Correlation With Blood Pressure of the Acetylcholine-Induced Endothelium-Derived Contracting Factor in the Rat Aorta

Yoshio Iwama, Toshio Kato, Masahito Muramatsu, Hiroshi Asano, Kiyokazu Shimizu, Yukio Toki, Yutaka Miyazaki, Kenji Okumura, Hidekazu Hashimoto, Takayuki Ito, and Tatsuo Satake

To examine a relation between the production of acetylcholine-induced endothelium-derived contracting factor and an increase in blood pressure, endothelium-dependent contraction and relaxation were evaluated by measuring the isometric tension of aortic rings from spontaneously hypertensive rats and Wistar-Kyoto rats at 5, 10, 20, and 30 weeks of age. In norepinephrine-precontracted rings, acetylcholine (10^-8 to 10^-5 M)-induced relaxations diminished at the doses of 10^-8 to 10^-5 M in both strains except at 5 weeks of age. Treatment with a thromboxane A2/prostaglandin H2 antagonist (ONO-3708) prevented this reduction in acetylcholine-induced relaxations in both strains and induced dose-dependent relaxations, which were completely inhibited by treatment with a nitric oxide inhibitor, N^G-nitro-L-arginine methyl ester. In aorta treated with N^G-nitro-L-arginine methyl ester without precontraction, acetylcholine induced dose-dependent contractions, which were greater in spontaneously hypertensive rats than in Wistar-Kyoto rats. These acetylcholine-induced contractions, which were observed only in rings with endothelium, were completely inhibited by treatment with ONO-3708 but not with a thromboxane A2 synthetase inhibitor (OKY-046). There was a statistically significant correlation between the acetylcholine-induced contractions and blood pressure. Release of 6-ketoprostaglandin F1a by acetylcholine from the aorta was greater in spontaneously hypertensive rats. In vivo administration of another thromboxane A2/prostaglandin H2 antagonist (ONO-8809) (10 or 30 μg per body per day) for 3 weeks (5–8 weeks of age) did not affect blood pressure in either rat strain. These results suggest that the production of acetylcholine-induced endothelium-derived contracting factor, which is most likely prostaglandin H2, is closely associated with the increase in blood pressure that occurs in young to adult rats. (Hypertension 1992;19:326–332)

KEY WORDS • endothelium • endothelium-derived contracting factor • endothelium-derived relaxing factor • prostaglandin H2 • spontaneously hypertensive rats

The endothelium produces various vasoactive substances and controls vascular smooth muscle tone. Since Furchgott and Zawadzki1 first reported the presence of endothelium-derived relaxing factor (EDRF) in 1980, many vessels were found to produce EDRF in response to a variety of stimuli.2,3 The primary component of EDRF was thereafter shown to be nitric oxide.4,5 It was also clarified that endothelium-derived contracting factor (EDCF) is also produced by stimulated vessels,6 and increased production of EDCF has been reported in anoxia,7 hypertension,8 and aging.9 The nature of EDCF varies with the species and the anatomical site of its production, but thromboxane A2 (TXA2),10 superoxide anion,11,12 endothelin,13 and prostaglandin H2 (PGH2)14 have been suggested as EDCF. The EDCF released from the rat thoracic aorta by acetylcholine stimulation is likely to be PGH2, as we have previously suggested.14

The present study was designed to confirm the hypothesis that increased production of the EDCF induced by acetylcholine in rat aorta coincides with the increase in blood pressure. For this purpose, we separately evaluated EDCF-induced contractile responses and EDRF-induced relaxations in the rat thoracic aorta under acetylcholine stimulation by inhibiting either EDRF or EDCF with a nitric oxide inhibitor or a TXA2/PGH2 receptor antagonist, respectively, and also by examining changes in the production of these factors in rats of various age groups. Furthermore, we examined whether blood pressure in rats in vivo was reduced by inhibition of the EDCF production.

Methods

Male spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats (Charles River Japan Inc., Atsugi, Japan) in 5-, 10-, 20-, and 30-week-old groups were used. Each group consisted of rats aged 5–6, 10–11, 20–22, and 30–32 weeks. Blood pressures were measured in conscious rats using the tail-cuff method. The rats were decapitated, the thoracic aorta was excised, and the connective tissue was removed in cooled Krebs-Henseleit solution with the following:...
Antagonists and a Nitric Oxide Inhibitor

Rings were treated with both L-NAME and ONO-3708, 30 minutes before precontraction.

Rings were treated with L-NAME or ONO-3708 15 minutes before precontraction with norepinephrine. When a plateau was reached, the rings were relaxed by rubbing the vascular lumen with a thin swab. Acetylcholine (10⁻⁷ M)-induced relaxation less than 5% of precontraction with norepinephrine (10⁻⁷ M) was considered to indicate the absence of endothelium.

Precontraction of the Arteries

Contractions in response to 10⁻⁷ M norepinephrine in aortic rings with and without endothelium were estimated by measuring the changes in tension of rings in each group of WKY rats and SHR.

Contractions to norepinephrine (10⁻⁸ to 10⁻⁵ M) in the rings without endothelium reached a plateau at the concentration of 10⁻⁵ M. Ratios of the contraction induced by 10⁻⁷ M norepinephrine to that induced by 10⁻³ M norepinephrine were as follows: 92.7±1.8% (5 weeks), 86.0±2.9% (10 weeks), 91.6±0.7% (20 weeks), and 91.7±1.1% (30 weeks) in WKY rats and 94.3±1.5% (5 weeks), 86.8±2.8% (10 weeks), 90.1±1.6% (20 weeks), and 89.5±3.1% (30 weeks) in SHR. Since these ratios were similar, the concentration of 10⁻⁷ M norepinephrine was considered an effective dose for all tissues.

To determine the optimal resting tension, contractions to 10⁻⁷ M norepinephrine in rings without endothelium were estimated under resting tension of 1 g, 2 g, and 3 g. Because contractions developed under these resting tensions were similar in each age group (data not shown), resting tension of 2 g was applied to all rings.

Responses to Acetylcholine and Effects of Thromboxane A₂/Prostaglandin H₂ Receptor Antagonists and a Nitric Oxide Inhibitor

The aortic rings with or without endothelium from SHR and WKY rats in 5-, 10-, 20-, and 30-week-old groups were contracted with norepinephrine (10⁻⁷ M). When a plateau was reached, the rings were relaxed by cumulative addition of 10⁻⁸ to 10⁻⁵ M acetylcholine.

The rings were treated with a TXA₂/PГH₄ receptor antagonist ONO-3708 (10⁻⁴ M) or a novel TXA₂/PГH₄ receptor antagonist ONO-NT-126 (3×10⁻⁸ M) 15 minutes before precontraction with norepinephrine. When the rings were treated with both a nitric oxide inhibitor, N⁶-nitro-l-arginine methyl ester (L-NAME) (10⁻⁵ M), and ONO-3708, L-NAME was added 30 minutes before precontraction.

In other experiments, 10⁻⁸ to 10⁻⁵ M acetylcholine was applied to the rings without precontraction. The rings were treated with L-NAME or ONO-3708 15 minutes before acetylcholine application. When the rings were treated with both L-NAME and ONO-3708, L-NAME was added 30 minutes before acetylcholine application.

When the rings were precontracted, the relaxations were expressed as percentages of the contraction induced by 10⁻⁷ M norepinephrine, and when the rings were not precontracted, they were expressed as percentages of the contraction induced by 10⁻⁷ M norepinephrine in another adjacent ring from the same rat.

Effects of a Thromboxane A₂ Synthetase Inhibitor

Under resting tension, 10⁻⁸ to 10⁻⁵ M acetylcholine was added cumulatively 15 minutes after the treatment with both 10⁻³ M L-NAME and 10⁻⁵ M OKY-046, a TXA₂ synthetase inhibitor, and vascular responses were examined.

Concentrations of 6-Ketoprostaglandin F₁α

Only a resting tension was applied to the aortic rings from SHR and WKY rats in the 30-week-old group, and 10⁻⁵ M acetylcholine was added 15 minutes after treatment with 10⁻³ M L-NAME. Before the treatment with L-NAME, immediately before the addition of acetylcholine and at the peak response to 10⁻⁵ M acetylcholine, 0.5 ml of the solution in the chamber was collected and the 6-ketoprostaglandin F₁α (6-keto-PGF₁α) concentration was determined by the method of Jaffe et al and Powell using a radioimmunoassay kit prepared by New England Nuclear, Boston, Mass.

Administration of a Thromboxane A₂/Prostaglandin H₂ Receptor Antagonist In Vivo

ONO-3708 is a short-acting agent in vivo, and ONO-8809 is a long-acting prodrug, which is transformed to ONO-NT-126 in vivo (unpublished observation). To evaluate the effect of inhibition of EDCF production on blood pressure, ONO-8809 (10 or 30 μg per body per day) was administered to 5-week-old WKY rats and SHR for 3 weeks. The systolic blood pressures of WKY rats and SHR were measured at 5 and 8 weeks of age by the tail-cuff method in the unanesthetized state.

Drugs

The following drugs from Sigma Chemical Co., St. Louis, Mo. were used: l-norepinephrine bitartrate, acetylcholine chloride, and N⁶-nitro-l-arginine methyl ester. Ono Pharmaceutical Company, Osaka, Japan, provided (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate (OKY-046), (9,11)(12)-dideoxa-9α, 11α-dimethylmethano-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19,20-pentanor-15-epi-TX A₂ (ONO-3708), 9,11-epithio-11,12-methano-TX A₂ (STA₂), n-decyl (Z)-6-[(1S,2S,3R,4R)-3-(4-bromobenzenesulfonylamino)methyl]bicyclo[2.2.1]hept-2-yl] hept-5-enoic acid (ONO-NT-126), STA₄, ONO-8809, and ONO-NT-126 were dissolved in ethanol. The final ethanol concentration in the bath solution was below 0.1%. Vehicle did not affect the acetylcholine-induced responses or the resting tension. The other drugs were dissolved in distilled water.
TABLE 1. Changes in Systolic Blood Pressure in Each Age Group

<table>
<thead>
<tr>
<th>Group</th>
<th>5 weeks</th>
<th>10 weeks</th>
<th>20 weeks</th>
<th>30 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>97±3</td>
<td>109±4</td>
<td>134±3</td>
<td>141±2</td>
</tr>
<tr>
<td>SHR</td>
<td>108±3</td>
<td>162±3</td>
<td>184±4</td>
<td>192±3</td>
</tr>
</tbody>
</table>

Results (mm Hg) are expressed as mean±SEM. n=6–9. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

Statistical Analysis

Results were expressed as mean±SEM. For statistical analysis, Student's t test for paired or unpaired observations and the Wilcoxon test were used. The regression line was determined by the least-squares method. Values of p<0.05 were considered to be significant.

Results

Blood Pressure

The changes in systolic blood pressure of SHR and WKY rats in each age group are shown in Table 1.

Contractions to Norepinephrine

Contractions to 10⁻⁷ M norepinephrine in aortic rings with and without endothelium are shown in Table 2. There were no age-dependent or hypertension-dependent changes in contractions to 10⁻⁷ M norepinephrine in either strain. These contractions were not affected by the presence of ONO-3708 and OKY-046, but in rings with endothelium, the contractions increased in the presence of L-NAME.

Responses to Acetylcholine

Figure 1A shows typical responses. When acetylcholine was added cumulatively after contraction induced by norepinephrine, aortic rings of 30-week-old SHR were relaxed at acetylcholine concentrations of 10⁻⁸ to 10⁻⁷ M, but relaxations were weaker at acetylcholine concentrations of 10⁻⁶ to 10⁻⁵ M. In the rings without endothelium, no relaxations to acetylcholine were observed in either SHR or WKY rats at all ages (data not shown).

In the 5-week-old group, both SHR and WKY rats showed dose-dependent relaxations to acetylcholine, but the relaxations decreased in the 10-week-old and older groups at acetylcholine concentrations of 10⁻⁶ to 10⁻⁵ M in both strains (Figure 2). The degree of these decreases was greater in SHR than in WKY rats at all ages between 10 and 30 weeks (Figure 2).

FIGURE 1. Typical records of acetylcholine (ACh)-induced responses in 30-week-old spontaneously hypertensive rat aortic rings. Panel A: Rings were contracted with 10⁻⁷ M norepinephrine (NE), and ACh was cumulatively added. Panel B: 10⁻⁴ M ONO-3708 was added 15 minutes before contraction by NE. Panel C: 10⁻³ M N⁰-nitro-L-arginine methyl ester (NNM, L-NAME in text) and 10⁻⁴ M ONO-3708 were added 30 and 15 minutes, respectively, before contraction by NE.

FIGURE 2. Line graphs show acetylcholine (ACh)-induced endothelium-dependent responses and the effect of ONO-3708 (10⁻⁴ M) in spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat aortic rings (n=6 rats). ACh was added cumulatively after contraction induced by 10⁻⁷ M norepinephrine. Relaxation was expressed as percent contraction induced by 10⁻⁷ M norepinephrine. Results are shown as mean±SEM. **p<0.01 between presence and absence of ONO-3708. †p<0.05, ††p<0.01 between SHR and WKY rats.
Effects of Thromboxane A₂/Prostaglandin H₂ Receptor Antagonists

Figure 1B shows typical responses of 30-week-old SHR aortic rings treated with ONO-3708. The decrease in the relaxation, which was observed at acetylcholine concentrations of $10^{-9}$ to $10^{-7}$ M without ONO-3708, disappeared in both SHR and WKY rats, and similar acetylcholine dose-dependent relaxations were observed at all ages (Figures 2 and 3). The aortic rings of 30-week-old SHR contracted with $10^{-7}$ M norepinephrine showed acetylcholine ($10^{-8}$ to $10^{-5}$ M) dose-dependent relaxations in the presence of ONO-NT-126 ($3 \times 10^{-6}$ M) as well as in the presence of ONO-3708 (data not shown).

Effects of a Nitric Oxide Inhibitor and a Thromboxane A₂ Synthetase Inhibitor

Figure 4A shows the typical acetylcholine dose-dependent contractile responses of 20-week-old SHR aortic rings after treatment with L-NAME under resting tension. These contractile responses increased with age and were always greater in SHR than in WKY rats (Figure 5).

In the rings without endothelium, no contractile responses were observed in either SHR or WKY rats at all ages (data not shown).

OKY-046 did not affect these contractile responses (data not shown).

Effects of a Thromboxane A₂/Prostaglandin H₂ Receptor Antagonist and a Nitric Oxide Inhibitor

Figure 1C shows the typical acetylcholine dose-dependent responses when 30-week-old SHR aortic rings were treated with both L-NAME and ONO-3708 before contraction induced by norepinephrine. The relaxations in response to the cumulative addition of acetylcholine disappeared completely in both SHR and WKY rats at all ages (Figure 3). Figure 4B shows the typical acetylcholine dose-dependent responses when 20-week-old SHR aortic rings were treated with both L-NAME and ONO-3708 under resting tension. No contractile relaxation was observed (data not shown).
sponses to acetylcholine were observed in either SHR or WKY rats at all ages (Figure 5).

Concentrations of 6-Keto-Prostaglandin F1α

The 6-keto-PG F1α concentration in the solution before addition of L-NAME and acetylcholine was similar at 128±12 pg/ml and 97±19 pg/ml for SHR and WKY rats, respectively. It was not affected by addition of L-NAME (data not shown). It was, however, 432±30 pg/ml in SHR and 313±29 pg/ml in WKY rats 10 minutes after addition of 10⁻⁵ M acetylcholine, being significantly higher in SHR (Figure 6).

Contractions to a Thromboxane A₂ Analogue

Contractions of aortic rings without endothelium to a thromboxane A₂ analogue, STA₂ (10⁻⁷ M), were determined in WKY rats and SHR (Table 3). There was no age-dependent change in either strain, and there was no difference between WKY rats and SHR in sensitivity to STA₂ except in the 10-week-old group.

Relation Between Endothelium-Dependent Contraction and Blood Pressure

Relation between 10⁻⁵ M acetylcholine-induced endothelium-dependent contraction and the systolic blood pressure is shown in Figure 7. There is a statistically significant correlation between acetylcholine-induced endothelium-dependent contraction and blood pressure.

Changes in Blood Pressure by Administration of a Thromboxane A₂/Prostaglandin H₂ Receptor Antagonist

After oral administration of ONO-8809, the systolic blood pressures in both strains were not reduced compared with control as shown in Table 4.

Discussion

The present study evaluated vascular responses to EDCF and EDRF in aortic rings of SHR and WKY rats after cumulative addition of acetylcholine. When the aortic rings of SHR and WKY rats were treated with ONO-3708 and then contracted with norepinephrine, rings from both strains showed similar acetylcholine dose-dependent relaxations in all age groups. Since ONO-3708 inhibits the action of the acetylcholine-stimulated EDCF, which is most likely PGH₂,¹⁴ and the relaxations due to inhibition of EDCF were completely suppressed by a nitric oxide inhibitor, L-NAME, the relaxations observed after treatment with ONO-3708 are considered to have been caused by acetylcholine-induced EDRF (nitric oxide). The degree of relaxation was similar in SHR and WKY rats from 5 to 30 weeks of age. The present findings indicate that EDRF production is maintained in rats from age 5 to 30 weeks, as has been noted in previous studies.⁸,⁹

Table 3. Contractions to 10⁻⁷ M Thromboxane A₂ Analogue in Rat Aortic Rings Without Endothelium in Each Age Group

<table>
<thead>
<tr>
<th>Group</th>
<th>5 weeks</th>
<th>10 weeks</th>
<th>20 weeks</th>
<th>30 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>0.78±0.04</td>
<td>0.90±0.04</td>
<td>0.80±0.04</td>
<td>0.80±0.01</td>
</tr>
<tr>
<td>SHR</td>
<td>0.72±0.03</td>
<td>0.72±0.03</td>
<td>0.73±0.03</td>
<td>0.78±0.07</td>
</tr>
</tbody>
</table>

Results (g) are expressed as mean±SEM. n=5. There is no age-dependent change in each strain. NS, statistically nonsignificant.

*p<0.05 between Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) at 10 weeks.
These observations suggest that the increase in EDCF in TXA2/PGH2 receptor antagonist, which inhibits the contraction to the EDCF (PGH2) as well as ONO-3708. In addition, the endothelium-dependent relaxations are reported to be normalized by antihypertensive treatments. Moreover, the blood pressure of SHR was not reduced by oral administration of ONO-8809, a novel TXA2/PGH2 receptor antagonist, which inhibits the contraction to the EDCF (PGH2) as well as ONO-3708. These observations suggest that the increase in EDCF in SHR is more likely to be a result than a cause of hypertension. In considering the close relation between the endothelium-dependent contraction and blood pressure, it is speculated that some genetic factor may promote the increase of both EDCF and blood pressure simultaneously. In any case, increased production of EDCF may be a deteriorating factor in increasing blood pressure.

Recently, endothelium-dependent relaxations to acetylcholine were reported to be reduced in the arteries of patients with essential hypertension. Also, the reduction in the endothelium-dependent relaxations in resistant arteries was suggested to act as a deteriorating factor in hypertension by producing injuries in various organs. The possible involvement of increased EDCF in conditions characterized by a reduction of such endothelium-dependent relaxations needs further clarification.

In conclusion, the degree of acetylcholine-induced endothelium-dependent relaxations in aortic rings was similar in SHR and WKY rats at 5–30 weeks of age, but the production of acetylcholine-induced EDCF, which is most likely PGH2, was consistently more enhanced at younger ages in SHR than in WKY rats. These findings suggest a close relation between increased EDCF production and the increase in blood pressure in young to adult rats.

Acknowledgment
We thank Keiko Sakai for her secretarial assistance.

References

Table 4. Changes in Systolic Blood Pressure of Rats After Oral Administration of ONO-8809

<table>
<thead>
<tr>
<th>Dose of agent</th>
<th>n</th>
<th>5 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONO-8809</td>
<td></td>
<td>110±1.3</td>
<td>149±3.7</td>
</tr>
<tr>
<td>(10 μg/body/day)</td>
<td>7</td>
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<td></td>
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<tr>
<td>ONO-8809</td>
<td></td>
<td>107±3.3</td>
<td>143±3.8</td>
</tr>
<tr>
<td>(30 μg/body/day)</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>107±2.5</td>
<td>144±2.8</td>
</tr>
<tr>
<td>WKY</td>
<td></td>
<td>97±1.6</td>
<td>109±2.2</td>
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<td>ONO-8809</td>
<td></td>
<td>98±4.0</td>
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<td>(10 μg/body/day)</td>
<td>6</td>
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<tr>
<td>ONO-8809</td>
<td></td>
<td>98±2.6</td>
<td>106±4.2</td>
</tr>
<tr>
<td>(30 μg/body/day)</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Five-week-old spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were orally administered ONO-8809 once a day for 3 weeks. Results (mm Hg) are expressed as mean±SEM. n, Number of rats.


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