-Infused Hypertensive Rats

In Ang II-infused hypertensive rats, spontaneous tone was evident by the oscillatory tension generated in the absence of added contractile agonists. In 11 of 12 hypertensive rats in which rings were equilibrated at the optimal resting tension for contraction (50 mN) in the presence of sodium nitroprusside ( $10^{-7}$  mol/L) or papaverine ( $10^{-5}$  mol/L), washing the rings to remove the vasodilator resulted in either persistent (Figure 1 and 2) or intermittent (Figure 3) spontaneous tone. Rapid oscillations in tone with an average frequency of 3 to 6 per minute were superimposed on the spontaneous tone in some, but not all rings. The peak tension achieved after washout of sodium nitroprusside was  $40 \pm 4.8$  mN ( $n=11$ ), which represented  $52 \pm 5.6\%$  of the maximal contractions to 120 mmol/L KCl (Table). In additional experiments using papaverine as the vasodilator, the peak spontaneous tone achieved after washout of papaverine was  $46 \pm 8.0\%$  ( $n=6$ ) of the maximal contraction to 120 mmol/L KCl, which was not significantly different from that observed with washout of sodium nitroprusside. Rings from sham-operated rats developed no significant tension ( $0.2 \pm 0.1$  mN [ $n=9$ ] after washing out sodium nitroprusside;  $0.3 \pm 0.3$  mN [ $n=9$ ] after washing out papaverine).

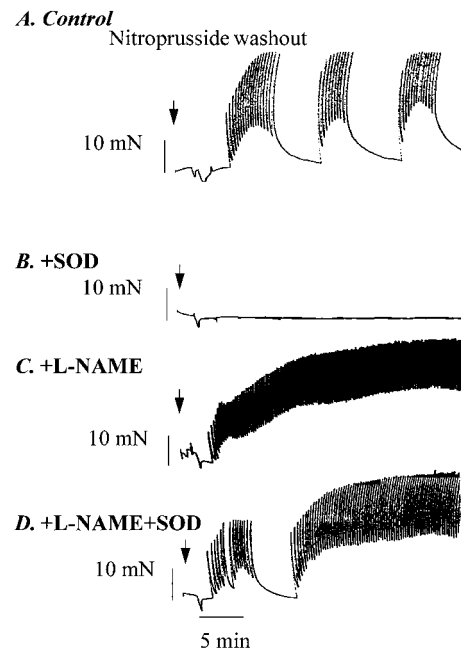
### Superoxide Anion Production by Lucigenin Chemiluminescence

Lucigenin (250  $\mu$ mol/L) chemiluminescence in rings from sham-operated normotensive rats was  $34 \pm 2.9$  milliunits  $\cdot$

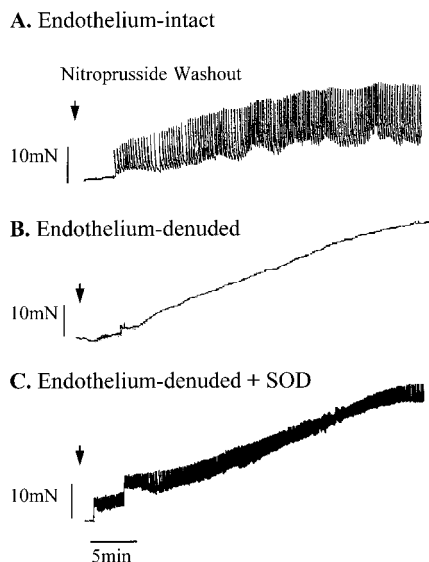


**Figure 2.** Effect of SOD and DPI on spontaneous tone in rings from Ang II-infused hypertensive rats. Trace A shows the responses following washout of nitroprusside from rings of Ang II-infused hypertensive rats. Trace B shows the effect of SOD (150 U/mL) treatment for the 90 minutes from the beginning of the tension measurement. Trace C shows the effect of DPI (10  $\mu$ mol/L) treatment for 90 minutes. SOD and DPI significantly inhibited the spontaneous tone.

$\text{mg}^{-1} \cdot \text{min}^{-1}$  ( $n=7$ ) and was significantly increased in the rings from Ang II-infused hypertensive rats ( $60 \pm 8.0$  milliunits  $\cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ ,  $n=9$ ,  $P<0.01$ ). After Tiron was added, chemiluminescence was reduced to  $17 \pm 0.8$  milliunits  $\cdot \text{mg}^{-1} \cdot \text{min}^{-1}$  in rings from normal rats and  $13 \pm 1.9$  milliunits  $\cdot \text{mg}^{-1} \cdot$



**Figure 3.** Effect of L-NAME on spontaneous tone and superoxide anion production. Trace A shows the effect of washout of nitroprusside from an aortic ring of an Ang II-infused hypertensive rat. Trace B shows the absence of spontaneous tone in a ring treated with SOD (150 U/mL). Traces C and D show the effect of washout of nitroprusside in the presence of L-NAME (300  $\mu$ mol/L) and both L-NAME (300  $\mu$ mol/L) and SOD (150 U/mL). SOD failed to inhibit spontaneous tone in the presence of L-NAME.



**Figure 4.** Effect of endothelium denudation on spontaneous tone. Trace A shows the effect of washout of nitroprusside from an aortic ring of an Ang II-infused hypertensive rat with intact endothelium. Trace B shows a similar level of the tone generated in a ring denuded of endothelium from the same hypertensive rat. Trace C shows that the spontaneous tone generated in another ring from the same rat denuded of endothelium was unaffected by SOD (150 U/mL).

min<sup>-1</sup> in rings from Ang II-infused rats,  $P > 0.05$ ). After subtracting lucigenin chemiluminescence in the presence of Tiron from that obtained in its absence, the calculated Tiron-quenchable chemiluminescence was significantly higher in rings from Ang II-infused rats ( $44 \pm 8.0$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>) than in the rings from sham-operated normotensive rats ( $18 \pm 2.8$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>,  $P < 0.01$ ).

Chemiluminescence measured with lucigenin (5  $\mu$ mol/L) in rings from sham-operated normotensive and Ang II-infused hypertensive rats was  $22 \pm 4.7$  and  $52 \pm 7.8$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>, respectively ( $n = 5$ ,  $P < 0.05$ ). After Tiron was added, the chemiluminescence was decreased to a similar level in rings from both groups of rats ( $21 \pm 4.6$  and  $17 \pm 3.2$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>, in sham-operated and Ang II-infused rats, respectively,  $P > 0.05$ ). The Tiron-quenchable chemiluminescence was 15-fold higher in the rings from Ang II-infused rats compared with those from sham-operated normotensive rats ( $35 \pm 7.9$  and  $2.3 \pm 1.3$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>, respectively,  $P < 0.01$ ).

#### Effect of Inhibitors of Enzymatic Sources of Superoxide Anion Production on Spontaneous Tone and Superoxide Anion Production

In the rings from sham-operated rats, SOD (150 U/mL) reduced Tiron-quenchable lucigenin (250  $\mu$ mol/L) chemiluminescence from  $18 \pm 2.8$  to  $2.8 \pm 2.0$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> ( $n = 8$ ,  $P < 0.01$ ), and there was no observable effect on tone.

In the rings from Ang II-infused hypertensive rats, SOD (150 U/mL) reduced spontaneous tone from  $39 \pm 5$  mN ( $n = 9$ ) to  $7 \pm 3$  mN ( $n = 8$ ,  $P < 0.01$ ), which represents  $12 \pm 4.1\%$  of the maximum contraction to KCl (Figure 2B). Tiron-quenchable lucigenin (250  $\mu$ mol/L) chemiluminescence production in the rings from hypertensive rats was also signifi-

cantly decreased from  $44 \pm 8.0$  to  $6.5 \pm 1.6$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> in the presence of SOD ( $n = 8$ ,  $P < 0.01$ , Figure 2C). The possibility that the inhibitory effect of SOD is due to its contamination by NH<sub>4</sub>Cl was considered by determining the effect of SOD, which was heated by autoclave for three 1-hour cycles. The autoclaved SOD had a significantly reduced inhibitory effect on spontaneous tone of aorta from Ang II-induced hypertensive rats ( $33 \pm 5.3\%$  KCl,  $n = 6$ ,  $P > 0.05$  compared with untreated aorta). Autoclaved SOD retained some activity to reduce Tiron-quenchable chemiluminescence, but this effect was also statistically smaller compared with control SOD ( $19 \pm 1.3$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>,  $n = 6$ ,  $P < 0.01$  compared with control SOD).

The effects of various inhibitors of enzymatic sources of superoxide anion generation were also tested. DPI (10  $\mu$ mol/L), an NAD(P)H oxidase inhibitor, effectively inhibited both spontaneous tone and superoxide production in rings from Ang II-infused hypertensive rats (spontaneous tone,  $13 \pm 13\%$  of the KCl contraction,  $n = 4$ ,  $P < 0.01$ , Figure 2C; Tiron-quenchable lucigenin (250  $\mu$ mol/L) chemiluminescence,  $2.4 \pm 0.9$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>,  $n = 4$ ,  $P < 0.05$ ). In contrast, neither the NADH dehydrogenase inhibitor, rotenone (10  $\mu$ mol/L), nor the xanthine oxidase inhibitor, oxypurinol (300  $\mu$ mol/L), decreased spontaneous tone or superoxide production in the rings from Ang II-infused hypertensive rats (spontaneous tone: oxypurinol,  $28.5 \pm 7.5\%$ ,  $n = 6$ ; rotenone,  $54 \pm 5.6\%$ ,  $n = 5$ ,  $P > 0.05$ ; Tiron-quenchable chemiluminescence: oxypurinol,  $55 \pm 5.2$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>,  $n = 6$ ,  $P > 0.05$  versus control; rotenone,  $48 \pm 8.7$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>,  $n = 5$ ,  $P > 0.05$ ).

#### Role of Extracellular Calcium in Spontaneous Tone and Superoxide Anion Production

Spontaneous tone was abolished in calcium-free buffer or by the L-type calcium channel blocker, nifedipine (100 nmol/L,  $n = 6$ , not shown). The blockade was reversible; replacement with buffer containing calcium (2.5 mmol/L) or washing out the nifedipine restored tone. Spontaneous tone was also prevented in rings pretreated with nifedipine from the beginning of the experimental protocol ( $3 \pm 3\%$  KCl contraction,  $n = 6$ ,  $P < 0.001$  versus control). In rings treated with nifedipine, Tiron-quenchable chemiluminescence was not significantly different compared with rings without nifedipine ( $32 \pm 10$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>,  $n = 6$ ,  $P > 0.05$ ).

#### Effect of L-NAME or Endothelium Denudation on Spontaneous Tone and Superoxide Anion Production

The role of endogenous nitric oxide in the modulation of spontaneous tone and superoxide anion levels was tested by the measurement of spontaneous tone in rings treated with L-NAME (Figure 3) or in rings from which the endothelium was denuded (Figure 4).

In the rings from sham-operated normotensive rats, L-NAME (300  $\mu$ mol/L) did not result in significant spontaneous tone or increase in superoxide anion production ( $17 \pm 6.8$  in control,  $19 \pm 4.1$  milliunits  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup> in L-NAME-treated rings,  $n = 4$ ,  $P > 0.05$ ). In endothelium-denuded rings of sham-treated rats, a small increase in

spontaneous tone developed after washout of sodium nitroprusside ( $2.3 \pm 0.7\%$  of the KCl contraction compared with  $0.3 \pm 0.2\%$  of the KCl contraction in endothelium-intact rings,  $P > 0.05$ ,  $n = 8$  and  $5$ , respectively).

In the rings from Ang II-infused hypertensive rats, L-NAME ( $300 \mu\text{mol/L}$ ) had no significant effect on spontaneous tone ( $58 \pm 6.8\%$  KCl,  $n = 6$ ,  $P > 0.05$ , Figure 3C) or on superoxide anion production ( $39 \pm 4.0$  milliunits  $\cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ ,  $n = 6$ ,  $P > 0.05$ ) compared with the untreated rings. In the presence of L-NAME, SOD ( $150 \text{ U/mL}$ ) failed to significantly inhibit the spontaneous tone ( $62 \pm 7.9\%$  of KCl contraction,  $P > 0.05$ , Figure 3D). SOD did however significantly decrease Tiron-quenchable superoxide anion production in rings treated with L-NAME to  $6.5 \pm 1.6$  milliunits  $\cdot \text{mg}^{-1} \cdot \text{min}^{-1}$  ( $P < 0.01$ ).

Endothelium denudation had no significant effect on the magnitude of spontaneous tone in rings from Ang II-infused hypertensive rats ( $69 \pm 8.6\%$  of KCl contraction,  $n = 5$ ,  $P > 0.05$  compared with endothelium-intact rings, Figure 4B). Tiron-quenchable lucigenin chemiluminescence also was not significantly different in rings of Ang II-infused hypertensive rats from which the endothelium was removed ( $31 \pm 5.8$ ,  $n = 6$ ,  $P > 0.05$  compared with intact rings). In endothelium-denuded rings of Ang II-infused hypertensive rats, the maximal spontaneous tone in rings treated with SOD ( $150 \text{ U/mL}$ ) was not significantly different from the values of untreated, endothelium-denuded rings ( $50 \pm 10\%$  KCl contraction,  $n = 5$ ,  $P > 0.05$ , Figure 4C). The reduction in Tiron-quenchable chemiluminescence caused by SOD was not significantly affected by removal of the endothelium ( $7.2 \pm 2.5$  milliunits  $\cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ ,  $n = 5$ ,  $P < 0.05$  compared with in the absence of SOD).

### Contractions to KCl

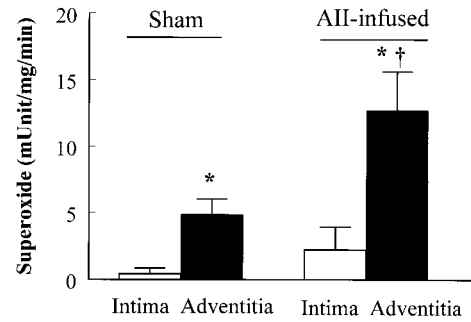
The contractions of aortic rings to  $120 \text{ mmol/L}$  KCl are shown in the Table. The contractions to KCl of the rings from Ang II-infused hypertensive rats were significantly greater than the rings from sham-operated normotensive rats. Removal of endothelium or L-NAME treatment did not have a significant effect on the contractions to KCl in the rings from either sham-operated normotensive rats or Ang II-infused hypertensive rats (Table).

SOD decreased the KCl-induced contractions in endothelium-intact, but not in endothelium-denuded rings from Ang II-infused hypertensive rats. SOD had no significant effect on KCl-induced tone in the sham-operated normotensive rats. After treatment with SOD, there was no significant difference in the contractions caused by KCl of endothelium-intact aortic rings from sham-operated normotensive and Ang II-infused hypertensive rats (Table).

Neither nifedipine nor DPI significantly decreased contraction to  $120 \text{ mmol/L}$  KCl (Table). Neither oxypurinol ( $300 \mu\text{mol/L}$ ) nor rotenone ( $10 \mu\text{mol/L}$ ) had any significant effect on the maximal contraction to  $120 \text{ mmol/L}$  KCl (Table).

### Adventitial Superoxide Anion Production Measured by Lucigenin Chemiluminescence

Lucigenin ( $5 \mu\text{mol/L}$ ) chemiluminescence in aortic rings in which the adventitia was facing outward was significantly

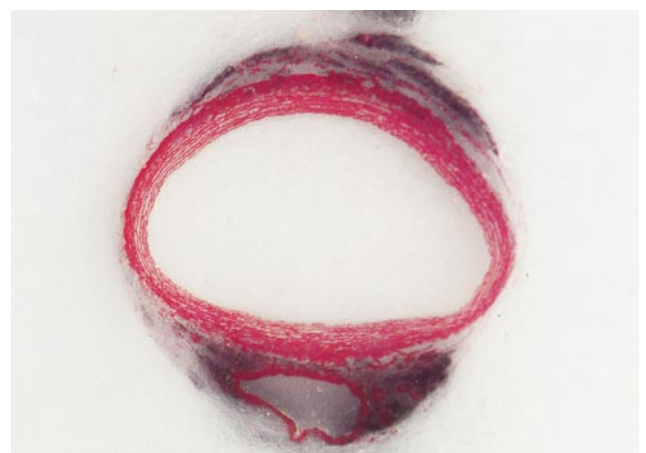


**Figure 5.** Chemiluminescence from luminal and adventitial aspects of the aorta of normotensive and Ang II-infused hypertensive rats. Lucigenin ( $5 \mu\text{mol/L}$ ) chemiluminescence was measured as described in methods from luminal and adventitial aspects. Greater chemiluminescence was detected from the adventitial compared with the luminal aspect of both normotensive sham and hypertensive rats. The adventitial chemiluminescence was significantly greater from the aorta of Ang II-infused rats. Values are expressed as mean  $\pm$  SEM. \* $P < 0.05$  compared with intima, † $P < 0.05$  compared with sham. Each group contains rings from 4 rats.

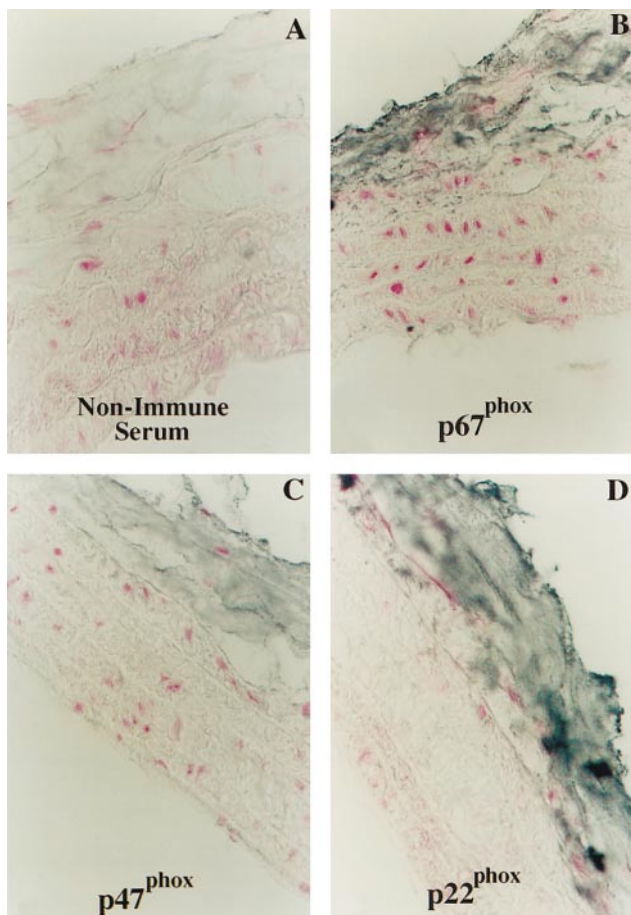
higher than in those in which the intimal surface was facing outward (Figure 5). The chemiluminescence measured from the adventitial side of aortic rings from Ang II-infused rats was 2.6-fold higher than in those from sham-operated rats ( $13 \pm 2.9$  and  $4.9 \pm 1.1$  milliunits  $\cdot \text{mg}^{-1} \cdot \text{min}^{-1}$  in Ang II-infused and in sham-operated rats, respectively,  $n = 4$  each,  $P < 0.05$ ). The chemiluminescence detected from the intimal side of aortic rings from hypertensive as well as sham-operated rats was significantly lower than that from the adventitial side (Figure 5).

### Localization of Tissue Site of Superoxide Anion Production by Reduction of Nitroblue Tetrazolium and by Immunohistochemistry of NAD(P)H Protein Subunits

Incubation of rings from 5 Ang II-infused rats with nitroblue tetrazolium resulted in blue staining predominantly of the adventitial layer. Figure 6 shows an example of a cross



**Figure 6.** Photomicrograph of a cross-section of a thoracic aorta of an Ang II-infused hypertensive rat stained with nitroblue tetrazolium. The extent of the media is delineated by the red eosin staining of the elastin lamellae. This image is representative of preparations from 5 rats. Magnification is  $\times 60$ .



**Figure 7.** Photomicrograph of cross-section of aortic ring from Ang II-infused rat aorta showing immunohistochemical staining of NAD(P)H oxidase subunits. Frozen sections of rat aorta were incubated with control nonimmune serum (panel A) or monoclonal antibodies against p67<sup>phox</sup> (panel B), p47<sup>phox</sup> (panel C), or p22<sup>phox</sup> (panel D), all at 20  $\mu\text{g/mL}$ , followed by gold-conjugated goat anti-mouse antibody). Magnification is  $\times 760$  for all panels.

section of an Ang II-treated rat aortic ring that had been incubated with nitroblue tetrazolium and counterstained with eosin. It was not possible to discriminate any difference in the intensity of nitroblue tetrazolium staining between the adventitia of Ang II-infused rats and that observed in normal rats (not shown).<sup>13</sup>

Immunohistochemistry performed with monoclonal antibodies against p67<sup>phox</sup> (Figure 7, panel B), p47<sup>phox</sup> (panel C), and p22<sup>phox</sup> (panel D) showed localization of each of these protein subunits of NAD(P)H oxidase to the adventitial layer. As with nitroblue tetrazolium staining, there was no evident difference between the intensity of staining observed in hypertensive versus normal rat aortic adventitia (not shown).<sup>13</sup> Nonimmune serum (panel A) showed no staining.

### Discussion

The major finding in this study is that the spontaneous tone of the isolated rat aorta of Ang II-infused hypertensive rats is dependent on an increased generation of superoxide anion radical. Localization of the adventitial site of generation of superoxide anion by lucigenin chemiluminescence and nitroblue tetrazolium, and the immunohistochemical localiza-

tion of NAD(P)H subunit proteins in the adventitia suggests a paracrine mechanism between cells of the adventitia with cells in the remainder of vascular wall that contributes to the regulation of smooth muscle tone.

Tone of resistance arteries, termed myogenic tone, is thought to normally mediate autoregulation of blood flow in response to changes in transmural pressure.<sup>21,22</sup> Spontaneous tone, such as that observed in this study, has been observed in isolated hypertensive resistance arteries and has been attributed to the increased transmural pressure present *in vivo*.<sup>22–24</sup> Spontaneous tone has also been noted in larger arteries of hypertensive animal models, including the hypertensive rat aorta which normally does not display such tone,<sup>23,24</sup> and parallels have been drawn between the mechanisms responsible for myogenic and spontaneous tone.<sup>21</sup> The spontaneous tone noted in the present study of the hypertensive rat aorta was observed after the artery was stretched to the optimal resting tension for contraction. The active nature of the tone was made evident by the fact that it was suppressed by sodium nitroprusside or papaverine. The tone depends critically on increased levels of superoxide anion as indicated by finding that (1) SOD, a specific scavenger of superoxide anion, and DPI, an inhibitor of NAD(P)H oxidase, prevented the tone; and (2) increased generation of superoxide anion was measured with lucigenin and suppressed by SOD. Initial observations made in this study with a higher concentration of lucigenin (250  $\mu\text{mol/L}$ ) were confirmed with a much lower concentration (5  $\mu\text{mol/L}$ ) that has been shown not to be associated with redox cycling.<sup>15</sup> In fact, the magnitude of the difference in superoxide anion production between sham-operated and Ang II-infused hypertensive rat aorta measured with the lower concentration of lucigenin was greater, perhaps because of redox cycling with the higher concentration.

Work by the groups of Griendling and Harrison<sup>2–4</sup> has shown that superoxide anion generation is increased in the aorta of Ang II-infused rats by way of increased activity of NAD(P)H oxidase. Our findings with DPI, an NAD(P)H oxidase inhibitor, are consistent with their results. The lack of effect of rotenone or oxypurinol suggests that mitochondrial NADH dehydrogenase or xanthine oxidase, respectively, were not the major source of superoxide anion in the aorta of Ang II-infused rats. Our results agree with earlier results that indicate that the majority of the superoxide anion produced is by an NAD(P)H oxidase.<sup>2–4</sup> Because Harrison and colleagues also showed that a superoxide anion scavenger decreased the elevated blood pressure,<sup>4</sup> they were able to implicate vascular superoxide anion generation at the level of resistance vessels as a significant mechanism that contributes to Ang II-induced hypertension. Because superoxide anion has been linked in this way to the mechanism of hypertension in this model, as well as in the SHR rat,<sup>25</sup> in which isolated arteries also display spontaneous tone,<sup>23,24,26</sup> it is important to determine the cellular mechanism by which the tone is mediated.

Influx of extracellular calcium through smooth muscle L-type calcium channels appears to be responsible for the tone in the aorta as indicated by the fact that nifedipine or removal of extracellular calcium prevented the tone. This finding agrees with previous work on myogenic tone in normal<sup>22,24,27</sup> and hypertensive<sup>23</sup> resistance arteries, which

shows the critical role of calcium influx through smooth muscle L-type calcium channels. As suggested by its effect on potassium-induced contractions (Table), DPI may have inhibited spontaneous tone, in part by its ability to inhibit L-type calcium channels.<sup>28</sup> However, even expressed as a percentage of the reduced contraction to KCl, DPI effectively inhibited the spontaneous tone. Although it is quite clear that calcium influx is responsible for the tone observed in the Ang II-infused hypertensive rat aorta, the generation of superoxide anion was not acutely affected by nifedipine. This suggests that the increased superoxide anion is responsible for the increased smooth muscle calcium influx and is consistent with our hypothesis that the increase in adventitial superoxide anion is causally related to the smooth muscle tone observed.

The spontaneous tone observed in the isolated rat aorta after 6 days of Ang II infusion contrasts with the contractions caused by Ang II in a normal rat aorta, because the latter are insensitive to SOD and are also not accompanied by a measurable increase in superoxide anion (H.D.W., unpublished observations, 1998). This is undoubtedly related to the fact that the increase in NAD(P)H oxidase activity and superoxide anion generation caused by Ang II requires significantly more time than was allowed in the acute exposure to Ang II. It is possible that a prolonged exposure to elevated levels of Ang II in vivo as in our studies results in the production of a number of growth and/or contractile factors that contribute to the regulation of superoxide anion production and might also be important in directly increasing smooth muscle cell tone.

The observation that increased superoxide anion generation in the Ang II-induced hypertensive rat aorta was responsible for abnormal endothelium-dependent relaxation to acetylcholine,<sup>2</sup> suggests that superoxide anion generation inactivates endothelium-derived nitric oxide in this model. It was therefore of interest to determine the role of nitric oxide in the spontaneous tone in these arteries. The fact that endothelium denudation or L-NAME did not increase the magnitude of the tone suggests that nitric oxide is either made in inadequate amounts under basal conditions, or it is not sufficiently active under these conditions to inhibit the tone of the hypertensive aorta. However, the fact that SOD did not inhibit tone in endothelium-denuded rings or in the presence of L-NAME suggests that the ability of SOD to decrease tone in endothelium-intact rings depends on the ability of the superoxide anion scavenger to increase the biological activity of endothelium-derived nitric oxide. This observation indicates that nitric oxide is inactivated by superoxide anion in the aorta of Ang II-infused rats, and is the reason that the spontaneous tone develops. Thus, superoxide anion appears to modulate smooth muscle tone in the hypertensive rat aorta predominantly by inactivating endothelium-derived nitric oxide, rather than by direct effects on the smooth muscle.

In our previous studies of the normal rat<sup>13</sup> and rabbit<sup>12</sup> aorta, it became evident that superoxide anion was generated to a major extent in the adventitia. A larger production of superoxide anion from the adventitial aspect of the vascular wall was demonstrated by lucigenin chemiluminescence in aorta of both normotensive,<sup>13</sup> and in this study, hypertensive rats. The endothelium apparently does not contribute a

significant amount to the measured vascular superoxide anion production because endothelial denudation did not affect the levels. The adventitial source of superoxide anion was also evident in nitroblue tetrazolium staining of the aorta of Ang II-infused hypertensive rats, which suggests the importance of induction by Ang II of NAD(P)H oxidase activity in adventitial fibroblasts, a fact that has been confirmed in cell culture.<sup>14</sup> Indeed, p22<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> components of neutrophil NAD(P)H oxidase were shown to be present in the aortic adventitia by immunohistochemical techniques in both normotensive,<sup>13</sup> and in this study, Ang II-infused rats. The absence of immunohistochemical staining in medial smooth muscle cells also indicates that the lower levels of superoxide anion detected by reduction of nitroblue tetrazolium is not likely caused by greater scavenging of superoxide anion by endogenous SOD but rather a paucity of the same enzymatic source of superoxide anion that is present in the adventitia of the intact aorta.

These observations suggest that increased quantities of superoxide anion and/or growth and contractile factors arising in the hypertensive aortic wall are responsible for contractile tone. However, the spontaneous tone in the hypertensive rat aorta is observed as a result of an interesting paracrine relationship between adventitial superoxide anion and the endothelium-derived nitric oxide that it inactivates. This is made possible by the diffusion distance for superoxide anion, which is estimated to be at least several microns,<sup>29</sup> and that of nitric oxide, whose diffusion distance is on the order of 30  $\mu\text{m}$  and is capable of pervading the entire vascular wall.<sup>29</sup> These observations suggest that novel paracrine relationships may exist between different components of the vascular wall of resistance arteries that have a role in hypertension. They also indicate that the adventitia is not a passive bystander during the development of hypertension but rather may play an important role in the regulation of smooth muscle tone.

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## Paracrine Role of Adventitial Superoxide Anion in Mediating Spontaneous Tone of the Isolated Rat Aorta in Angiotensin II-Induced Hypertension

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