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 N\textsuperscript{ω}-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 N\textsuperscript{ω}-monomethyl L-arginine, like those in untreated rings, were endotheli-um-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. (Hypertension 1992;19:442–445)

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

Acetylcholine causes the simultaneous release of endothelium-derived relaxing and contracting factors in aortas from spontaneously hypertensive rats (SHR).\textsuperscript{1,2} Only relaxing factors are released from normotensive controls, Wistar-Kyoto (WKY) rats.\textsuperscript{1,2} In rings contracted with norepinephrine, the concomitant release of endothelium-derived contracting factor from SHR aortas leads to reduced endothe-lium-dependent relaxations compared with aortas from WKY rats.\textsuperscript{1} In quiescent rings, acetylcholine causes endothelium-dependent contractions only in aortas from SHR but not from WKY rats.\textsuperscript{1} The nature of the contracting factors has not been identified so far. It appears to be an unstable product of cyclooxygenase (most likely endoperoxides) that stimulates thrombox-ane A\textsubscript{2}/prostaglandin H\textsubscript{2} receptors on vascular smooth muscle.\textsuperscript{1,3–5} The purpose of the present study was to determine whether endothelium-dependent contrac-tions to acetylcholine are modified by the simultaneous release of endothelium-derived relaxing factor.

Methods

The experiments were performed on the thoracic aorta from male, normotensive WKY rats and age- and weight-matched SHR (30–34 weeks old; weight, WKY 381±7 g, n=5, SHR 391±4 g, n=16) (Harlan Sprague Dawley, Indianapolis, Ind.). Systolic blood pressure was determined by an indirect tail-cuff method in the un-anesthetized animal before the experiment (WKY 122±8 mm Hg, SHR 202±4 mm Hg; p<0.05). The thoracic aorta was excised under anesthesia with sodium pentobarbital (50 mg/kg i.p.) and placed into ice-cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, CaCl\textsubscript{2} 2.5, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25.0, edetate calcium disodium 0.026, glucose 11.1 (control solution). Up to four rings (6-mm length) were obtained from each animal. In some rings the endothelium was removed deliberately by gently rubbing the intimal surface with the tip of a small forceps. The rings were suspended in organ chambers between a clip and a force transducer (UTC-2, Gould Inc., Cleveland, Ohio) by two stainless steel wires inserted into the lumen. The organ chambers were filled with 25 ml control solution, were kept at 37°C, and were aerated with a 95% O\textsubscript{2}/5% CO\textsubscript{2} gas mixture. Changes in isometric force were measured. The preparations were set individually at the optimal point of their length–tension relation as determined by repeated exposure to norepinephrine (3×10\textsuperscript{-7} M) at different levels of stretch. The presence of the endothelium was confirmed by the occurrence of relaxations to
acetylcholine ($10^{-7}$ M) in rings contracted with norepinephrine ($3 \times 10^{-7}$ M). Endothelium-dependent contractions were evoked with acetylcholine ($10^{-8}$ to $10^{-4}$ M) after 1 hour of equilibration. N$\text{G}^{\text{O}}$-Monomethyl l-arginine (L-NMMA) ($5 \times 10^{-5}$ M) or its stereoisomer N$\text{G}^{\text{O}}$-monomethyl d-arginine (D-NMMA) was added 60 minutes before acetylcholine. Oxyhemoglobin ($10^{-5}$ M), methylene blue ($10^{-5}$ M), superoxide dismutase (SOD) (150 units/ml), or deferoxamine ($10^{-4}$ M) was added after 30 minutes of equilibration. Acetylcholine ($10^{-8}$ to $10^{-4}$ 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$_2$CO$_3$ ($10^{-5}$ M). All concentrations are expressed as final molar (M) bath concentrations.

Oxyhemoglobin was prepared by adding 10 mM sodium dithionate to a 1 mM solution of hemoglobin. The sodium dithionate 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$\text{G}^{\text{O}}$-Monomethyl l-Arginine

Incubation with L-NMMA ($5 \times 10^{-5}$ 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 ($5 \times 10^{-5}$ 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 ($10^{-6}$ 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 ($10^{-5}$ 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 ($10^{-6}$ M) had no effect on basal tone. Acetylcholine ($10^{-4}$ to $10^{-4}$ M) did not cause contractions in rings without endothelium (data not shown).

Treatment with indomethacin ($10^{-3}$ 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.¹⁴ The inhibitor of nitric oxide synthase, L-NMMA; the scavenger of nitric oxide, oxyhemoglobin; and the inhibitor of soluble guanylate cyclase, methylene blue,⁶,¹⁰ 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,¹⁰-¹⁵ 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.³,⁸,¹⁰,¹¹. The inhibitory effect of basally released endothelium-derived relaxing factor on the contractile response of the rat aorta is well documented³ and is comparable in the SHR and WKY aorta.¹⁴

The main finding of the present study is that L-NMMA and oxyhemoglobin, which interfere with the production and the transfer, respectively,⁶-⁹ 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 ¹-²-¹⁵,¹⁶ 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,¹⁰ and in the rat aorta in particular,⁵,¹⁷-¹⁹) does not augment significantly 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>Control</th>
<th>SOD (150 units/ml)</th>
<th>Hb (10⁻⁴ M)</th>
<th>SOD+Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁷</td>
<td>3×10⁻⁷</td>
<td>10⁻⁶</td>
<td>3×10⁻⁵</td>
</tr>
<tr>
<td></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>
</tr>
<tr>
<td></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>
</tr>
<tr>
<td></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>
</tr>
<tr>
<td></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>
</tr>
</tbody>
</table>

Data are expressed as increases in tension in percent and shown as mean±SEM (n=4). SOD, superoxide dismutase; Hb, oxyhemoglobin.

**Figure 2.** Line plot shows contractions to acetylcholine in quiescent rings with endothelium from spontaneously hypertensive rats (SHR) (●), normotensive controls (WKY) (●), and SHR in the presence of indomethacin (10⁻⁵ M) (●). Data shown are mean±SEM of three to five experiments.
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 vasconstrictor prostanoids. 20 Endothelium-derived relaxing factor and nitric oxide interact chemically with oxygen-derived free radicals. 21,22 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. 21-23 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. 6,10 It is made untenable by the observation that SOD, a scavenger of superoxide anions, does not prevent the potentiating 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. 1

The present observations confirm that SOD (the scavenger of superoxide anion) does not prevent the endothelium-dependent contractions to acetylcholine in the SHR aorta 4 and, thus, that superoxide anions cannot be regarded as an endothelium-derived contracting factor 24,25 in this blood vessel. It is likely that endoperoxides are the endothelium-derived contracting factor in the SHR aorta and that they activate prostaglandin H 2/thromboxane A 2 receptors in vascular smooth muscle. 5,26 The potential importance of cyclooxygenase-dependent vasoconstrictor responses in the pathogenesis of hypertension is supported by the demonstration that inhibitors of prostaglandin H 2/thromboxane A 2 receptors prevent antihypertensive properties in different models of the disease. 27,28

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.
W Auch-Schwelk, Z S Katusic and P M Vanhoutte

doi: 10.1161/01.HYP.19.5.442

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/19/5/442