

Superoxide Anion From the Adventitia of the Rat Thoracic Aorta Inactivates Nitric Oxide

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Abstract—The purpose of this study was to determine whether superoxide anion is produced endogenously in the rat aortic adventitia and whether sufficient superoxide anion is produced to interfere with the response of the rat aorta to nitric oxide. Relaxation was measured in rings of the rat thoracic aorta, which were oriented so that the adventitial or luminal surface could be preferentially exposed to nitric oxide or sodium nitroprusside. To accomplish this, the rings were mounted (1) with the adventitia facing outward, (2) with the adventitia facing inward after inverting, or (3) with the adventitia facing outward after inverting twice (to control for the inverting procedure). The relaxation to nitric oxide, but not to sodium nitroprusside, was less in rings with the adventitia facing outward compared with those in which it faced inward. In contrast, the response to nitric oxide via either surface was similar when extracellular superoxide anion was scavenged with superoxide dismutase. Incubation of rings with nitro blue tetrazolium (NBT) resulted in blue formazan staining of the adventitia, and lucigenin chemiluminescence was significantly greater when detected from the adventitial compared with the intimal aspect of the artery. The reduction of NBT in intact aortic rings was $30 \pm 2 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and was significantly decreased by superoxide dismutase to $19 \pm 2 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and by a synthetic superoxide dismutase mimic, Euk-8, to $11 \pm 2 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The NADPH oxidase inhibitor, diphenyleneiodonium, decreased NBT reduction to $9 \pm 1 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, whereas inhibitors of xanthine oxidase, mitochondrial oxidases, and nitric oxide synthase were ineffective. Immunohistochemical staining indicated the localization of NADPH oxidase proteins gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} almost exclusively in the adventitia of the rat aorta with no substantial staining in the media. These results indicate that NADPH oxidase located in the adventitia of rat thoracic aorta generates sufficient extracellular superoxide anion to constitute a barrier capable of inactivating nitric oxide. This study suggests that adventitial superoxide anion can play a role in the pathophysiology of the arterial wall. (*Circ Res.* 1998;82:810-818.)

Key Words: NADPH oxidase ■ nitro blue tetrazolium ■ lucigenin ■ diphenylene iodonium

Superoxide anion production in intact blood vessels can be increased by inhibiting endogenous SOD activity¹⁻³ and by vascular disease.⁴⁻⁶ The production of this free radical may interfere with vasodilation of blood vessels by inactivating nitric oxide⁷ and by altering calcium levels,⁸ gene expression,⁹ and growth of tissue.¹⁰ Different cell types contain various superoxide anion-generating systems. Xanthine oxidase¹¹ and an NADH oxidase¹² are present in endothelial cells, and an NADPH oxidase is present in dermal fibroblasts and leukocytes^{13,14} and smooth muscle cells.^{15,16} The physiological importance of these different sources whose activity may be altered during vascular disease is currently being studied intensively.

We found that in the aorta of normal rabbits, the greatest amount of superoxide anion is produced in the adventitia by an NADPH oxidase that is localized to the plasma membrane

fraction.¹⁷ In the normal rabbit aorta, the superoxide anion activity is nearly completely scavenged by endogenous SOD activity, being easily measured only after SOD activity is inhibited by diethyldithiocarbamate.² When SOD activity is inhibited, the levels of superoxide anion rise to levels sufficient to inhibit nitric oxide-induced relaxation¹ and to induce alterations in gene expression.⁹

Steinhorn et al¹⁸ showed that delivery of nitric oxide to either the intimal or adventitial surfaces of rabbit pulmonary artery produced markedly different responses. There was a reduced vasodilatory response when nitric oxide was added to the adventitial compared with the intimal surface. Although the mechanism for this difference in vasodilator response was not explained, these authors observed that the route of administration did not lead to differences in relaxation to carbon monoxide or sodium nitroprusside.

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Selected Abbreviations and Acronyms

DPI	= diphenyleioidonium
L-NAME	= <i>N</i> ^o -nitro-L-arginine methyl ester
NBT	= nitro blue tetrazolium
SOD	= superoxide dismutase

The purpose of the present study was to determine whether superoxide anion is produced endogenously in the rat aortic adventitia and whether sufficient superoxide anion is produced to interfere with the response of the rat aorta to nitric oxide. To quantify the low levels of superoxide anion produced by the aorta, we used NBT, which becomes gradually reduced by superoxide anion during incubation, accumulating the product, blue formazan. This allows not only for quantification of superoxide anion production but also for histological localization of the site of production. We also confirmed that superoxide anion was being produced primarily by the adventitia by measuring lucigenin chemiluminescence and by showing that NADPH oxidase protein subunits were localized almost exclusively in the adventitia. The data presented here indicate that the primary site of superoxide anion production in the normal rat aorta is the adventitia, which constitutes a significant barrier to exogenous nitric oxide reaching the smooth muscle.

Materials and Methods

Preparation of Rings of Rat Thoracic Aorta

Male Wistar rats (175 to 251 g) were killed by injection of pentobarbital sodium (50 mg/kg IP). Sodium heparin (100 U) was given intravenously to prevent coagulation of the blood. The thoracic aorta was removed immediately after death and placed in ice-cold buffer containing (mmol/L) NaCl 118.3, KCl 4.7, MgSO₄ 0.6, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, Na₂-EDTA 0.026, and glucose 5.5. The aorta was cleaned of adherent fat.

The endothelium was removed by inserting a polyethylene tube (PE-50) into the lumen of the aorta and rolling the vessel on moistened gauze. After cutting a segment from the aorta, the aorta was inverted by tying the vessel onto one end of the tubing and then pulling the aorta over the tubing. After another segment was cut off, the aorta was reinverted by pulling it back over the tubing. Rings 5 mm long were cut from the segments and mounted on triangular stirrups for isometric tension recording in organ chambers containing 25 mL of buffer. This resulted in three groups of rings. One group of rings was oriented with the adventitia facing outward. The second group was oriented with the intimal surface facing outward. The third group of rings, which had been inverted twice, was oriented with the adventitia facing outward and served as a control for potential cellular injury caused by stretching the smooth muscle during the inverting procedure. The rings were then mounted on the triangular stirrups and suspended in organ baths. Rings were maintained at 37°C and pH 7.4 by gassing with 95% O₂/5% CO₂. After equilibration for 60 minutes at the optimal resting tension (5 g), the aortic smooth muscle was contracted with phenylephrine hydrochloride. After achieving a stable contraction, cumulative concentrations of nitric oxide (10⁻¹⁰ to 10⁻⁶ mol/L) or sodium nitroprusside (10⁻¹⁰ to 10⁻⁵ mol/L) were added in the presence or absence of SOD (150 U/mL). Experiments with sodium nitroprusside were routinely performed in the dark. At the end of the experiment, acetylcholine (10⁻⁷ to 10⁻⁶ mol/L) was added to assess the functional integrity of the endothelium. Rings that did not respond to acetylcholine were considered effectively denuded of endothelium. This procedure provided a method for the delivery of drugs preferentially to the intimal and adventitial aspects of the aorta. When suspended under

the optimal resting tension, the ring is stretched between the two stirrups approximating the opposing sides of the ring, which limits diffusion of nitric oxide to the apposing faces. This procedure was found to be more feasible for demonstrating the functional role of the adventitia than were attempts to strip the adventitia from the media, which caused more damage and total loss of the ability of the smooth muscle to contract.

Detection of Superoxide Anion by NBT Reduction

Aortic rings were incubated with NBT in order to allow superoxide anion generated by the tissue to reduce the NBT to blue formazan.¹⁹ The site of superoxide anion could be visualized in the intact ring, and the amount of the superoxide anion generated by the vessel could be quantified by measuring the absorbance of blue formazan extracted from the tissue. Furthermore, the enzymatic source of superoxide anion in the vessels was determined pharmacologically by using different antagonists. Aortic rings were cut into transverse rings 6 mm in length. The rings were then transferred into test tubes containing 5 mL of buffer and maintained at 37°C and gassed with 95% O₂/5% CO₂ for 30 minutes in the presence or the absence of Euk-8 (100 μmol/L, a synthetic SOD mimic^{20,21}), SOD (150 U/mL), DPI (100 μmol/L, an NADPH oxidase inhibitor), oxypurinol (300 μmol/L, a xanthine oxidase inhibitor), rotenone (50 μmol/L, a mitochondrial oxidase inhibitor), or L-NAME (300 μmol/L, a nitric oxide synthase inhibitor). The concentration of Euk-8 that was used has an approximate SOD activity of 100 U/mL, an activity comparable to that of the authentic SOD used. Rings were then placed in 5 mL buffer composed of (mmol/L) NaCl 119, HEPES 20, KCl 4.6, MgSO₄ 1.0, Na₂HPO₄ 0.15, KH₂PO₄ 0.4, NaHCO₃ 5, CaCl₂ 1.2, and glucose 5.5 (pH 7.4) and containing NBT (100 μmol/L) in the presence or absence of the scavengers or the enzyme inhibitors for 1 to 1.5 hours. Five milliliters of 0.5N HCl was added to stop the reaction. In each case in which an inhibitor was used, a simultaneous control ring was used for comparison.

Some rings were then fixed in 4% formalin and embedded in paraffin. The surface of the paraffin block was cut with a microtome, and the block was placed on a microscope stage and photographed directly with epi-illumination and transillumination. Efforts to cut and prepare histological sections resulted in significant solubilization and leaching of blue formazan from the tissue.

As described by Pourcyrous et al,¹⁹ NBT reduction was quantified in some rings treated with SOD or Euk-8 after they were minced and homogenized in a mixture of 0.1N NaOH and 0.1% SDS in water containing 40 mg/L of diethylenetriaminepentaacetic acid. The mixture was centrifuged at 20 000g for 20 minutes. The resultant pellet was resuspended in 1.5 mL of pyridine during heating at 80°C for 1 to 1.5 hours to extract formazan. The mixtures were subjected to a second centrifugation at 10 000g for 10 minutes as previously described.¹⁹ The absorbance of formazan was determined spectrophotometrically at 540 nm. The extinction coefficient of blue formazan is 0.72 L · mmol⁻¹ · mm⁻¹. The quantity of formazan was calculated as follows: NBT reduction = $A \times V / (T \times Wt \times \epsilon \times l)$, where A is the absorbance of blue formazan at 540 nm, V is the volume of the solution, T is the time period during which rings were incubated with NBT, Wt is the blotted wet weight of the artery ring, ϵ is the extinction coefficient, and l is the length of the light path. Results are reported as picomoles per minute per milligram wet weight of artery. Extraction efficiency was nearly 100%, as estimated from extracting homogenates of normal aortic rings, which were mixed with known quantities of blue formazan.

To determine the amount of NBT that was nonenzymatically reduced,²² we incubated a ring of boiled aorta with NBT under conditions similar to the above; this value is reported in the results but is not subtracted from the reported values for intact rings. The SDS and NaOH in the extraction media appeared to reduce NBT to blue formazan during the extraction only in rings treated with DPI. Therefore, extractions of rings from experiments with all the enzyme inhibitors (Figure 6) were performed without SDS and NaOH. Omitting SDS and NaOH did not significantly affect the recovery of the blue color from the aortic pellet from the rings. When extracted with SDS and NaOH, the NBT reduction for a control rat aortic ring

was 27 ± 7 pmol \cdot min⁻¹ \cdot mg tissue⁻¹ (n=11), and the value for rings extracted without SDS and NaOH was 23 ± 7 pmol \cdot min⁻¹ \cdot mg tissue⁻¹ (n=11). This indicates that the recovery of blue formazan was not significantly different for the two extraction methods used.

Tests of the specificity of this assay showed that NBT reduction is >600-fold less sensitive to H₂O₂ than to superoxide anion. Optical density at 550 nm 15 seconds after adding 1 and 100 mmol/L H₂O₂ was 0.001 and 0.030, respectively, whereas that for 1 mmol/L potassium superoxide was 0.620. The small reduction of NBT by 100 mmol/L H₂O₂ was unaffected by catalase, whereas that caused by potassium superoxide was reduced by SOD (150 U/mL) to 0.024. This substantiates that the NBT assay is specific for superoxide anion rather than H₂O₂.

Detection of Superoxide Anion by Lucigenin Chemiluminescence

The details of this assay have been published previously.¹⁷ Briefly, aortic rings were cut into transverse rings 7 mm in length. The rings were cut longitudinally, flattened, and tied with 3-0 silk suture to a small black plastic plate either with adventitial or intimal surfaces facing outward. The rings were then transferred into test tubes containing 5 mL of buffer, maintained at 37°C, and gassed with 95% O₂/5% CO₂ for 45 minutes. Rings were then placed in 1 mL of HEPES buffer containing lucigenin (250 μmol/L) and maintained at 37°C. The luminometer was set to report arbitrary units of light emitted and integrated over a 30-second interval; repeated measurements were made over 5 minutes and averaged. Tiron (10 mmol/L), a nonenzymatic scavenger of superoxide anion, was then added to quench the chemiluminescence; readings from the last 90 seconds of an additional 5-minute period were averaged.

Immunohistochemistry

To identify the source of superoxide production in the adventitia, we analyzed frozen sections of rat aorta using immunohistochemistry with monoclonal antibodies recognizing four NADPH oxidase proteins known to be essential for NADPH oxidase activity in leukocytes.²³ Thoracic aorta was removed sterilely from male Wistar rats, embedded in OCT, and snap-frozen on dry ice. Immunohistochemistry was performed using previously characterized monoclonal antibodies specifically recognizing gp91^{phox},²⁴ p22^{phox},²⁴ p47^{phox},²⁵ and p67^{phox}.²⁵ Frozen aortas were cryosectioned at 5 μm. The sections were air-dried for 1 hour, fixed in cold acetone for 10 minutes, air-dried again for 1 hour, washed with blotto (5% skim milk+0.1% Tween 20 in Dulbecco's PBS, pH 7.6), and blocked with 10% goat serum in blotto for 30 minutes. The sections were then incubated overnight with control nonimmune mouse serum or monoclonal antibodies (all at ≈20 μg/mL) in Dulbecco's PBS containing 1% bovine serum albumin, 0.1% Tween 20, 0.1% NaN₃, and 1% goat serum. After washing five times for 5 minutes with blotto, the sections were incubated for 60 minutes at 25°C with 5-nm gold-conjugated goat anti-mouse antibody (Goldmark Biologicals) diluted 1:50 in the same buffer as used for the primary antibodies.²⁶ The sections were rinsed with H₂O, developed using silver acetate enhancement in the dark,²⁶ and counterstained using Nuclear Fast Red (Vector Laboratories).

Drugs

Acetylcholine chloride, lucigenin, phenylephrine hydrochloride, L-NAME, NBT, oxypurinol, rotenone, sodium nitroprusside, and Tiron were purchased from Sigma Chemical Co. SOD was purchased from Fluka. DPI was purchased from Biomol. Euk-8 was generously supplied by Eukarion Inc. All drugs were made fresh just before each experiment. Drugs were added to the incubation solution in aliquots of <1% of the solution volume. DPI and rotenone were prepared in pure dimethyl sulfoxide. Oxypurinol was solubilized in 0.5N NaOH and diluted to a stock concentration in distilled water. All other drugs were prepared as stock solutions in distilled water.

Data Analysis

ANOVA for repeated measures was applied to the data for all the concentration-response curves by using the SAS software package. Simultaneous multiple comparisons were based on post hoc comparison procedures using a Student-Newman-Keuls test. A paired *t* test was applied to the data for the NBT reduction and lucigenin chemiluminescence.

Results

Isometric Tension Responses to Phenylephrine and Nitric Oxide

The contractions of aortic rings to phenylephrine are summarized in the Table. The contractions of the inverted rings whose adventitia was oriented inward were significantly reduced compared with contractions of the control rings, which were not inverted ($P < .001$). The contractions were also significantly reduced in rings inverted twice, with the adventitia oriented outward, compared with control rings ($P < .01$). The contractions were not significantly different between the inverted rings with the adventitia oriented inward and those that were reinverted with the adventitia again oriented outward ($P > .05$). Rings inverted twice provided a comparison with control rings, in which the adventitia was oriented similarly, and were studied to determine whether the smaller contraction or adventitial orientation resulted in changes in relaxation.

Figure 1 shows examples of recorded changes in tension (1) in rings that were mounted with the adventitia facing outward (adventitia out), (2) in rings that were mounted with the adventitia positioned inside after inverting the ring (lumen out, inverted), or (3) in rings that were inverted twice to control for the inverting procedure (adventitia out, rein-

Contractions Induced by Phenylephrine

Treatment Groups	Adventitia Out (Control), mN	Lumen Out (Inverted), mN	Adventitia Out (Reinverted), mN
Nitric oxide	34 ± 5.3 (6)	20 ± 2.7 (6)	23 ± 3.7 (7)
SOD and nitric oxide	34 ± 4.0 (6)	18 ± 3.8 (5)	23 ± 3.2 (5)
SNP	39 ± 5 (8)	18 ± 2.9 (6)	22 ± 3.4 (5)
SOD and SNP	37 ± 4 (8)	ND	ND
Papaverine	41 ± 3 (8)	ND	ND
Papaverine and SOD	37 ± 2 (8)	ND	ND

ND indicates not determined. Phenylephrine (10⁻⁷ to 10⁻⁶ mol/L) was administered to induce contractions for subsequent relaxation. The contraction of rings under control conditions was approximately equal to 80% of the maximal contraction of the smooth muscle to 120 mmol/L potassium chloride. The contractions to phenylephrine of inverted rings (lumen out, n=17), in which the luminal surface was oriented outward, were not significantly different from those of rings that were twice inverted and in which the adventitia faced outward (adventitia out and reinverted, n=17; $P > .05$). Contractions of both groups for which the data are shown in Figures 1 and 2 (top) were significantly smaller than the contractions of control rings to which they were compared, which were not inverted and in which the adventitia faced outward (control, n=20; $P < .001$ and $P < .01$ compared with inverted and reinverted rings, respectively). Contractions are also shown for groups of rings for which the data are shown in Figure 3B and 3C. SOD pretreatment had no significant effects on contractions before administering nitric oxide, sodium nitroprusside (SNP), or papaverine. SOD had no significant effect on the contraction caused by phenylephrine. Numbers in parentheses indicate the number of rats in each individual treatment group. Values are mean ± SEM.

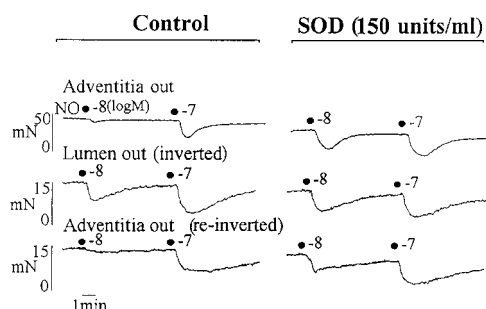


Figure 1. Typical examples of recorded changes in tension (1) in rings that were mounted with the adventitia facing outward (adventitia out), (2) in rings with the adventitia positioned inside after inverting the ring and with the lumen out (inverted), and (3) in rings with the adventitia out (re-inverted) that were inverted twice to control for the inverting procedure. The traces at left represent the responses to nitric oxide (10^{-8} and 10^{-7} mol/L) in the absence of exogenous SOD. The traces at right are the responses to nitric oxide in the presence of SOD (150 U/mL). Nitric oxide administered to the inverted rings whose luminal surface was oriented outward caused more relaxation compared with the response to nitric oxide administered to the rings whose adventitial surface was oriented outward (inverted twice or not inverted). In the presence of SOD, the responses to nitric oxide were improved in the rings with the adventitia oriented outward and were similar to those of rings with the luminal surface oriented outward.

verted). Nitric oxide (10^{-8} mol/L) caused a small relaxation in rings in which the luminal surface was oriented outward but not in the rings whose adventitial surfaces were exposed. Nitric oxide (10^{-7} mol/L) still induced a larger relaxation in the inverted rings compared with the rings whose adventitia was oriented outward (inverted twice or not inverted). In the presence of SOD (150 U/mL), the nitric oxide-induced relaxation was improved more in the rings whose adventitial surfaces were oriented outward than in the inverted rings.

The relaxations to nitric oxide expressed as a percentage of the contractions induced by phenylephrine are shown in Figure 2. Nitric oxide (10^{-10} to 10^{-8} mol/L)-induced relaxations were significantly shifted to the right in the rings whose adventitial side was oriented outward (either before or after inverting twice) compared with relaxations in the rings whose luminal surface was directed outward ($n=6$ for adventitia-out rings, $n=6$ for lumen-out rings, and $n=7$ for adventitia-out reinverted rings; $P<.01$). At nitric oxide concentrations of 10^{-9} , 10^{-8} , or 10^{-7} mol/L, respective relaxation values were (on average) 15%, 32%, and 43% lower in the rings whose adventitial surface was oriented outward compared with the rings whose luminal surface was outward ($P<.01$). At the higher concentration of nitric oxide (10^{-6} mol/L), there were no significant differences observed among the groups. The relaxation to nitric oxide of the rings whose adventitial surface was exposed before, or after inverting twice, were not significantly different.

In the presence of SOD (150 U/mL), the responses to nitric oxide were similar among the groups regardless of whether the adventitial or luminal surface was oriented outward ($n=6$ for rings with adventitia out, $n=5$ for inverted rings, and $n=5$ for reinverted rings; $P>.05$).

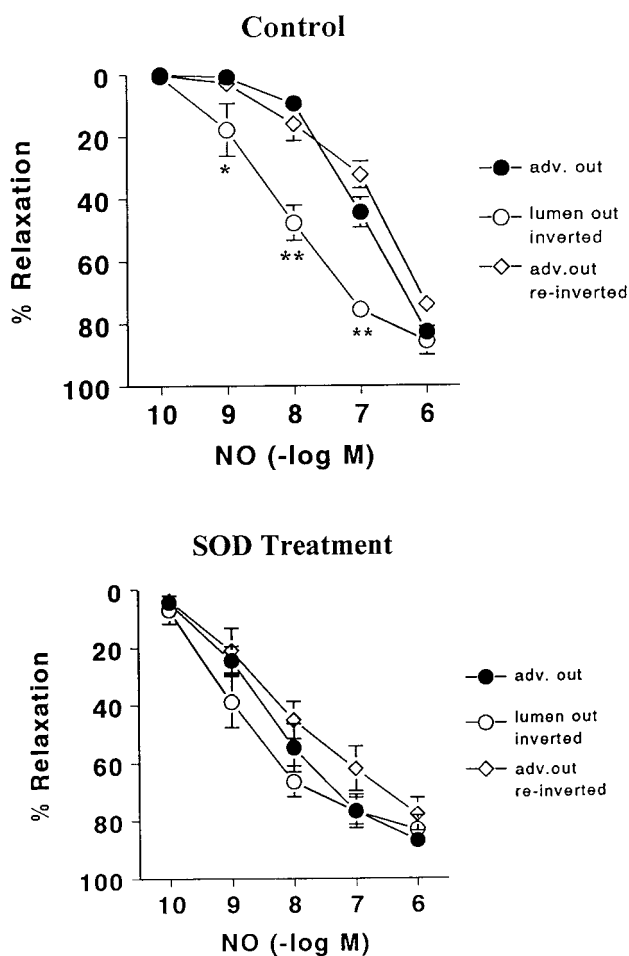


Figure 2. Comparison of nitric oxide concentration-response curves among rings in which either the adventitial or luminal surface faced outward in the absence (control) or presence of SOD (150 U/mL). Rings in organ chambers were mounted either with the adventitia facing outward (adv. out) or with the luminal surface facing outward after inverting the rings (lumen out inverted), or rings were inverted twice to control for the inverting procedure, returning the adventitia to its outward facing position (adv. out reinverted). The responses to nitric oxide in inverted rings whose luminal surface was oriented outward were significantly shifted to the left compared with the corresponding responses in rings whose adventitial surface was oriented outward (inverted twice or not inverted; $P<.01$, ANOVA for repeated measures). In the presence of SOD, the responses to nitric oxide in rings whose luminal surface was oriented outward were similar to responses in rings whose adventitial surface was oriented outward (before or after inverting). Values are expressed as mean \pm SEM percent relaxation. Each group contains responses of aortic rings from 5 to 7 rats. Contractions to phenylephrine are found in the Table. $*P<.05$ and $**P<.01$ compared with the responses in the rings with adventitia facing outward (normal rings and rings inverted twice).

In contrast to the results obtained with nitric oxide, sodium nitroprusside caused similar relaxations in rings whether the adventitial surface (before or after inverting twice) or luminal surface (inverted once) was oriented outward (Figure 3) ($n=5$ for rings with adventitia out and $n=6$ for rings with lumen out, $P>.05$). In rings with the adventitia oriented outward, SOD (150 U/mL) had no significant effects on relaxation caused by sodium nitroprusside ($n=8$) or papaverine ($n=8$), a nitric oxide-independent vasodilator (Figure 3).

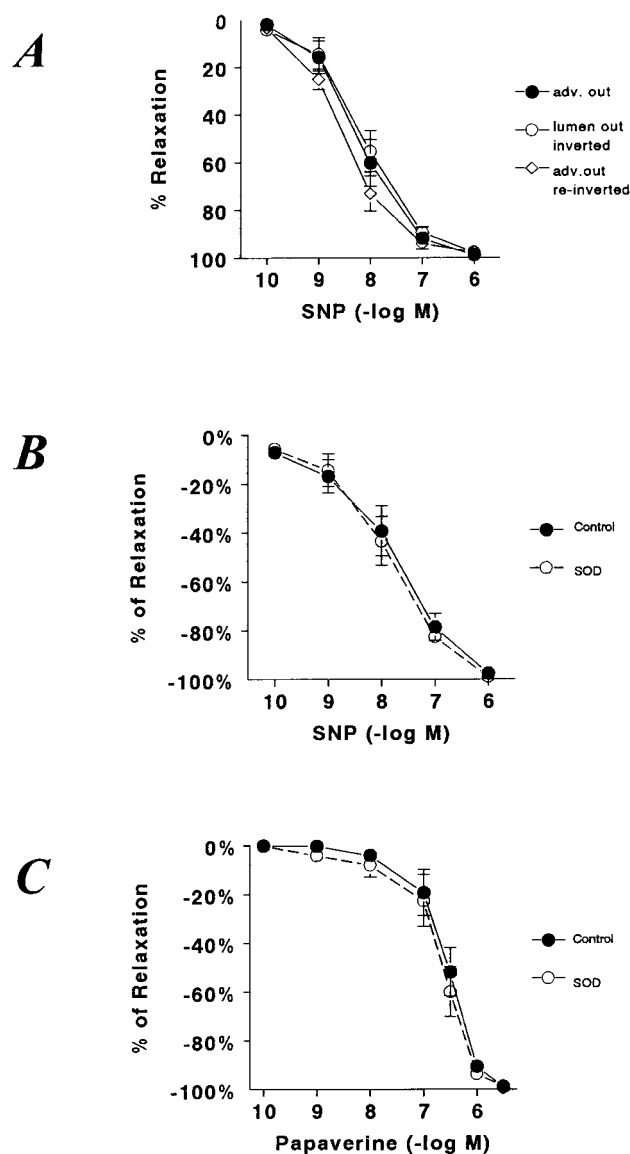


Figure 3. Influence of orientation of the adventitia and SOD on concentration-response curves to sodium nitroprusside (SNP) and papaverine. A, Rings in organ chambers were mounted either with the adventitia facing outward (adv. out) or with the luminal surface facing outward after inverting the rings (lumen out inverted), or rings were inverted twice to control for the inverting procedure, leaving the adventitia facing outward (adv. out re-inverted). The response of rings whose luminal surface was oriented outward was similar in rings whose adventitial surface was oriented outward (before or after inverting; $P > .05$, ANOVA for repeated measures). B and C, The effect of SOD was evaluated on relaxations caused by SNP and papaverine in rings in which the adventitia was oriented outward. SOD had no significant effect on the response to either SNP or papaverine. Values are expressed as mean \pm SEM. Each group contains individual responses of rings from 5 to 8 rats. Contractions to phenylephrine are found in the Table.

Tissue Site and Quantification of Superoxide Anion Production by NBT Reduction

Incubation of rings with NBT resulted in blue staining of the adventitia (Figure 4). In rings that were inverted, the adventitia was also stained blue, despite its being oriented inward, indicating that superoxide anion was generated mainly in the adventitia regardless of its physical orientation. The superox-

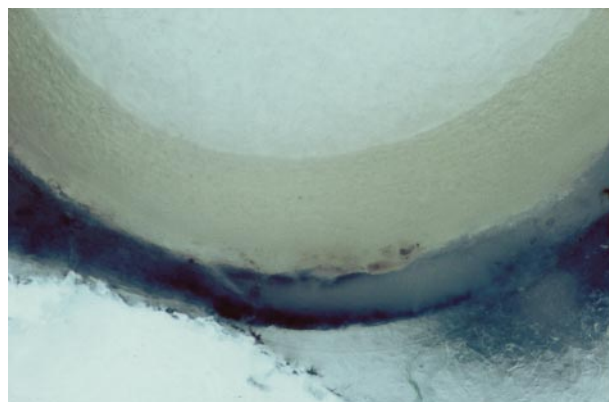


Figure 4. Photomicrograph of a cross section of a rat thoracic aorta stained with NBT. The aortic ring was incubated with NBT for 1.5 hours, embedded in paraffin, and photographed directly with epi-illumination and transillumination as described in "Materials and Methods." Staining by blue formazan, the reduction product of NBT, was mainly accumulated in the adventitial layer, whereas the media was unstained. This image is representative of preparations from 6 rats. Magnification $\times 60$.

ide scavengers, SOD and Euk-8, visibly decreased the intensity of the blue staining (not shown). The effect of Euk-8 was greater than the effect of SOD.

The NBT reduction quantified in aortic rings is shown in Figure 5. In control aortic rings, the NBT reduction was 31 ± 2 pmol \cdot min $^{-1}$ \cdot mg wet wt $^{-1}$. SOD and Euk-8 significantly decreased the NBT reduction to 19 ± 2 and 11 ± 2 pmol \cdot min $^{-1}$ \cdot mg wet wt $^{-1}$, respectively ($n=7$, $P < .01$ compared with control). NBT reduction was inhibited more by Euk-8 than by SOD ($P < .05$). Nonenzymatic NBT reduction determined in a boiled ring (10 pmol \cdot min $^{-1}$ \cdot mg wet wt $^{-1}$) was similar to that obtained in rings treated with Euk-8, suggesting that NBT reduction persisting in the presence of Euk-8 was nonenzymatic.

The effects of inhibitors of different enzymatic sources of superoxide anion are shown in Figure 6. DPI (100 μ mol/L), an NADPH oxidase inhibitor, significantly reduced NBT reduction from 27 ± 2 to 9 ± 1 pmol \cdot min $^{-1}$ \cdot mg wet wt $^{-1}$, a value similar to that obtained with boiled tissue. In contrast, neither the NADH dehydrogenase inhibitor, rotenone (50 μ mol/L), nor the xanthine oxidase inhibitor, oxypurinol (300 μ mol/L), significantly decreased NBT reduction. L-NAME (300 μ mol/L), a nitric oxide synthase inhibitor, also did not significantly affect NBT reduction ($P > .05$).

Detection of Superoxide Anion Production by Lucigenin Chemiluminescence

Lucigenin chemiluminescence was significantly higher in aortic rings in which the adventitia was facing outward than in aortic rings in which the intimal surface was facing outward (13 ± 1.7 versus 7.6 ± 0.6 mU/mg wet wt, respectively; $P < .05$). Chemiluminescence after adding Tiron was reduced and similar in aortic rings with the adventitia or intima facing outward (6 ± 0.6 versus 6.4 ± 0.7 mU/mg wet wt, respectively). After subtracting lucigenin chemiluminescence in the presence of Tiron from that obtained in its absence, the Tiron-quenchable chemiluminescence was significantly higher with the adventitia facing outward (7.3 ± 1.8 versus 1.1 ± 0.3 mU/mg wet wt, respectively; $P < .05$).

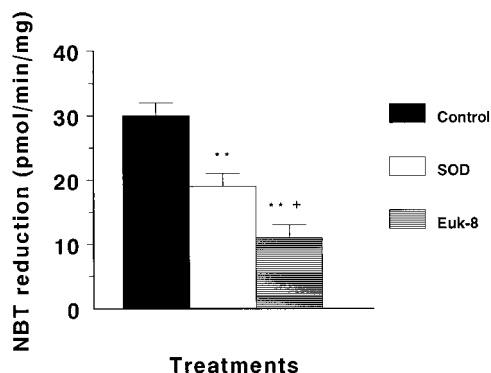


Figure 5. Comparison of NBT reduction between aortic rings in the absence or presence of SOD (150 U/mL) or Euk-8 (100 μ mol/L). Values are expressed as picomoles per minute per milligram blotted weight of aortic rings (mean \pm SEM). Each group contains rings from 6 rats. ** $P < .01$ compared with the control measured in the absence of SOD or Euk-8. + $P < .05$ compared with the response with SOD.

Immunohistochemical Detection of NADPH Oxidase Proteins in Rat Aortic Adventitia

As shown in Figure 7, immunohistochemistry of sections of rat aorta revealed specific labeling of the adventitia with monoclonal antibodies against human phagocyte p22^{phox} and gp91^{phox} and recombinant p47^{phox} and p67^{phox}, whereas no substantial staining was observed in other areas of the aorta.

Discussion

Results reported in the present study indicate that extracellular superoxide anion produced by the adventitia of the rat thoracic aorta inactivates exogenously applied nitric oxide. The hypothesis that the superoxide anion generated by rat aorta might be mainly derived from the adventitia was based on the demonstration that the adventitia was the site of greatest NADPH oxidase activity in the rabbit aorta.¹⁷ This hypothesis was first tested in rings of rat aorta that were

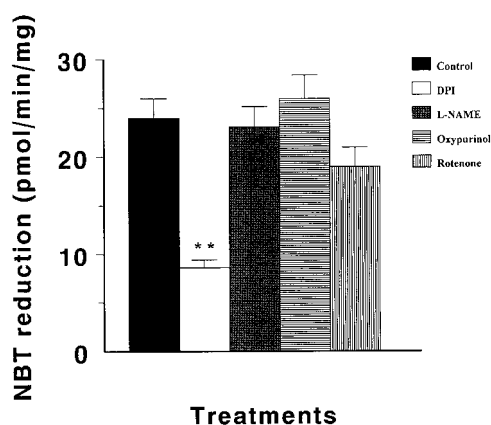


Figure 6. Comparison of NBT reduction among rings in the absence or presence of different enzyme inhibitors: DPI, an NADPH oxidase inhibitor; L-NAME, a nitric oxide synthase inhibitor; oxypurinol, a xanthine oxidase inhibitor; and rotenone, a mitochondrial NADH oxidase inhibitor. Among all the inhibitors, only DPI significantly decreased the NBT reduction ($P < .01$). Values are expressed as picomoles per minute per milligram blotted weight of the rings (mean \pm SEM). Each group contains from 5 to 12 different rats. ** $P < .01$ compared with the response in the control group.

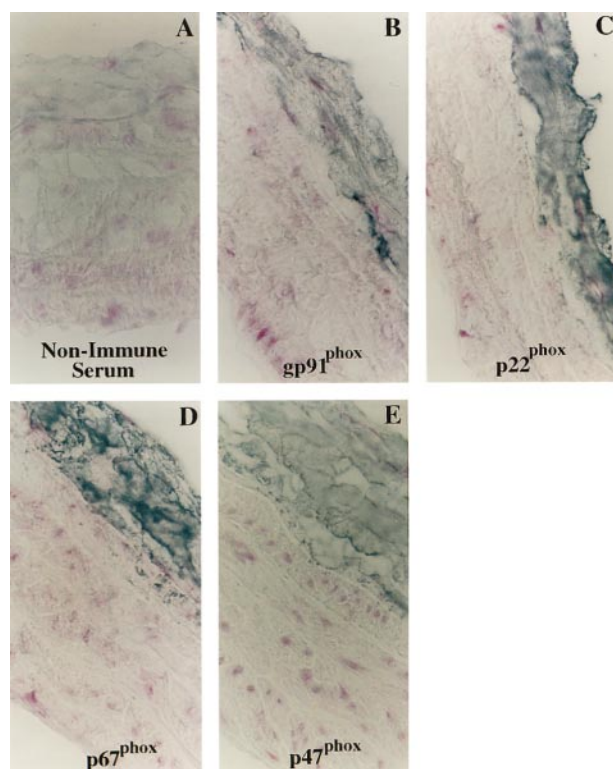


Figure 7. Immunohistochemical localization of neutrophil NADPH oxidase subunit proteins in the rat aorta. Frozen sections of the rat aorta were incubated with control nonimmune serum (A) or monoclonal antibodies against p22^{phox} (B), gp91^{phox} (C), p67^{phox} (D), and p47^{phox} (E), followed by gold-conjugated goat anti-mouse antibody. The sections were developed using silver acetate enhancement in the dark and counterstained using Nuclear Fast Red as described in "Materials and Methods." The sections shown are representative of at least two different stained sections for each antibody. Magnification $\times 760$ for all panels.

oriented so that the adventitial or luminal surface was preferentially exposed to nitric oxide. The relaxation to nitric oxide administered to the adventitial surface was shifted to the right compared with that administered to the luminal surface. In contrast, in the presence of SOD, the responses to nitric oxide administered via either adventitial or luminal surfaces were the same and were similar to the responses of rings oriented with the lumen oriented outward but in the absence of SOD. This suggests that superoxide anion can inactivate and decrease the effect of nitric oxide on adjacent smooth muscle cells. The superoxide anion that limits nitric oxide-induced relaxation is evidently released into the extracellular space in the adventitia, where it is accessible to the added SOD.²⁷

It might be argued that the influence of orientation on the response to nitric oxide of aortic rings might be related to the reduced contractile response to phenylephrine that occurred as a result of inverting the arterial rings. However, the responses to nitric oxide in the rings that were inverted twice and in which contractile force was reduced to a similar extent were also shifted to the right compared with the responses of the rings whose luminal surfaces were exposed. This indicates that it is the orientation rather than the altered smooth

muscle contraction that results in the difference in response to nitric oxide.

Because it is difficult not to damage the endothelium when inverting the aorta, the present experiments were carried out in endothelium-denuded rings. The relaxation to nitric oxide of rings with an intact endothelium and the adventitia oriented outward was similar to that of rings without endothelium (data not shown), indicating that removal of the endothelium was not responsible for the reduced sensitivity to nitric oxide of rings oriented with the lumen oriented inward.

Unlike authentic nitric oxide, sodium nitroprusside induced similar relaxations when administered to rings regardless of the orientation of the adventitial or luminal surface. Steinhorn et al¹⁸ also found that the adventitia of the rabbit pulmonary artery interfered with relaxation caused by nitric oxide but not sodium nitroprusside. This difference may be due to the fact that if care is taken to perform experiments in the dark, sodium nitroprusside primarily releases nitric oxide intracellularly in smooth muscle cells,²⁸ where it would be inaccessible to extracellular superoxide anion. The similar relaxation to sodium nitroprusside administered to either aspect of the vascular wall, as well as the lack of an effect of SOD on the relaxation, therefore supports the suggestion that it is extracellular superoxide anion in the adventitia that acts as a barrier to exogenous authentic nitric oxide. The equal relaxations to sodium nitroprusside also exclude a nonspecific difference arising as a result of the differences in orientation or differences in contraction of the inverted rings. Finally, the lack of effect of SOD on sodium nitroprusside or papaverine-induced relaxations indicates that SOD lacks nonspecific effects, which might have otherwise accounted for its effect on nitric oxide-induced relaxation.

The adventitial source of superoxide anion was demonstrated by blue formazan staining of rings incubated with NBT. The blue staining of the adventitia was decreased by SOD, consistent with the localization of extracellular superoxide anion as the functional barrier to nitric oxide administered to the adventitial aspect of the blood vessel. The staining of the adventitia was not related to better NBT penetration of the adventitia oriented outwardly, as demonstrated by similar staining of the adventitia in inverted rings. Incubation of the rabbit thoracic aorta with NBT also resulted in blue formazan staining mainly in the adventitia, but in that blood vessel, the staining occurred only after endogenous SOD was inhibited with diethyldithiocarbamate.²⁹ In the rat, sufficient superoxide anion was generated in the aortic adventitia to be detected under normal conditions. This could be due to increased production or decreased scavenging of the free radical.

The production of superoxide anion in the adventitia could be quantified by measuring the amount of NBT reduction. We used this indicator in the present study because NBT reduction is very sensitive to low levels of superoxide anion as a result of the accumulation of blue formazan in the tissue over time. It is therefore an integrative measurement of the very low production of superoxide anion produced under basal conditions. The specificity of the NBT assay was further indicated by showing that the superoxide anion scavengers, SOD and Euk-8, decreased the reduction of NBT and that

H₂O₂ did not significantly reduce NBT by comparison. SOD decreased NBT reduction to a lesser extent than did Euk-8. This may be due to the indication that Euk-8 is a cell-permeant superoxide scavenger²⁰ and that it may gain access to superoxide anion produced intracellularly. In contrast, at least in the short term, exogenous SOD only has access to superoxide anion generated extracellularly, since it is repelled from cells because of its negative charge.²⁷ The difference in the effects of SOD and Euk-8 may suggest that approximately half of the measurable superoxide anion is extracellular. The amount of NBT reduction in Euk-8-treated tissue was similar to that in boiled tissue, suggesting that this residual was nonenzymatic, possibly resulting from reduction of NBT by thiols or other tissue chemical constituents.²²

DPI decreased NBT reduction to the same levels as found after Euk-8 treatment or boiling, suggesting that the majority of NBT reduction is accounted for by superoxide anion production by an NADPH oxidase in rat aortic adventitia. This finding is consistent with previous results in rabbit thoracic aorta.¹⁷ That rotenone had no significant effect indicates that the major source of superoxide anion is not mitochondrial NADH dehydrogenase. We can also exclude xanthine oxidase as a significant source of superoxide anion in the aorta, because oxypurinol did not decrease NBT reduction. We confirmed this pharmacological and biochemical evidence by showing the presence of neutrophil-like NADPH oxidase subunit proteins localized almost exclusively in the adventitia. This further establishes the adventitia as being a site of superoxide anion production.

It is possible that the inducible form of nitric oxide synthase is expressed in the adventitia of rat aorta by endotoxin,³⁰ to which the blood vessel is exposed after removal from the rat. Because nitric oxide synthases, like other NADPH-dependent cytochrome P₄₅₀ reductases, can reduce NBT,^{31,32} it is possible that this enzyme could account for the NBT staining observed.³³ This possibility is, however, unlikely to explain our findings, because neither L-NAME nor *N*^ω-nitro-L-arginine (data not shown) had a significant effect on NBT reduction in the adventitia. In addition, treatment of the rat aorta with cycloheximide (20 μmol/L) throughout the course of experiments similar to those reported here failed to affect the development of NBT staining (not shown). Because this protein synthesis inhibitor blocks the expression of inducible nitric oxide synthase,³⁴ this, or other inducible proteins, cannot explain the superoxide production by the adventitial cells observed in the present study.

The greater adventitial production of superoxide anion observed in the present study was confirmed by lucigenin chemiluminescence, which was shown to be detectable predominantly from the adventitial aspect. Lucigenin is a more specific but, as a consequence of the fact that it reflects steady-state levels, a less sensitive indicator than NBT of low levels of superoxide anion produced under basal conditions. It should be pointed out that previously published results using lucigenin chemiluminescence in intact vascular rings have commonly detected light emission from the adventitial aspect of the blood vessel; therefore, our results suggest that the values reported may be weighted toward reporting adventitial production of the free radical.

We previously found that the normal rabbit aortic endothelium does not produce a sufficient concentration of nitric oxide or that it is not localized sufficiently close to the adventitia to reduce the levels of superoxide anion produced there.² It would also appear unlikely that superoxide anion produced in the adventitia decreases the biological activity of nitric oxide produced by endothelial cells in normal arteries. This is suggested by the fact that scavenging of superoxide anion with SOD does not affect endothelium-dependent relaxations in normal arteries, including the rat aorta.³⁵⁻³⁸

It is quite possible that in disease states, such as hypertension,⁵ diabetes mellitus,³⁵ or atherosclerosis,^{6,39} during which increased superoxide anion levels inhibit endothelium-dependent relaxation, increased adventitia-derived superoxide anion may play a role. In angiotensin II-induced hypertension, for instance, it has been reported that superoxide anion production is increased and that this impairs acetylcholine-induced endothelium-dependent relaxation.⁵ In our own recent studies of this hypertensive model, which have confirmed the elevation in superoxide anion levels, the production of the free radical and the expression of NADPH oxidase proteins remain primarily localized in the adventitia.⁴⁰

When superoxide anion reacts with nitric oxide, it produces the less potent vasodilators, peroxynitrite, nitrite, and nitrate.²⁹ Thus, oxidative degradation likely explains the reduced relaxation to exogenously added nitric oxide when administered to the adventitial side of the rat aorta. In other studies (not shown), we have attempted to detect peroxynitrite both with immunohistochemical techniques directed toward nitrotyrosine⁴¹ and with luminol chemiluminescence, which can detect peroxynitrite.⁴² Neither technique detected peroxynitrite formed after exposing the normal rat aorta to nitric oxide, probably because peroxynitrite would be expected to be very short-lived and because nitrotyrosine was not formed in sufficient amounts. It is possible that increased levels of superoxide anion in disease states could react with nitric oxide to form significant amounts of peroxynitrite.⁴³ This might be expected in particular if the expression of nitric oxide synthase also increased in the adventitia as part of the disease process, as it does after exposure to endotoxin.³⁰

In addition to its role in inhibiting the activity of nitric oxide, it is possible that superoxide anion is involved in regulating growth or inflammation in the adventitia. Several recent studies have shown that the adventitia is the site of cellular proliferation after intimal injury and that adventitial fibroblasts migrate and participate in the formation of the neointima.⁴⁴⁻⁴⁶ In addition, the synthesis and action of growth factors may be regulated by superoxide anion. For instance, the secretion of insulin-like growth factor-1 is increased by superoxide anion.⁴⁷ As demonstrated by *in situ* hybridization, the production of this growth factor is localized in the adventitia of the normal rat aorta,⁴⁸ where its production could be under the influence of the superoxide anion shown in the present study to be produced there.

In conclusion, these data support the hypothesis that an NADPH oxidase produces superoxide anion in the vascular adventitia. The local production of superoxide anion explains the observation that the adventitia is a functional barrier to

nitric oxide¹⁸ and suggests that adventitial production of superoxide anion may contribute to the regulation of vascular function.

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