Prostaglandin H₂ May Be the Endothelium-Derived Contracting Factor Released by Acetylcholine in the Aorta of the Rat

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The present experiment was performed to identify endothelium-derived contracting factor produced by acetylcholine stimulation in the aorta of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats. The rings of the thoracic aorta were obtained from age-matched SHR and WKY rats, and changes in isometric tension were recorded. The relaxant responses to acetylcholine in the aortic rings from SHR were significantly weaker than those from WKY rats. The relaxant responses to acetylcholine were significantly enhanced by pretreatment with a cyclooxygenase inhibitor (indomethacin) or thromboxane A₂/prostaglandin H₂ receptor antagonist (ONO-3708) in aortic rings from both SHR and WKY rats. A thromboxane A₂ synthetase inhibitor (OKY-046) did not affect the acetylcholine-induced relaxation in the aortic rings from SHR or WKY rats. In the organ bath solution, after acetylcholine stimulation, prostaglandin E₂ and 6-keto-prostaglandin F₁α concentrations increased but not prostaglandin F₂α and thromboxane B₂ concentrations. Exogenous prostaglandin H₂, a stable analogue of thromboxane A₂, and prostaglandin F₂α induced contractions of the SHR rings at a lower concentration than prostaglandin E₂, prostaglandin D₂, and prostaglandin I₂. These contractile responses to various prostaglandins were markedly inhibited by pretreatment with ONO-3708. A prostacyclin synthetase inhibitor did not affect the relaxant responses to acetylcholine in the SHR rings. These results show that endothelium-derived contracting factor is produced and released by acetylcholine stimulation not only in the aorta of SHR but also in those of WKY rats and suggest that prostaglandin H₂, a precursor of the released prostaglandins, is a strong candidate for endothelium-derived contracting factor produced by acetylcholine stimulation. (Hypertension 1990;15:475–481)
Recently, endothelin was identified as an EDCF. This substance is the most potent mammalian vasoconstrictor peptide and has characteristic constricting effects. The endothelin-induced contraction is resistant to cyclooxygenase inhibitors, lipoxygenase inhibitors, and α-adrenergic, histaminergic, and serotoninergic antagonists.\(^{15}\) In the aorta of spontaneously hypertensive rats (SHR), acetylcholine causes the simultaneous release of EDRF and an EDCF that differs from endothelin, which is a peptide, and is considered to be a cytooxygenase product.\(^{16}\) However, the details remain obscure. We performed the present study to identify the EDCF, which is produced by acetylcholine stimulation in the SHR aorta.

### Methods

The thoracic aorta from male SHR and normotensive Wistar-Kyoto (WKY) rats matched for age (30–32 weeks) and body weight (330–400 g) was used. Blood pressure measured by the tail-cuff method in the unanesthetized state was 191.9 ± 3.4 mm Hg in SHR (n = 8) and 140.6 ± 1.5 mm Hg in WKY rats (n = 8).

The rats were decapitated, and the thorax was opened. The thoracic aorta was immediately removed and placed in cold Krebs-Henseleit solution with the following composition (mM): NaCl 118, KCl 4.7, CaCl\(_2\) 2.55, MgSO\(_4\) 1.18, KH\(_2\)PO\(_4\) 1.18, NaHCO\(_3\) 24.88, glucose 11.1, and Ca-2Na EDTA 0.026. The fat and connective tissues on the vascular surface were removed, and the vessel was cut into rings (5 mm in length) with scissors. After two stainless steel wires were inserted into the vascular lumen, the rings were suspended in an organ chamber, which contained 30 ml buffer solution. The rates of relaxation were expressed as percentages to the contraction induced by 10\(^{-7}\) M norepinephrine.

All the following experiments were done in the aortic rings from SHR alone.

### Concentrations of Prostaglandins in the Bath Solution

In the SHR rings, changes in the concentrations of various prostaglandins (PGs) in the bath solution before and after addition of acetylcholine were evaluated. Immediately before the induction of contractions by norepinephrine, at the peak response to 10\(^{-7}\) M norepinephrine and at the peak response to 10\(^{-5}\) M acetylcholine after cumulative addition of 10\(^{-8}\) to 10\(^{-5}\) M acetylcholine, the organ bath solution (1 ml) was replaced at 30-minute intervals. The resting tension was adjusted to 1.0 g. The intimal surface was removed by gentle rubbing with a swab inserted into the vascular lumen. Removal of the intima was confirmed by the absence of responses to 10\(^{-7}\) M acetylcholine.

### Responses to Norepinephrine

Norepinephrine was cumulatively added to the bath solution for the aortic rings from SHR or WKY rats at concentrations of 10\(^{-8}\) to 10\(^{-3}\) M, and changes in tension were evaluated. In the rings from SHR, the contraction induced by 10\(^{-7}\) M norepinephrine was 77.2 ± 3.2% (n = 6) of the maximum contraction (10\(^{-6}\) M norepinephrine). In the rings from WKY rats, the contraction induced by 10\(^{-7}\) M norepinephrine was 75.7 ± 2.0% (n = 6) of the maximum contraction (10\(^{-6}\) M norepinephrine). Because there was no significant difference between these values, the rings from both SHR and WKY rats were contracted with 10\(^{-7}\) M norepinephrine.

### Responses to Acetylcholine and Effects of Inhibitors and an Antagonist

The aortic rings with and without endothelium from SHR and WKY rats were contracted with 10\(^{-7}\) M norepinephrine. After the contraction reached a plateau, acetylcholine was added at cumulative concentrations of 10\(^{-8}\) to 10\(^{-5}\) M to relax the rings. Fifteen minutes before the induction of contractions by norepinephrine, indomethacin,\(^{17}\) which is a cyclooxygenase inhibitor (10\(^{-5}\) M), OKY-046,\(^{18}\) which is a TXA\(_2\) synthetase inhibitor (10\(^{-5}\) M), and ONO-3708,\(^{19}\) which is a TXA\(_2\)/prostaglandin H\(_2\) (PGH\(_2\)) receptor antagonist (10\(^{-6}\) M) were each added separately to the bath solution, and each effect on relaxant responses to acetylcholine was evaluated. The rates of relaxation were expressed as percentages to the contraction induced by 10\(^{-7}\) M norepinephrine.

### Responses to Exogenous Prostaglandins

PGH\(_2\), PGF\(_{2\alpha}\), and TXB\(_2\) were added at cumulative concentrations of 10\(^{-9}\) to 10\(^{-6}\) M and PGF\(_{2\alpha}\) was added at cumulative concentrations of 10\(^{-9}\) to 10\(^{-5}\) M to the bath solution for the SHR aortic rings with and without endothelium at the basal state and at the precontracted (by norepinephrine 10\(^{-7}\) M) state, and vascular responses and the effects of ONO-3708 (10\(^{-6}\) M) on these responses were evaluated. The rate of contraction was expressed as percentages to the contraction induced by 10\(^{-7}\) M norepinephrine. The tests were performed at 40-minute intervals with replacement of the buffer every 20 minutes.

### Effects of a Prostacyclin Synthetase Inhibitor

Tranylcypromine,\(^{22}\) a prostacyclin synthetase inhibitor, was added to the bath solution at a concentration of 10\(^{-4}\) M 15 minutes before the induction of contraction by norepinephrine, and its effects on relaxant responses to acetylcholine in the rings from the SHR were evaluated.
Drugs

The following drugs from Sigma Chemical Co. (St. Louis, Missouri) were used: l-norepinephrine bitartrate, acetylcholine chloride, indomethacin, transeptone, sodium nitroprusside, and PGI2. Ono Pharmaceutical Co. (Osaka, Japan) provided the PGE2, PGE2, PGD2, PGE2, 9,11-epithio-11,12-methano-TxA2 (STA2), (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate (OKY-046) (a TXA2 synthetase inhibitor), and (9,11),(11,12)-dideoxa-9α,11α-dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19,20-pentanor-15-epi-TxA2 (ONO-3708) (aTXA2 antagonist). Indomethacin was dissolved in distilled water containing 3×10−2 M Na2CO3. PGI2, PGF2α, PGE2, PGD2, PGH2, and STA2 were dissolved in ethanol. The final ethanol concentration in the bath solution was 0.1% or less. The other drugs were dissolved in distilled water. Vehicle did not affect the acetylcholine-induced relaxations or the resting tension. Results were expressed as mean±SEM. For statistical analysis, Student’s t test for paired or unpaired observations and the Wilcoxon test were used. Values of p<0.05 were considered to be significant.

Results

Responses to Acetylcholine

In the aortic rings from both SHR and WKY rats, the maximum relaxation was observed with 10−7 M acetylcholine. At higher concentrations, acetylcholine induced contractions (Figure 1A, Figure 2). In the rings without endothelium from SHR and WKY rats, responses to acetylcholine were negligible (Figure 1B, Figure 2). The acetylcholine-induced relaxation in rings from the SHR was significantly weaker than that in rings from the WKY rats at acetylcholine concentrations of 10−7 to 10−5 M, and contractile responses observed at high acetylcholine concentrations disappeared (Figure 3). Similarly, in rings from the WKY rats relaxant responses were significantly enhanced at acetylcholine concentrations of 10−6 to 10−5 M, resulting in similar responses in rings from both SHR and WKY rats (Figure 3).

OKY-046 (10−5 M) did not affect the relaxant responses to acetylcholine in rings from the SHR or WKY rats (Figure 4).

To determine whether TXA2 is involved, the effects of ONO-3708 (10−6 M) were evaluated. In aortic rings from the SHR, relaxant responses were enhanced by pretreatment with ONO-3708 at acetylcholine concentrations of 10−7 to 10−5 M, and contractile responses observed at high acetylcholine concentrations disappeared (Figures 5 and 6). Similarly,
FIGURE 3. Line graph showing effects of indomethacin (10^{-5} M) on acetylcholine-induced responses in spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat aortic rings with endothelium. Rate of relaxation was expressed as percentages to contraction induced by 10^{-7} M norepinephrine. Results are shown as mean±SEM. **p<0.01 between the presence and absence of indomethacin.

Indomethacin, OKY-046, and ONO-3708 did not significantly affect the contractions induced by 10^{-7} M norepinephrine in the rings from SHR and WKY rats. ONO-3708 (10^{-6} M) did not significantly affect the relaxant responses to sodium nitroprusside in the rings from SHR and WKY rats (data not shown).

Concentrations of Prostaglandins in the Bath Solution

In rings from the SHR, the concentrations of PGE_2 and 6-keto-PGF_1α increased about threefold to fourfold after acetylcholine stimulation. The concentration of PGE_2 in the bath solution after acetylcholine stimulation was about 10^{-10} M, and that of 6-keto-PGF_1α was about 10^{-9} M. The concentrations of PGF_2α and TXB_2 did not change significantly (Figure 7).

Responses to Exogenous Prostaglandins

In the SHR aortic rings with endothelium, PGE_2, STA_2, PGH_2, PGE_2, and PGI_1 induced only contractions at the basal state. STA_2, PGE_2, and PGH_2 induced contractions at 10^{-1} M or more; on the other hand, PGE_2 and PGD_2 induced contractions only at a high concentration of 10^{-6} M and PGI_2 at 10^{-5} M (Figure 8). ONO-3708 at a concentration of 10^{-6} M inhibited all contractile responses to the various PGs (Figure 8). In the SHR aortic rings without endothelium, results similar to those in the SHR aortic rings with endothelium were obtained (data not shown).

In the SHR aortic rings with and without endothelium, PGE_2, STA_2, PGH_2, PGE_2, and PGD_2 also induced only contractions at the precontracted state. Results similar to those at the basal state were obtained, although the percentage of contraction was reduced (data not shown).

Effects of a Prostacyclin Synthetase Inhibitor

The contractions in the rings from SHR induced by PGI_2 at high concentrations presented the possibility that PGI_2 is EDCF. Therefore, the effects of tranylcypromine (10^{-4} M) on relaxant responses to acetylcholine in rings from the SHR were evaluated. Because tranylcypromine reduced the norepinephrine-induced contractions, the rings were contracted with 10^{-6} M norepinephrine to obtain responses comparable with those in the control group. Tranylcypromine had no significant effect on acetylcholine-induced relaxation in rings from the SHR (Figure 9).

PGI_2 is known to have potent vasodilating effects. In the SHR aortic rings with and without endothelium at the basal state, contractions were induced with 10^{-5} M PGI_2, but there were no significant changes at 10^{-9} to 10^{-6} M. At the precontracted state, negligible or only slight relaxations were observed at PGI_2 concentrations of 10^{-9} to 10^{-6} M. Contractions were induced at 10^{-5} M (data not shown).

Discussion

Acetylcholine-induced endothelium-dependent relaxations have been reported to be weaker in the
aorta of SHR than in those of WKY rats. These weaker relaxations in the SHR aorta have been suggested to be due to EDCF produced and released by acetylcholine stimulation in the SHR aorta. The present study confirmed significantly weaker relaxant responses to acetylcholine at concentrations of $10^{-7}$ to $10^{-5}$ M in the SHR aortic rings than in the WKY rat aortic rings. Indomethacin and ONO-3708 enhanced the relaxations at acetylcholine concentrations of $10^{-7}$ to $10^{-5}$ M in the SHR rings and at $10^{-6}$ to $10^{-5}$ M in the WKY rat aortic rings, resulting in similar responses in the SHR and WKY rat aortic rings. ONO-3708 did not affect the relaxations to sodium nitroprusside and the contractions induced by norepinephrine. Therefore, the relaxant responses to acetylcholine would not be enhanced by the direct effect of ONO-3708 on the vascular smooth muscle. These results suggest that a substance that is inhibited by indomethacin and ONO-3708 is produced and released by acetylcholine stimulation in the endothelium. This substance (EDCF) produced and released simultaneously with EDRF seems to weaken acetylcholine-induced relaxations. EDCF has been considered to be present only in SHR. However, our results suggest that EDCF is also produced and released by acetylcholine stimulation in the WKY rat aorta. Endothelium-dependent relaxations have been reduced in vascular smooth muscle of the rat with increasing age. The existence of EDCF in WKY rats suggests that EDCF may participate in the reduced endothelium-dependent relaxations with increasing age. Therefore, age and blood pressure may promote endothelium-dependent contractions in the aorta of the rat. Whether the difference in response between the SHR and WKY rat aortic rings is caused by a difference in the amount of EDCF produced by acetylcholine stimulation or that in the sensitivity of the smooth muscle to EDCF remains to be clarified. Reports have suggested a substance in the cyclooxygenase system to be the EDCF produced and released by acetylcholine stimulation in the endothelium. Solution was obtained and analyzed by radioimmunoassay immediately before induction of contractions by norepinephrine (NE) (control), when contraction induced by $10^{-7}$ M NE was stabilized, and when response induced by $10^{-4}$ M acetylcholine (ACh) reached a peak after cumulative ACh addition at $10^{-9}$ to $10^{-3}$ M. Results are expressed as mean±SEM. Between concentration before ACh addition and that after ACh addition, TX, thromboxane.
released from the rat aorta. This was confirmed by the present study. To identify EDCF, the possibility of TXA2 involvement was evaluated at first. This EDCF was not inhibited by OKY-046, a TXA2 synthetase inhibitor, but was inhibited by ONO-3708, a TXA2/PGH2 receptor antagonist. ONO-3708, which inhibits the actions of TXA2 and PGH2, also inhibited contractions induced by PGF2α, PGE2, PGD2, and PGI2. This result shows that ONO-3708 is not a selective antagonist to TXA2 and PGH2. Consequently, EDCF seems to be a cyclooxygenase product or products other than TXA2 as reported previously.

Next, the concentrations of various PGs in the organ bath solution were determined. After acetylcholine stimulation, the concentrations of PGE2 and 6-keto-PGF1α increased about threefold to fourfold. The concentration of PGE2 in the bath solution after acetylcholine stimulation was about 10^{-10} M and that of 6-keto-PGF1α (i.e., PGI2) was about 10^{-9} M. Lüscher et al. have obtained similar results. The effects of various exogenous PGs on the blood vessel were then evaluated. PGF2α, STA2, PGH2, PGE2, PGD2, and PGI2 caused contractions. PGE2 and PGD2 induced contractions only at a high concentration of 10^{-6} M and PGI2 at 10^{-5} M. The concentrations of PGE2 and PGI2 measured in the bath solution were very low, and the concentrations that induced contractions were about 10^2 times higher. Although the degree of transfer of a substance produced in the tissue to the solution is not known, the local concentrations of the PGs released from the endothelium within the blood vessel wall would be much higher than those measured in the organ chamber. Tranylcypromine, a prostacyclin synthetase inhibitor of acetylcholine-induced relaxations, had no effect, which is consistent with the result of the previous report, which suggests that PGI2 is not increased in the tissue to the degree that induces vascular contractions. Therefore, there is only a slight possibility that PGI2 or PGE2 is EDCF. In addition, there was no increase in the concentrations of PGF2α and TXB2 in the solution excludes the possibility that PGF2α or TXA2 is EDCF. These results suggest a cyclooxygenase product or products other than TXA2, PGE2, PGI2, or PGF2α as the EDCF produced and released by acetylcholine stimulation in the SHR and WKY rat aorta.

PGI2 is produced in endothelial cells and has potent vasodilating effects. When administered exogenously, it is known to induce biphasic responses in some types of blood vessels: relaxation is observed at low concentrations and contraction at high concentrations. After acetylcholine stimulation, the concentration of PGI2 in the solution increased. The degree of the involvement of this increased PGI2 in relaxations in the rat aorta was evaluated. The aortic rings at the basal state showed no changes in tension at PGI2 concentrations of 10^{-9} to 10^{-6} M. Contractions were induced at 10^{-5} M. The rings contracted with 10^{-7} M norepinephrine showed neg-
ligible change or only slight relaxation at PGI₂ concentrations of 10⁻¹⁰ to 10⁻⁶ M. At 10⁻⁵ M, contractions were induced. Therefore, PGI₂ increased by acetylcholine stimulation is not likely to be involved in not only contractions but also relaxations in the rat aorta.

In the present experiment, we could not identify EDCF produced by acetylcholine stimulation in the rat aorta but found that: 1) There is a very slight possibility that the final product in the cyclooxygenase system is an EDCF. 2) Exogenous PGH₂ induced contractions at 10⁻⁷ M or more. 3) The contractions induced by PGH₂ were inhibited by ONO-3708. 4) The concentrations of PGI₂ and PGE₂ increased after acetylcholine stimulation in the organ bath solution. The concentration of PGI₂ in the bath solution after acetylcholine stimulation was about 10⁻⁹ M and that of PGE₂ was about 10⁻¹⁰ M. 5) The volume of the aortic ring was about 1/10³ to 1/10⁴ of that of the organ bath solution. Taking the concentrations of PGs measured in the organ bath solution and a volume ratio of the vascular tissue to the organ bath solution into consideration, the concentration of PGH₂, a precursor of the released PGs, would be at least 10⁻⁵ M or more in the vascular tissue. Therefore, it seems that the concentration of PGH₂ produced in the tissue is sufficient to induce vascular contractions. These observations suggest that PGH₂ is a strong candidate for EDCF produced by acetylcholine stimulation in the rat aorta.

Acknowledgment

We thank Miss Keiko Sakai for secretarial assistance.

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Key Words: endothelium • acetylcholine • prostaglandin synthase • aorta • spontaneously hypertensive rats • Wistar-Kyoto rats
Prostaglandin H2 may be the endothelium-derived contracting factor released by acetylcholine in the aorta of the rat.
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Hypertension. 1990;15:475-481
doi: 10.1161/01.HYP.15.5.475

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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