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 F2, 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)

All blood vessels are lined by the endothelium, and the important role of an organic or functional abnormality of endothelial cells in the pathogenesis and pathophysiology of various diseases has attracted attention. In 1980, Furchgott et al1 found that acetylcholine induced endothelium-dependent relaxation in the rabbit aorta. Since then, various substances have been reported to induce endothelium-dependent relaxation in most of the blood vessels in mammals.2,3 Several pharmacological observations have strongly suggested that there is more than one endothelium-derived relaxing factor (EDRF)4,5 and that nitric oxide is one of these EDRFs.6,7 After the discovery of EDRF, studies by Vanhoutte and coworkers8-11 have shown that endothelial cells produce not only EDRF but also endothelium-derived contracting factors (EDCF) after various stimulations. Several substances have been suggested as EDCFs. In isolated rabbit intrapulmonary arteries, acetylcholine-induced endothelium-dependent vasoconstriction is caused by thromboxane A2 (TXA2)-mediated contraction.12 In canine basilar arteries, calcium ionophore A23187, arachidonic acid, and acetylcholine caused endothelium-dependent contractions. In the case of A23187 and arachidonic acid, TXA2 contributes to the endothelium-dependent contractions.13 A recent publication has suggested that one of the EDCF may be the superoxide anion.14
Responses to Norepinephrine

Contraction induced by 10⁻⁷ M norepinephrine was bath solution for the aortic rings from SHR or WKY rats at concentrations of 10⁻⁸ to 10⁻⁵ M, and changes in tension were evaluated. In the rings from SHR, 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 TXA₂ synthetase inhibitor (10⁻⁵ M), and ONO-3708,¹⁹ which is a TXA₂/prostaglandin H₂ (PGH₂) 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.

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 10⁻⁴ M acetylcholine after cumulative addition of 10⁻⁵ to 10⁻³ M acetylcholine, the organ bath solution (1 ml) was obtained, and the concentrations of PGE₂, 6-keto-PGF₁α, and TXB₂ using a radioimmunoassay (RIA) kit prepared by New England Nuclear (Boston, Massachusetts) and PGF₂α, using an RIA kit prepared by Clinical Assays (Cambridge, Massachusetts) were determined by the RIA method of Jaffe et al.²⁰ and Powell.²¹

Responses to Exogenous Prostaglandins

PGE₃, PGF₂α, a stable analogue of TXA₂ (STA₂),¹⁹ PGE₂, and PGD₂ were added at cumulative concentrations of 10⁻⁹ to 10⁻⁶ M and PGF₁α at 10⁻⁴ to 10⁻² 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 (10⁻⁴ 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.
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Figure 1. Typical records of acetylcholine (ACh)-induced responses in spontaneously hypertensive rat aortic rings with (panel A) and without (panel B) endothelium. Rings were contracted with 10^-7 M norepinephrine (NE), and ACh was cumulatively added at 10^-8 to 10^-3 M. Wo, wash out.

Drugs
The following drugs from Sigma Chemical Co. (St. Louis, Missouri) were used: l-norepinephrine bitartrate, acetylcholine chloride, indomethacin, tranylcypromine, sodium nitroprusside, and PGI2. Ono Pharmaceutical Company (Osaka, Japan) provided the PGF2α, PGE2, PGD2, PGH2, 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-pentano-15-epi-TXA2 (ONO-3708) (a TXA2/PGI2 antagonist). Indomethacin was dissolved in distilled water containing 3 x 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,
in the rings from the WKY rats relaxant responses were enhanced at acetylcholine concentrations of $10^{-6}$ to $10^{-5}$ M, resulting in relaxation similar in degree to that of rings from SHR and WKY rats (Figure 6). The degree of the enhancement of relaxation was comparable with that after pretreatment with 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$_{1a}$ 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$_{1a}$ was about $10^{-9}$ M. The concentrations of PGF$_{2a}$ and TXB$_2$ did not change significantly (Figure 7).

Responses to Exogenous Prostaglandins

In the SHR aortic rings with endothelium, PGF$_{2a}$, STA$_2$, PGH$_2$, PGE$_2$, PGD$_2$, and PGI$_2$ induced only contractions at the basal state. STA$_2$, PGF$_{2a}$, and PGH$_2$ induced contractions at $10^{-7}$ 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 at the basal state, 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, PGF$_{2a}$, 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 $10^{-4}$ M (data not shown).

Discussion

Acetylcholine-induced endothelium-dependent relaxations have been reported to be weaker in the
Figure 5. Representative record of effects of ONO-3708 (10^{-6} M) on acetylcholine (ACh)-induced responses in spontaneously hypertensive rat rings with endothelium. NE, norepinephrine; Wo, wash out.

Figure 6. Line graph showing effects of ONO-3708 (10^{-6} 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 ONO-3708.

Figure 7. Bar graphs showing changes in concentrations of various prostaglandins (PG) in organ bath solution in spontaneously hypertensive rat (SHR) aortic rings. 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^{-5} M acetylcholine (ACh) reached a peak after cumulative ACh addition at 10^{-4} to 10^{-3} M. Results are expressed as mean±SEM. **p<0.01 between concentration before ACh addition and that after ACh addition. TX, thromboxane.
referred to as endothelium-derived contracting factor (EDCF). On the other hand, TXA_{2} and PGH_{2} are known to be involved in the control of vascular tone. The present study was conducted to identify EDCF released from the rat aorta. This was confirmed by the present study. To identify EDCF, the possibility of TXA_{2} involvement was evaluated at first. This EDCF was not inhibited by OKY-046, a TXA_{2} synthetase inhibitor, but was inhibited by ONO-3708, a TXA_{2}/PGH_{2} receptor antagonist. ONO-3708, which inhibits the actions of TXA_{2} and PGH_{2}, also inhibited contractions induced by PGF_{2α}, PGE_{2}, PGD_{2}, and PGI_{2}. This result shows that ONO-3708 is not a selective antagonist to TXA_{2} and PGH_{2}. Consequently, EDCF seems to be a cyclooxygenase product or products other than TXA_{2} as reported previously.

Next, the concentrations of various PGs in the organ bath solution were determined. After acetylcholine stimulation, the concentrations of PGE_{2} and 6-keto-PGF_{1α} increased about threefold to fourfold. The concentration of PGE_{2} in the bath solution after acetylcholine stimulation was about 10^{-10} M and that of 6-keto-PGF_{1α} (i.e., PGI_{2}) 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. PGF_{2α}, STA_{2}, PGH_{2}, PGE_{2}, PGD_{2}, and PGI_{2} caused contractions. PGE_{2} and PGD_{2} induced contractions only at a high concentration of 10^{-6} M and PGI_{2} at 10^{-5} M. The concentrations of PGE_{2} and PGI_{2} measured in the bath solution were very low, and the concentrations that induced contractions were about 10^{4} 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 PGI_{2} is not increased in the tissue to the degree that induces vascular contractions. Therefore, there is only a slight possibility that PGI_{2} or PGE_{2} is EDCF. In addition, that there was no increase in the concentrations of PGF_{2α} and TXB_{2} in the solution excludes the possibility that PGF_{2α} or TXA_{2} is EDCF. These results suggest a cyclooxygenase product or products other than TXA_{2}, PGE_{2}, PGI_{2}, or PGF_{2α} as the EDCF produced and released by acetylcholine stimulation in the SHR and WKY rat aorta.

PGE_{2} 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 PGI_{2} in the solution increased. The degree of the involvement of this increased PGI_{2} in relaxations in the rat aorta was evaluated. The aortic rings at the basal state showed no changes in tension at PGI_{2} 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 PG\(_I_2\) concentrations of 10\(^{-10}\) to 10\(^{-6}\) M. At 10\(^{-5}\) M, contractions were induced. Therefore, PG\(_I_2\) 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 PGH\(_2\) induced contractions at 10\(^{-7}\) M or more. 3) The contractions induced by PGH\(_2\) were inhibited by ONO-3708. 4) The concentrations of PG\(_I_2\) and PGE\(_2\) increased after acetylcholine stimulation in the organ bath solution. The concentration of PG\(_I_2\) in the bath solution after acetylcholine stimulation was about 10\(^{-9}\) M and that of PGE\(_2\) was about 10\(^{-10}\) M. 5) The volume of the aortic ring was about 1/10\(^3\) to 1/10\(^4\) 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 PGH\(_2\), 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 PGH\(_2\) produced in the tissue is sufficient to induce vascular contractions. These observations suggest that PGH\(_2\) is a strong candidate for EDCF produced by acetylcholine stimulation in the rat aorta.

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