Role of Endothelium in Endothelin-Evoked Contractions in the Rat Aorta

Stefano Taddei and Paul M. Vanhoutte

We designed experiments to determine the role of endothelium-derived contracting factor or factors in the response to endothelin-1 and endothelin-3 in the aorta of normotensive and hypertensive rats. Rings of thoracic aortas, with and without endothelium, from normotensive and spontaneously hypertensive rats were suspended in organ chambers for recording of isometric tension in the presence of nitro-L-arginine, an inhibitor of nitric oxide synthase. The removal of endothelium decreased the contractions evoked by both endothelins in the aorta of spontaneously hypertensive but not of normotensive rats. Indomethacin (inhibitor of cyclooxygenase), dazoxiben (inhibitor of thromboxane synthase), and SQ-29,548 (antagonist of thromboxane A<sub>2</sub> receptors) reduced, in aortic rings of spontaneously hypertensive rats, the contractions to endothelins in rings with but not in those without endothelium, whereas their effect was not endothelium-dependent in tissues of normotensive rats. BQ-123, a selective endothelin-A receptor antagonist, shifted the concentration-response curve to endothelin-1 to the right in a concentration-dependent manner and abolished the endothelium-dependent component of the contractions evoked by the peptide. The presence of the endothelium increased the basal and endothelin-stimulated release of thromboxane B<sub>2</sub>, the stable metabolite of thromboxane A<sub>2</sub> in aortas of spontaneously hypertensive rats but not in those of normotensive rats. These data suggest that endothelium-derived thromboxane A<sub>2</sub> contributes to contractions evoked by endothelin-1 and endothelin-3 in the aorta of the spontaneously hypertensive rat but not in that of the normotensive rat. Both the receptors on the endothelial cells (mediating the release of thromboxane A<sub>2</sub>) and those on vascular smooth muscle belong to the endothelin-A subtype. (*Hypertension* 1993;21:9-15)

**KEY WORDS** • endothelins • thromboxane A<sub>2</sub> • indomethacin • rats, inbred SHR • rats, inbred WKY • endothelium • aorta

In most isolated arteries, acetylcholine affects the tone of vascular smooth muscle through the release of endothelium-derived relaxing factor<sup>1-3</sup>. However, in the aorta of spontaneously hypertensive rat (SHR), the endothelium-dependent relaxations to acetylcholine are blunted by the concomitant release of a cyclooxygenase-dependent endothelium-derived contracting factor, which most likely is an endoperoxide.<sup>4,5</sup> In the same preparation, with endothelium from both the SHR and normotensive Wistar-Kyoto (WKY) rat, the direct constrictor effect of endothelin-1 (ET-1) and endothelin-3 (ET-3) on the vascular smooth muscle is blunted by the release of endothelium-derived relaxing factor (most likely nitric oxide).<sup>6,7</sup> In addition, in aorta with endothelium from the hypertensive but not from the normotensive strain, contractions to ET-1 are reduced by the inhibitor of cyclooxygenase indomethacin, suggesting that the peptide causes the release of a cyclooxygenase-dependent endothelium-derived contracting factor.<sup>8</sup> We designed the present experiments to determine the nature of the cyclooxygenase-dependent endothelin-derived contracting factor released by endothelin in SHR aorta and, because two different endothelin receptor subtypes exist (endothelin-A and endothelin-B), to identify the endothelin receptor subtype involved.

**Methods**

**Contractile Responses**

Experiments were performed on the thoracic aortas from male, normotensive WKY rats and age- and weight-matched SHRs (28-32 weeks old; weight: WKY, 394±9.3 g; SHR, 403±10.2 g; n=24 for each group; Harlan Sprague Dawley, Inc., Indianapolis, Ind.). All procedures followed were in accordance with the guidelines of the Animal Protocol Review Committee of Baylor College of Medicine. Systolic blood pressure was determined by an indirect tail-cuff method in the unanesthetized animal before the experiment (WKY, 128±9 mm Hg; SHR, 212±11 mm Hg; p<0.001). The thoracic aorta was excised with rats under sodium pentobarbital anesthesia (50 mg/kg i.p.) and was placed into ice-cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118.3, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, EDTA 0.026, and glucose 11.1 (control solution). Up to eight rings (3-4-mm length) were obtained from...
Radioimmunoassay of Thromboxane B$_2$

Rings, with and without endothelium, of aorta were placed in a siliconized glass tube containing 1 ml control solution, oxygenated with 95% O$_2$–5% CO$_2$, and incubated for 60 minutes at 37°C in a shaking water bath. The incubation buffer was then replaced with 1 ml fresh buffer containing nitro-L-arginine (10$^{-4}$ M; inhibitor of cyclooxygenase), dazoxiben (10$^{-4}$ M; inhibitor of thromboxane synthase), and SQ-29,548 (10$^{-7}$ M; antagonist of thromboxane A$_2$/prostaglandin H$_2$ receptors). In another series of experiments, the effect of BQ-123 (3×10$^{-8}$, 3×10$^{-7}$, 3×10$^{-6}$ M; 20 minutes of incubation$^{19}$; selective antagonist of endothelin-A receptors$^{20}$) was evaluated.

Contractile Responses

Experiments were performed in the presence of nitro-L-arginine to eliminate the production of nitric oxide. In some rings, this agent caused contractions. When the contraction exceeded 1 g, the experiment was discarded to maintain comparable basal tension levels among experimental groups. For the preparations reported, the increases in basal tension induced by nitro-L-arginine averaged 0.24±0.09 g.

In SHR and WKY aortas, with and without endothelium, ET-1 (10$^{-10}$ to 10$^{-7}$ M) caused concentration-dependent increases in tension that were slow in onset and reached a stable plateau after approximately 15 minutes. At 10$^{-7}$ M (the highest concentration tested), ET-3 evoked significantly smaller contractions than ET-1; the threshold concentration of ET-3 was significantly larger than that of ET-1. In SHR aorta, removal of the endothelium significantly reduced the contraction to the highest concentrations tested of both ET-1 and ET-3, by 31.2% and 33.3%, respectively (Figure 1 and Table 1). When the endothelial component of the response to endothelins in SHR aorta was compared, the concentration–response curve to ET-3 was significantly smaller than that of ET-1 (Figure 2). In WKY aorta, the contractions evoked by ET-1 and ET-3 were minimally affected by the removal of endothelium (Figure 1 and Table 1).

In SHR rings with endothelium, indomethacin, dazoxiben, or SQ-29,548 caused a rightward shift of the concentration–response curve and a reduction of the response to 10$^{-7}$ M ET-1 and ET-3 (Figure 3 and

**Results**

**Contractile Responses**

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**Calculations and Statistical Analysis**

Increases in tension are expressed as percent of the maximal response to phenylephrine (10$^{-6}$ M). Results are presented as mean±SEM; $n$ represents the number of rats studied. For experiments with BQ-123, Schild plots$^{11}$ were constructed; the dose ratios of concentrations of ET-1 eliciting 50% of the contraction to phenylephrine in the presence and absence of BQ-123 were used. The $p_A_2$ values were calculated if the slope of the plot did not differ significantly from unity (determined by linear regression analysis). Subtraction of the data obtained in paired rings with and without endothelium of the same arteries was performed to evaluate the endothelium-dependent component of the response to endothelins.$^{12}$

Statistical evaluation of the data was performed by Student's $t$ test for paired and unpaired observations, and analysis of variance was used to check the difference between different treatments. Differences were considered to be statistically significant at a value of $p<0.05$.

**Drugs**

Acetylcholine hydrochloride, indomethacin, phenylephrine, and nitro-L-arginine were purchased from Sigma Chemical Co., St. Louis, Mo.; ET-1 and ET-3 were purchased from Peninsula Laboratories, Inc., Belmont, Calif.; SQ-29,548 was generously supplied by Bristol-Myers Squibb, Princeton, Pa.; BQ-123 was supplied by Banyu Pharmaceuticals, Tokyo, and dazoxiben by Pfizer, Groton, Conn. Drugs were prepared daily in distilled water, except for indomethacin, which was dissolved by sonication in an equimolar concentration of Na$_2$CO$_3$ (final bath concentration, 10$^{-5}$ M).
FIGURE 1. Line graphs show contractions evoked by endothelin-1 in aorta from spontaneously hypertensive rat (SHR) (left panel) and Wistar-Kyoto (WKY) rat (right panel) with and without endothelium. All experiments were performed in the presence of nitro-L-arginine (10^{-4} M). Results are mean±SEM (n=6 for each experiment) and expressed as percent of maximal contractions to phenylephrine. *Significant difference between rings with and without endothelium (p<0.05).

Table 1). The inhibitors of the arachidonic acid cascade abolished the endothelium-dependent component of the response to endothelins (Figure 3 and Table 1). In SHR rings without endothelium, the compounds caused a slight but significant shift to the right of the concentration-response curve to both ET-1 and ET-3, without reducing the maximal response to the peptides (Figure 3 and Table 1). In WKY aorta, indomethacin, dazoxiben,

<table>
<thead>
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<th>Treatment</th>
<th>SHR aortas</th>
<th>WKY aortas</th>
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<tr>
<td><strong>Endothelin-3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelin-3</td>
<td>10^{-9} M</td>
<td>10^{-6} M</td>
</tr>
<tr>
<td>Control</td>
<td>3.47±1.18</td>
<td>9.77±3.46</td>
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<tr>
<td>Indomethacin, 10^{-5} M</td>
<td>1.52±0.96</td>
<td>5.55±1.97</td>
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<td>Dazoxiben, 10^{-4} M</td>
<td>1.75±0.80</td>
<td>5.10±1.87</td>
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<tr>
<td>SQ-29,548, 10^{-7} M</td>
<td>2.19±1.38</td>
<td>5.81±2.60</td>
</tr>
<tr>
<td>Control</td>
<td>1.66±1.12</td>
<td>5.78±2.50†</td>
</tr>
<tr>
<td>Indomethacin, 10^{-5} M</td>
<td>0.00±0.00</td>
<td>2.78±1.60</td>
</tr>
<tr>
<td>Dazoxiben, 10^{-4} M</td>
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<td>4.93±2.57</td>
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<tr>
<td>SQ-29,548, 10^{-7} M</td>
<td>1.61±0.75</td>
<td>5.01±2.35</td>
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SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats. All experiments performed in the presence of nitro-L-arginine (10^{-4} M). Results are mean±SEM (n=6) and are expressed as percent of maximal contraction to phenylephrine. *Significant difference between control and treated rings. †Significant difference between rings with and without endothelium.
FIGURE 2. Line graph shows contractions evoked by endothelin-1 (ET-1) and endothelin-3 (ET-3) in aorta from spontaneously hypertensive rat. All experiments were performed in the presence of nitro-L-arginine (10^{-4} M). Data are presented as subtraction curves (response of ring without endothelium minus response of paired ring with endothelium). Results are mean±SEM (n=6 for each experiment) and expressed as percent of maximal contractions to phenylephrine. *Significant difference between rings with and without endothelium (p<0.05).

ben, or SQ-29,548 produced a comparable inhibition of the contractions evoked by ET-1 and ET-3, which was not significantly different between rings with or without endothelium (Figure 4 and Table 1).

In SHR aorta with endothelium, BQ-123 caused a parallel, concentration-dependent shift to the right of the concentration–response curve to ET-1, as well as a reduction in the response to the highest concentration of the peptide (Figure 5). The endothelin-A antagonist abolished the endothelium-dependent component of the response to ET-1 (Figure 6). In WKY aorta with endothelium, BQ-123 induced a shift to the right in the concentration–response curve to ET-1 without affecting the maximal response (Figure 5); removal of the endothelium did not significantly alter the effect of BQ-123 (data not shown). In all groups, the slope of the Schild plot for the endothelin-A antagonist was not significantly different from unity (Figure 7 and Table 2). BQ-123 did not affect the concentration–response curve to phenylephrine (from 10^{-11} to 10^{-4} M) in rings (with endothelium) from either SHR or WKY aortas (n=2; data not shown).

Release of Thromboxane B_{2}

In SHR aorta but not in WKY aorta, the presence of endothelium significantly increased the basal release of thromboxane B_{2}. At 10^{-7} M, ET-1 and ET-3 augmented the production of thromboxane B_{2}, which was increased further by the presence of endothelium in SHR aorta (Figure 8). In WKY aorta, the presence of endothelium did not affect the increased production of thromboxane B_{2} evoked by the peptides (Figure 8). Dazoxiben abolished both the basal and endothelin-induced release of thromboxane B_{2} (data not shown).

Discussion

In rat aorta, ET-1 and ET-3 can release endothelium-derived relaxing factor, and the removal of the endothelium or treatment with antagonists of nitric oxide synthase augment contractions evoked by endothelins.6,7 Because this effect is more pronounced in arteries from hypertensive than those from normotensive animals, all the experiments of the present study were performed in the presence of nitro-L-arginine, a noncompetitive inhibitor of nitric oxide synthase.13 Inhibitors of this enzyme also potentiate endothelium-dependent contractions in SHR aorta, most likely by...
Figure 4. Line graphs show effect of indomethacin, SQ 29548, and dazoxiben on contractions evoked by endothelin-1 in aorta from Wistar-Kyoto (WKY) rat with (left panel) and without (right panel) endothelium. All experiments were performed in the presence of nitro-L-arginine (10^{-4} M). Results are mean±SEM (n=6 for each experiment) and expressed as percent of maximal contractions to phenylephrine. *Significant difference between control and treated rings (p<0.05).

In the present study, ET-1 and ET-3 evoked contractions in SHR aorta that were blunted by the removal of the endothelium. In confirmation of earlier findings with ET-1, the inhibitor of cyclooxygenase indomethacin inhibited the response to the peptides. This finding indicates that endotheilns induce the production of a cyclooxygenase-dependent endothelium-derived contracting factor or factors in the aorta of hypertensive rats. The observation that dazoxiben, an inhibitor of thromboxane synthase, and SQ-29,548, an antagonist of thromboxane A_{2} receptors, have an inhibitory effect comparable to that of indomethacin suggests that thromboxane A_{2} rather than endoperoxides is the cyclooxygenase-dependent endothelium-derived contracting substance that contributes to the response to endotheilns. The greater inhibition of the response to the peptide induced by the treatment with indomethacin, SQ-29,548, and dazoxiben as compared with the removal of the endothelium indicates that a certain amount of thromboxane A_{2} must be produced in the smooth muscle cells. This interpretation is strengthened by the observation (in confirmation of previous reports) that in the aortas of normotensive animals, the removal of the endothelium did not modify the response to endotheilns, and that indomethacin, dazoxiben, and SQ-29,548 blunted the contractions evoked by endothelin-1 in aorta from spontaneously hypertensive rat (SHR) (left panel) and Wistar-Kyoto (WKY) rat (right panel) with endothelium. All experiments were performed in the presence of nitro-L-arginine (10^{-4} M). Results are mean±SEM (n=4 for each experiment) and expressed as percent of maximum to phenylephrine.

Figure 5. Line graphs show effect of treatment with BQ-123 (from 3×10^{-8} to 3×10^{-6} M) on contractions evoked by endothelin-1 in aorta from spontaneously hypertensive rat (SHR) (left panel) and Wistar-Kyoto (WKY) rat (right panel) with endothelium. All experiments were performed in the presence of nitro-L-arginine (10^{-4} M). Results are mean±SEM (n=4 for each experiment) and expressed as percent of maximum to phenylephrine.
the peptides to the same extent in rings with and without endothelium. As reported previously, in rings without endothelium from SHR aorta, the contractions to endothelins were reduced as compared with WKY aorta. Treatment with indomethacin, SQ-29,548, and dazoxiben blunted the response to endothelins in WKY aorta without endothelium, whereas it was less effective in the same preparations from the SHR. This finding indicates that in aorta of normotensive rats endothelins cause the production of vasoconstrictor products of cyclooxygenase in the vascular smooth muscle cells.

Taken in conjunction, the present data indicate that a component of the contraction to endothelins in the rat aorta is mediated by the generation of a cyclooxygenase-dependent substance, presumably thromboxane A₂, which in the hypertensive aorta is produced mainly by the endothelium, whereas in the normotensive blood vessel wall, it originates in another structure, most likely the vascular smooth muscle.

As in previous reports, the present findings demonstrate that, in the SHR aorta, ET-1 is a more potent contracting agent than ET-3, particularly in terms of threshold concentration. This is the case in rings both with and without endothelium. The response to both peptides is blunted by endothelium removal, which does not cause a rightward shift of the concentration-response curves to ET-1 and ET-3 but only results in a decrease in response to the highest concentration of the peptides. The most likely explanation for these observations is that, at lower concentrations, the effect of the peptides on the smooth muscle is responsible for the contraction, whereas the release of endothelium-derived contracting factor only occurs when higher concentrations of endothelins are reached.

The fact that ET-1 is more potent than ET-3 in inducing endothelium-dependent contractions, and presumably the underlying release of thromboxane B₂, suggests that the receptor involved at the endothelial cells belongs to the endothelin-A subtype. This interpretation is supported by the finding that BQ-123, a selective antagonist of this subtype of endothelin receptors, abolishes the endothelial component of the response. An identical interpretation can be reached for the subtype mediating the direct effect or effects of endothelins on vascular smooth muscle. Indeed, in both SHR and WKY aorta without endothelium, BQ-123 caused a rightward shift of the concentration-response

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<th>SHR</th>
<th>WKY</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>With endothelium</td>
<td>Without endothelium</td>
</tr>
<tr>
<td>pA₂</td>
<td>8.19±0.09</td>
<td>7.83±0.08</td>
</tr>
<tr>
<td>Slope</td>
<td>0.98</td>
<td>0.98</td>
</tr>
</tbody>
</table>

SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

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by ET-1 and ET-3 is mediated by the thromboxane A$_2$ which the incubation buffer was assayed for thromboxane B$_2$ (TBXB$_2$) from rings with (E+) and without (E-) endothelium in both strains; however, there was a 10-fold difference between the values obtained in the SHR and WKY rat.

In conclusion, the present observations indicate that in SHR aorta, a component of the contractions evoked by ET-1 and ET-3 is mediated by the thromboxane A$_2$ that is produced by endothelial cells.

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