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 A2 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 B2, the stable metabolite of thromboxane A2, in aortas of spontaneously hypertensive rats but not in those of normotensive rats. These data suggest that endothelium-derived thromboxane A2 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 A2) and those on vascular smooth muscle belong to the endothelin-A subtype. (Hypertension 1993;21:9-15)

KEY WORDS • endothelins • thromboxane A2 • 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.1-3 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.4,5 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).6,7 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.8 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, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, EDTA 0.026, and glucose 11.1 (control solution). Up to eight rings (3–4-mm length) were obtained from...
each animal. In some rings, the endothelium was removed 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 stirrups inserted into their lumen. The organ chambers were filled with 25 ml control solution, kept at 37°C, and aerated with a 95% O₂-5% CO₂ gas mixture. Changes in isometric force were recorded. The preparations were set individually at the optimal point of their length–tension relation as determined by repeated exposure to phenylephrine (10⁻⁴ M) at different levels of stretch. The presence of the endothelium was confirmed by the occurrence of relaxations to acetylcholine (10⁻⁶ M) in rings contracted with phenylephrine (10⁻⁴ M). After that, all experiments were performed in the presence of nitro-L-arginine (10⁻⁴ M) to prevent the production of nitric oxide, which reduces endothelium-dependent contractions. Rings with and without endothelium of the same aorta were studied in parallel.

After 1 hour of equilibration, concentration–response curves (10⁻¹¹ to 10⁻⁷ M) to ET-1 or ET-3 were obtained by cumulative addition of the peptides. Responses to ET-1 and ET-3 were obtained both under control conditions and after incubation (45 minutes) with indomethacin (10⁻⁴ M; inhibitor of cyclooxygenase), dazoxiben (10⁻⁴ M; inhibitor of thromboxane synthase), and SQ-29,548 (10⁻⁷ M; antagonist of thromboxane A₂/prostaglandin H₂ receptors). In another series of experiments, the effect of BQ-123 (3 x 10⁻⁶ M; selective antagonist of endothelin-A receptors) was evaluated.

Radioimmunoassay of Thromboxane B₂

Rings, with and without endothelium, of aorta were placed in a siliconized glass tube containing 1 ml control solution, oxygenated with 95% O₂-5% CO₂, 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) and, where indicated, dazoxiben (10⁻⁴ M). After 15 minutes, the buffer was replaced once more with fresh solution containing the drugs, and the preparations were allowed to equilibrate for 30 minutes before an aliquot was collected for the determination of the basal generation of thromboxane B₂. At that time, the buffer was changed, and ET-1 (or ET-3) was added. A second aliquot was removed after 30 minutes of incubation. Rings were then lyophilized for the determination of dry weight. The generation of thromboxane B₂ was quantitated by radioimmunoassay using a commercially available kit (Du Pont, Boston). The average intra-assay coefficient of variation was 6.2%, and the interassay coefficient of variation was 5.9%. The sensitivity of thromboxane B₂ in this radioimmunoassay was 4 pg per assay tube. The cross-reactivity of the assay was 0.28% for prostaglandin D₂, 0.003% for prostaglandin E₂, 0.04% for prostaglandin F₂α, 0.002% for prostaglandin E₁, 0.01% for 6-ketoprostaglandin F₂α, 0.001% for 15-dehydroepi prostaglandin E₂, 0.002 for prostaglandin A₁, and 0.001% for prostaglandin B₂.

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 Na₂CO₃ (final bath concentration, 10⁻⁵ M).

Calculations and Statistical Analysis

Increases in tension are expressed as percent of the maximal response to phenylephrine (10⁻⁴ M). Results are presented as mean±SEM; n represents the number of rats studied. For experiments with BQ-123, Schild plots 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 pA₂ 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.²

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.

Results

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 to the right of that to 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
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, dazoxibene, and SQ-29,548 abolished the endothelium-dependent component of the response to endothelins (Figure 3 and Table 1). In SHR aorta, indomethacin, dazoxibene, and SQ-29,548 abolished the endothelium-dependent component of the response to endothelins (Figure 3 and Table 1). In WKY aorta, indomethacin, dazoxibene, and SQ-29,548 abolished the endothelium-dependent component of the response to endothelins (Figure 3 and Table 1).

**TABLE 1. Effect of Indomethacin, Dazoxiben, and SQ-29,548 on Contractions Evoked by Endothelin-3 in Rat Aorta With and Without Endothelium**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SHR aortas</th>
<th>WKY aortas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10$^{-9}$ M</td>
<td>10$^{-8}$ M</td>
</tr>
<tr>
<td>Rings with endothelium</td>
<td></td>
<td></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>
</tr>
<tr>
<td>Dazoxiben, 10$^{-4}$ M</td>
<td>1.75±0.80</td>
<td>5.10±1.87</td>
</tr>
<tr>
<td>SQ-29,548, 10$^{-2}$ M</td>
<td>2.19±1.38</td>
<td>5.81±2.60</td>
</tr>
<tr>
<td>Rings without endothelium</td>
<td></td>
<td></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>
<td>2.03±0.95</td>
<td>4.93±2.57</td>
</tr>
<tr>
<td>SQ-29,548, 10$^{-2}$ M</td>
<td>1.61±0.75</td>
<td>5.01±2.35</td>
</tr>
<tr>
<td>Rings with endothelium</td>
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</tr>
<tr>
<td>Control</td>
<td>0.00±0.00</td>
<td>1.12±0.72</td>
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<tr>
<td>Indomethacin, 10$^{-5}$ M</td>
<td>0.00±0.00</td>
<td>0.95±0.95</td>
</tr>
<tr>
<td>Dazoxiben, 10$^{-4}$ M</td>
<td>0.69±0.69</td>
<td>2.63±1.25</td>
</tr>
<tr>
<td>SQ-29,548, 10$^{-2}$ M</td>
<td>0.69±0.69</td>
<td>1.97±1.25</td>
</tr>
<tr>
<td>Rings without endothelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.00±0.00</td>
<td>1.65±1.05</td>
</tr>
<tr>
<td>Indomethacin, 10$^{-5}$ M</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Dazoxiben, 10$^{-4}$ M</td>
<td>0.40±0.40</td>
<td>1.19±1.19</td>
</tr>
<tr>
<td>SQ-29,548, 10$^{-2}$ M</td>
<td>0.30±0.30</td>
<td>1.06±0.68</td>
</tr>
</tbody>
</table>

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).

FIGURE 3. Line graphs show effect of indomethacin, SQ 29548, and dazoxiben on contractions evoked by endothelin-1 in aorta from spontaneously hypertensive rat (SHR) 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).

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,\textsuperscript{8} the inhibitor of cyclooxygenase indomethacin inhibited the response to the peptides. This finding indicates that endothelins induce the production of a cyclooxygenase-dependent endothelium-derived contracting factor or factors in the aorta of hypertensive rats.\textsuperscript{8} The observation that dazoxiben, an inhibitor of thromboxane synthase, and SQ-29,548, an antagonist of thromboxane A\textsubscript{2} receptors, have an inhibitory effect comparable to that of indomethacin suggests that thromboxane A\textsubscript{2} rather than endoperoxides\textsuperscript{5} is the cyclooxygenase-dependent endothelium-derived contracting substance that contributes to the response to endothelins. 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\textsubscript{2} must be produced in the smooth muscle cells. This interpretation is strengthened by the observation (in confirmation of previous reports\textsuperscript{14,15}) that in the aortas of normotensive animals, the removal of the endothelium did not modify the response to endothelins, and that indomethacin, dazoxiben, and SQ-29,548 blunted the contractions evoked by

FIGURE 5. Line graphs show effect of treatment with BQ-123 (from 3x10^{-8} to 3x10^{-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.
FIGURE 6. Bar graph shows effect of treatment with BQ-123 (bars from left to right: control, BQ-123 at 3×10⁻⁸ M, BQ-123 at 3×10⁻⁷ M, BQ-123 at 3×10⁻⁶ M) on contractions evoked by endothelin-1 in aorta from spontaneously hypertensive rat with endothelium. All experiments were performed in the presence of nitro-L-arginine (10⁻⁴ M). Data are presented as subtraction bars (response of ring without endothelium minus response of paired ring with endothelium). 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. This interpretation is supported by the demonstration that the presence of endothelium increased the basal release of thromboxane B₂ (the stable metabolite of thromboxane A₂) in aortic rings of the SHR but not in those of the WKY rat. Moreover, ET-1 and ET-3 caused an increased release of thromboxane B₂ that was amplified by the presence of endothelium in the aorta of the SHR but not of the WKY rat. Expectedly, dazoxiben prevented both the basal and endothelin-induced release of thromboxane B₂. Earlier work has demonstrated that endothelium-dependent contractions in response to agonists such as acetylcholine and serotonin are prominent in the SHR compared with the WKY aorta.

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 curves to ET-1 and ET-3.

### Table 2. pA₂ Values and Slopes for BQ-123 in Rat Aorta With and Without Endothelium

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SHR</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td>pA₂</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.
curve to ET-1, whereby the slopes of the Schild plots were not different from unity, suggesting the competitive nature of the antagonism in both strains. The pA2 values were not significantly modified by removal of endothelin in both strains; however, there was a 10-fold difference between the values obtained in the SHR and WKY rat. This suggests that there may be different receptor subtypes involved in the response to ET-1 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 A2 that is produced by endothelial cells.

Figure 8. Bar graphs show release of thromboxane B2 (TBXB2) from rings with (E+) and without (E-) endothelium of aorta from spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat. Rings were incubated with either vehicle (basal, closed bars) or endothelin-1 (ET-1) or endothelin-3 (ET-3) (10^(-7) M, shaded bars) for 30 minutes, after which the incubation buffer was assayed for thromboxane B2.

Results are mean±SEM (n=4). Asterisks denote significant differences (p<0.05); stars denote significant difference between rings with and without endothelium (p<0.05).

Acknowledgments

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