Nitric Oxide Inactivates Endothelium-Derived Contracting Factor in the Rat Aorta

Wolfgang Auch-Schwelk, Zvonimir S. Katušić, and Paul M. Vanhoutte

Acetylcholine evokes the simultaneous release of endothelium-derived relaxing and contracting factors in aortas from spontaneously hypertensive rats. Only relaxing factors are released in aortas from normotensive controls. Experiments were designed to determine whether inhibitors of endothelium-dependent relaxations modify endothelium-dependent contractions. Rings of thoracic aortas of normotensive and spontaneously hypertensive rats, with and without endothelium, were suspended in organ chambers for isometric tension recording. Oxyhemoglobin (a scavenger of endothelium-derived relaxing factor) and Nω-monomethyl l-arginine (an inhibitor of nitric oxide formation) augmented the contractions to acetylcholine. Methylene blue (an inhibitor of soluble guanylate cyclase) and superoxide dismutase (a scavenger of superoxide anions) did not modify these contractions. The contractions in the presence of oxyhemoglobin or Nω-monomethyl l-arginine, like those in untreated rings, were endothelium-dependent; they only occurred in aortas from spontaneously hypertensive rats and were abolished by indomethacin. The contractions to acetylcholine in the presence of oxyhemoglobin were not affected by superoxide dismutase or deferoxamine. These data suggest that endothelium-derived relaxing factor inhibits endothelium-dependent contractions to acetylcholine in the spontaneously hypertensive rat aorta, probably by chemical inactivation of the endothelium-derived contracting factor rather than by stimulation of guanylate cyclase or scavenging of oxygen-derived free radicals.

KEYWORDS • acetylcholine • hemoglobins • arginine • nitric oxide • spontaneously hypertensive rats

Received February 15, 1991; accepted in revised form January 13, 1992.

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Results of these experiments have been previously reported in part in abstract form (J Am Coll Cardiol 1990;15:67A).

Supported by grant Au 76/1-1 from the Deutsche Forschungsgemeinschaft, grant HL-35614 from the National Institutes of Health, and an unrestricted research grant from Bristol Myers Squibb.

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acetylcholine (10^{-7} \text{M}) in rings contracted with norepinephrine (3 \times 10^{-7} \text{M}). Endothelium-dependent contractions were evoked with acetylcholine (10^{-9} \text{ to } 10^{-4} \text{M}) after 1 hour of equilibration.

\(N^G\)-Monomethyl L-arginine (L-NMMA) (5 \times 10^{-5} \text{M}) or its stereoisomer \(N^G\)-monomethyl D-arginine (D-NMMA) was added 60 minutes before acetylcholine. Oxyhemoglobin (10^{-6} \text{M}), methylene blue (10^{-5} \text{M}), superoxide dismutase (SOD) (150 units/ml), or deferoxamine (10^{-4} \text{M}) was added after 30 minutes of equilibration. Acetylcholine (10^{-8} \text{ to } 10^{-4} \text{M}) was added in a cumulative manner after the response to the antagonist had reached a plateau or after 60 minutes of equilibration (in control rings). In some rings, the antagonists caused strong contractions (two of seven with oxyhemoglobin, one of six with L-NMMA, two of seven with methylene blue); when the contractions exceeded more than 1 g, the experiment was discarded to keep basal tension levels comparable between experimental groups. For the remaining preparations, the increases in basal tension averaged 0.7±0.14 g for oxyhemoglobin, 0.14±0.07 g for L-NMMA, and 0.5±0.3 g for methylene blue.

**Drugs**

The following drugs were used: acetylcholine hydrochloride, indomethacin, (L)-norepinephrine, hemoglobin, methylene blue, L-NMMA, D-NMMA (both generous gifts from Dr. S. Moncada, Wellcome Laboratories, Beckenham, Great Britain), SOD, and deferoxamine. Unless otherwise specified all drugs were obtained from Sigma Chemical Co., St. Louis, Mo.; they were prepared daily in distilled water except for indomethacin, which was dissolved by sonication in Na_2CO_3 (10^{-5} \text{M}). All concentrations are expressed as final molar (M) bath concentrations.

Oxyhemoglobin was prepared by adding 10 mM sodium dithionite to a 1 mM solution of hemoglobin. The sodium dithionite was removed by dialysis in distilled water (containing 0.001% EDTA) for 2 hours at room temperature. The concentration of oxyhemoglobin in the final solution was determined spectrophotometrically (70±4\%, n=7).

**Calculations and Statistical Analysis**

Increases in tension are expressed as percent of the maximal response to KCl (60 mM), which was comparable in all groups. Data are shown as mean±SEM; \( n \) refers to the number of rats from which vessels were taken. Statistical evaluation was done by Student's \( t \) test for paired observations since the effects of the antagonists were always compared with a control ring from the same rat. Comparisons between experiments in SHR and WKY rats were done by unpaired Student's \( t \) test. When \( p<0.05 \), the means were considered to be statistically significantly different.

**Results**

\(N^G\)-Monomethyl L-Arginine

Incubation with L-NMMA (5 \times 10^{-5} \text{M}) caused a transient and endothelium-dependent increase in tension, which reached a plateau at 2±2\% of the response to KCl (60 mM, \( n=5 \)). In the presence of L-NMMA the contraction evoked by acetylcholine was significantly larger compared with control rings from the same rat (Figure 1). Incubation with the stereoisomer D-NMMA (5 \times 10^{-5} \text{M}) was without effect on acetylcholine-induced contractions (\( n=2 \), data not shown). In WKY rings with endothelium treated with L-NMMA, acetylcholine did not cause contractions (data not shown).

**Oxyhemoglobin**

The addition of oxyhemoglobin (10^{-6} \text{M}) caused a transient contraction in rings with endothelium from SHR aortas (3±2\% of the response to 60 mM KCl). The response to oxyhemoglobin was not affected by indomethacin (10^{-5} \text{M}; data not shown). In the presence of oxyhemoglobin, the contractions to acetylcholine were augmented significantly compared with control rings from the same rat; they were comparable in magnitude to those observed in the presence of L-NMMA (Figure 1). In rings without endothelium from SHR aortas oxyhemoglobin (10^{-6} \text{M}) had no effect on basal tone. Acetylcholine (10^{-4} \text{ to } 10^{-3} \text{M}) did not cause contractions in rings without endothelium (data not shown).

Treatment with indomethacin (10^{-3} \text{M}) abolished the contractions to acetylcholine in rings with endothelium (Figure 2), in the presence of oxyhemoglobin.
In the presence of oxyhemoglobin (10^{-6} M) acetylcholine caused a small contraction in WKY rat aortic rings with endothelium (Figure 2); the tension at the maximum was not statistically different from rings without endothelium (data not shown).

**Methylene Blue**

Methylene blue (10^{-3} M) caused a transient contraction in rings with endothelium from SHR that was followed by a relaxation. Basal tone after stabilization was elevated to 10±6% of the contraction evoked by 60 mM KCl.

Acetylcholine caused contractions in rings with endothelium, but those contractions were not different from those observed in control rings from the same rat (Figure 1).

**Scavengers of Oxygen-Derived Free Radicals**

SOD (150 units/ml) did not significantly affect the response to acetylcholine in rings with endothelium in the absence or presence of oxyhemoglobin (Table 1). Likewise, the contractions of rings with endothelium incubated with oxyhemoglobin were not affected significantly by deferoxamine (10^{-4} M; data not shown).

**Discussion**

The present study confirms earlier findings that acetylcholine causes endothelium-dependent contractions in the aorta of the SHR but not in that of the WKY rat. As in earlier work, the contractions evoked by acetylcholine were abolished by indomethacin, illustrating that they can be attributed to the release by the cholinergic transmitter of cyclooxygenase-dependent endothelium-derived contracting factor or factors. The inhibitor of nitric oxide synthase, L-NMMA; the scavenger of nitric oxide, oxyhemoglobin; and the inhibitor of soluble guanylate cyclase, methylene blue, all caused increases in tension in quiescent rings of rat aorta. These contractions must reflect the basal release of endothelium-derived relaxing factor, most likely nitric oxide, which eventually, through activation of soluble guanylate cyclase and the resulting accumulation of cyclic GMP, inhibits the inherent propensity of the vascular smooth muscle to develop intrinsic tone. The inhibitory effect of basally released endothelium-derived relaxing factor on the contractile response of the rat aorta is well documented and is comparable in the SHR and WKY aorta.

The main finding of the present study is that L-NMMA and oxyhemoglobin, which interfere with the production and the transfer, respectively, of endothelium-derived nitric oxide, augment the endothelium-dependent contractions evoked by acetylcholine. These observations could mean that either basally released endothelium-derived relaxing factor or the stimulated release of the factor by acetylcholine curtails the action of the cyclooxygenase-dependent contracting factor on the vascular smooth muscle of the SHR aorta. However, this interpretation is not tenable in view of the fact that methylene blue (which inhibits soluble guanylate cyclase and thus the action of endothelium-derived relaxing factor on vascular smooth muscle in general and in the rat aorta in particular) does not significantly affect the endothelium-dependent contractions to acetylcholine. An alternative explanation for the lack of effect of methylene blue could be that it inhibits the production of the contracting factor, an action that could affect any augmentation of the endo-

**TABLE 1. Effect of Superoxide Dismutase on the Response to Acetylcholine in the Spontaneously Hypertensive Rat Aorta With Endothelium**

<table>
<thead>
<tr>
<th>Acetylcholine (M)</th>
<th>10^{-7}</th>
<th>3×10^{-7}</th>
<th>10^{-6}</th>
<th>3×10^{-5}</th>
<th>10^{-5}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-3.3±2.5</td>
<td>-3.6±1.1</td>
<td>6.0±2.0</td>
<td>12.5±3.3</td>
<td>9.1±4.7</td>
</tr>
<tr>
<td>SOD (150 units/ml)</td>
<td>-1.1±0.6</td>
<td>-1.7±0.3</td>
<td>4.0±0.9</td>
<td>8.3±1.0</td>
<td>7.6±1.8</td>
</tr>
<tr>
<td>Hb (10^{-6} M)</td>
<td>-6.1±4.7</td>
<td>-8.1±5.5</td>
<td>6.7±6.0</td>
<td>20.2±6.5</td>
<td>20.7±8.3</td>
</tr>
<tr>
<td>SOD+Hb</td>
<td>0.1±0.9</td>
<td>-0.5±0.5</td>
<td>10.5±5.8</td>
<td>23.2±7.8</td>
<td>26.3±7.8</td>
</tr>
</tbody>
</table>

Data are expressed as increases in tension in percent and shown as mean±SEM (n=4). SOD, superoxide dismutase; Hb, oxyhemoglobin.
thelium-dependent contractions due to inhibition of soluble guanylate cyclase. However, although methylene blue inhibits the production of prostacyclin in isolated arteries, it does not affect that of vasoconstrictor prostanoioids. Endothelium-derived relaxing factor and nitric oxide interact chemically with oxygen-derived free radicals. The present observations with hemoglobin would be explained if it were to release oxygen-derived free radicals leading to the destruction of endothelium-derived nitric oxide with reduced inhibition of the contractions caused by endothelium-derived coronary factors. This interpretation is hard to reconcile with the lack of potentiation with methylene blue, which also releases superoxide anions and curtails the inhibitory effect of endothelium-derived relaxing factor on vascular smooth muscle. It is made untenable by the observation that SOD, a scavenger of superoxide anions, does not prevent the potentiation effect of oxyhemoglobin. Thus, the logical conclusion to be reached from the present findings is that endothelium-derived nitric oxide neutralizes the cyclooxygenase-dependent endothelium-derived contracting factor (or vice versa) before it reaches the underlying vascular smooth muscle. An interpretation in the sense of a chemical interaction between endothelium-derived nitric oxide and endoperoxides in the SHR aorta would also explain why inhibitors of cyclooxygenase restore normal endothelium-dependent relaxations to acetylcholine in the SHR aorta.

The present observations confirm that SOD (the scavenger of superoxide anion) does not prevent the endothelium-dependent contractions to acetylcholine in the SHR aorta and thus, that superoxide anions cannot be regarded as an endothelium-derived contracting factor in this blood vessel. It is likely that endoperoxides are the endothelium-derived contracting factor in the SHR aorta and that they activate prostaglandin H2/thromboxane A2 receptors in vascular smooth muscle. The potential importance of cyclooxygenase-dependent vasoconstrictor responses in the pathogenesis of hypertension is supported by the demonstration that inhibitors of prostaglandin H2/thromboxane A2 receptors potentiate antihypertensive properties in different models of the disease.

The present findings demonstrate that inhibitors of the production or the transfer of endothelium-derived nitric oxide optimize the conditions to study endothelium-dependent, cyclooxygenase-dependent contractions in the SHR aorta.

References
Nitric oxide inactivates endothelium-derived contracting factor in the rat aorta.
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doi: 10.1161/01.HYP.19.5.442

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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