Role of Endothelium in the Contractions Induced by Norepinephrine and Clonidine in Rat Aorta

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Abstract—The inhibitory effects of endothelium-derived relaxing factor (EDRF) on the contractions induced by norepinephrine and clonidine in rat aorta were examined. Carbachol induced a relaxation of norepinephrine-induced contraction in rat aorta with endothelium. Removal of endothelium inhibited the carbachol-induced relaxation and increased the magnitude of norepinephrine-induced contraction. Quinacrine, a phospholipase A2 inhibitor, methylene blue, a guanylate cyclase inhibitor and tetraethylammonium, a potassium permeability inhibitor, inhibited carbachol-induced relaxation and augmented the magnitude of norepinephrine-induced contraction only when endothelium was present. Clonidine induced a contraction when endothelium was removed or muscle was treated with methylene blue. The contractions induced by norepinephrine and clonidine were equally sensitive to prazosin and equally less sensitive to yohimbine. Clonidine inhibited the norepinephrine-induced contraction, whereas it potentiated the angiotensin II- or 12 mM K-induced contractions in the aorta with endothelium. The inhibitory effect of clonidine on the norepinephrine-induced contraction was reduced by endothelium-removal and by methylene blue but not by yohimbine. These results suggest that norepinephrine has a strong direct stimulating action and clonidine has a weak one on vascular smooth muscle cells possibly mediated by \(\alpha_1\)-adrenoceptors, and their contractile effects are inhibited by the spontaneously released EDRF.

In rabbit aorta, Furchgott and Zawadzki (1) reported that relaxation induced by acetylcholine is due to release of endothelium-derived relaxing factor (EDRF). They suggested that acetylcholine induces Ca influx into endothelial cells and the cellular Ca activates phospholipase A2, releases arachidonic acid from cell membrane, and EDRF is produced by lipoxygenase therefrom. Relaxation of vascular smooth muscle induced by histamine (2), 5-hydroxytryptamine (3), bradykinin (4), adenosine (5) and nucleotides (6) seems to be also due to the same mechanism. In most of the vascular smooth muscles, norepinephrine is a potent stimulant which induces contraction mainly by increasing Ca influx (7, 8). If norepinephrine can also increase Ca influx in endothelial cells, EDRF would be released. However, there are conflicting reports on the role of endothelium on norepinephrine-induced contraction in rat aorta. Fortes et al. (9) and Eglème et al. (10) reported that removal of endothelium increased maximum tension level and decreased EC50 (concentration to induce half maximum contraction) for norepinephrine, whereas Allan et al. (11) reported an increase in EC50 with no change in maximum response. Recently, Eglème et al. (10) have reported that removal of endothelium from aorta enhanced the contractile response to clonidine and suggested that clonidine activates \(\alpha_2\)-adrenoceptors in endothelium which results in an inhibition of contraction due to \(\alpha_2\)-adrenoceptors on smooth muscle. In the present experiments, we examined in rat aorta 1) the role of endothelium in the contractions induced by
norepinephrine and clonidine, 2) the effects of several inhibitors of EDRF on these contractions and 3) the effects of a selective inhibitor of α2-adrenoceptor, yohimbine, in order to know if norepinephrine and clonidine release EDRF and if these effects are mediated by α2-adrenoceptors on the endothelium. Some of the experiments were repeated in rabbit aorta. A part of this work has been presented at the Japanese Pharmacological Society Meeting (12).

Materials and Methods
Preparations: Male Wistar rats, weighing about 200 g, were stunned and bled. The thoracic aorta was dissected out and strips, 2–3 mm wide and 5–8 mm long, were prepared. Male New Zealand rabbits, weighing 2.0–2.5 kg, were killed by a rapid injection of sodium pentobarbital (25 mg/kg) and air into an ear vein. The thoracic aorta was rapidly removed and cut into helical strips, and muscle strips, 3–4 mm wide and 4–8 mm long, were prepared. Great care was taken not to injure the endothelium. In some experiments, the endothelium was removed by gently rubbing the intimal surface with a finger moistened with physiological solution (1, 2, 6, 10, 11, 13–16).

Solutions: The normal bathing solution (pH 7.4, 37°C) contained 136.9 mM NaCl, 2.7 mM KCl, 1.5 mM CaCl2, 1.0 mM MgCl2, 23.8 mM NaHCO3 and 5.5 mM glucose. High K solution was made by hyperosmotically adding 12 mM or 60 mM KCl to the above solution.

Tension recordings: Each strip was attached to a holder under a resting tension of 1 g and equilibrated for 60 min in the bathing solution aerated with a 95% O2 and 5% CO2 mixture. The contractile tension of the muscle strip was recorded isometrically with a force-displacement transducer connected to a Nihon Kohden polygraph. High K-induced contraction was used for the standard because this contraction was not affected by the removal of the endothelium (see Results). Every preparation was checked to determine if carbachol induces a transient relaxation of norepinephrine-induced contraction in order to know the functional integrity of the endothelium. Results were expressed as the mean±S.E. of n experiments.

Drugs: Drugs used in this experiment were l-norepinephrine bitartrate (Wako), carbamylcholine chloride (carbachol, Tokyo Kasei), quinacrine dihydrochloride (Sigma), methylene blue trihydrate (Wako), tetraethylammonium chloride (TEA, Nakarai), angiotensin II (Hypertensin, Ciba-Geigy), indomethacin (Sigma), 2-(2,6-dichloroanilino)-2-imidazoline hydrochloride (clonidine, Tokyo Kasei), yohimbine hydrochloride (Sigma) and prazosin hydrochloride (Pfizer).

Results
A. Role of endothelium in high K- and norepinephrine-induced contractions: The magnitude of the contraction induced by 60 mM K was not affected by removing endothelium in rat and rabbit aortae (Table 1). Further, 10⁻⁶ M carbachol did not relax the contraction induced by 60 mM K in rat aorta without endothelium and relaxed it only slightly (10.0±1.4%, n=6) in rat aorta with endothelium (Fig. 1). Therefore, we used the magnitude of 60 mM K-induced contraction as the reference. Addition of 10⁻⁶ M carbachol to the 10⁻⁷ M and 10⁻⁶ M norepinephrine-pretreated rat aorta with endothelium transiently relaxed the contraction by 69.2±2.4% (n=8) and 52.2±3.9% (n=10), respectively. In contrast, no relaxation was observed in rat aorta without endothelium (Fig. 1). Concentration-response curves for norepinephrine in aortae with or without endothelium are shown in Fig. 2. Maximum contractile tension induced by

| Table 1. Magnitude of high K-induced contraction in aortae with or without endothelium |
|------------------------------------------|-------------|-----------------------------|
|                                        | With endothelium | Endothelium removal |
|                                        | g tension±S.E. (n) | g tension±S.E. (n)           |
| Rat aorta                              | 0.99±0.10 (6)    | 0.99±0.06 (6)               |
| Rabbit aorta                           | 1.49±0.05 (4)    | 1.47±0.06 (4)               |
norepinephrine was enhanced (Figs. 1 and 2), and EC50 was decreased by removing endothelium in both rat and rabbit aortae (Table 2). Inhibitory effect of carbachol on norepinephrine-induced contraction did not change after repeated applications of 60 mM K, suggesting that high K does not modify the function of endothelium.

B. Effect of quinacrine, methylene blue, TEA and indomethacin: As shown in Fig. 1, 10^{-6} M quinacrine (added 10 min before and washed out on the application of norepinephrine), 10^{-5} M methylene blue (added 60 min before) and 1 mM TEA (added 10 min before) inhibited the relaxation induced by 10^{-6} M carbachol in rat aorta with endothelium. These agents also increased the magnitude of norepinephrine-induced contraction in rat aorta with endothelium: maximum contractile tension increased to 122.2% by quinacrine, to 137.7% by methylene blue and to 108.8% by TEA as shown in Table 3. However, these agents did not or only slightly (in the case of quinacrine) potentiated the norepinephrine-induced contraction in rat aorta without endothelium (Table 3). Also in rabbit aorta, methylene blue potentiated the 10^{-6} M norepinephrine-induced contraction to 127.3±1.4% (n=4). In contrast, 10^{-5} M indomethacin changed neither the norepinephrine-induced contraction nor the carbachol-induced relaxation in rat aorta with endothelium (P<0.05, n=6 each).

C. Role of endothelium on clonidine-induced contraction: Clonidine in the concentrations of 10^{-9} M to 10^{-4} M did not induce contraction in rat aorta with endothelium. However, in the aorta without endothelium, clonidine induced a contraction with an EC50 of 2.92×10^{-7} M, and the maximum contraction induced by 3×10^{-6} M clonidine averaged 71.5±3.8% (n=11) of the 60 mM K-induced contraction. In methylene blue-pretreated aorta, clonidine also induced a contraction with a magnitude similar to that in the aorta without endothelium. These contractions were not affected by carbachol.
D. Effects of yohimbine and prazosin: In rat aorta without endothelium, the sustained contraction induced by $10^{-6}$ M norepinephrine was inhibited by cumulative applications of prazosin with an IC50 (concentration needed to induce 50% inhibition) of $8.55 \times 10^{-9}$ M. Yohimbine in the concentrations of $10^{-9}$ M to $10^{-7}$ M did not show any effect on norepinephrine-induced contraction. Higher concentrations of yohimbine inhibited the norepinephrine-induced contraction with an IC50 of $1.45 \times 10^{-5}$ M.
Table 2. EC50 (concentration needed to induce half maximum contraction) for norepinephrine

<table>
<thead>
<tr>
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<th>With endothelium</th>
<th>Endothelium removal</th>
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<tbody>
<tr>
<td>Rat aorta</td>
<td>$\left( 8.70 \pm 0.15 \right) \times 10^{-9} \text{ M (4) }$</td>
<td>$\left( 6.70 \pm 0.16 \right) \times 10^{-8} \text{ M (4)}$</td>
</tr>
<tr>
<td>Rabbit aorta</td>
<td>$\left( 1.10 \pm 0.31 \right) \times 10^{-6} \text{ M (4) }$</td>
<td>$\left( 9.73 \pm 0.88 \right) \times 10^{-8} \text{ M (4) }$</td>
</tr>
</tbody>
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Values are the mean±S.E. * and †: significantly different from the control (with endothelium) with P<0.01 and P<0.05, respectively.

Table 3. Effects of quinacrine, methylene blue and TEA on 10^{-6} M norepinephrine-induced contraction in rat aorta with or without endothelium

<table>
<thead>
<tr>
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<th>With endothelum</th>
<th>Endothelium removal</th>
</tr>
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<tbody>
<tr>
<td>10^{-4} M Quinacrine</td>
<td>122.2±3.6% (5)†</td>
<td>106.2±0.4% (4)†</td>
</tr>
<tr>
<td>10^{-5} M Methylene blue</td>
<td>137.7±11.7% (4)†</td>
<td>103.3±3.2% (4)</td>
</tr>
<tr>
<td>1 mM TEA</td>
<td>108.8±1.7% (5)</td>
<td>98.6±3.8% (4)</td>
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Experiments were done as shown in Fig. 1. 10^{-6} M Norepinephrine-induced contraction before the application of each drug was taken as 100% and the relative contractile tension induced by norepinephrine in the presence of each drug is shown. Values are the mean±S.E. * and †: significantly different from the control (with endothelium) with P<0.01 and P<0.05, respectively.

Table 4. IC50 (concentration needed to induce a 50% inhibition) for yohimbine and prazosin in the contraction induced by either norepinephrine or clonidine in rat aorta

<table>
<thead>
<tr>
<th></th>
<th>Yohimbine</th>
<th>Prazosin</th>
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<tbody>
<tr>
<td>10^{-6} M Norepinephrine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelium (+)</td>
<td>(3.68±0.26)×10^{-5} M (5)</td>
<td>(6.25±0.17)×10^{-9} M (6)</td>
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<tr>
<td>Endothelium (−)</td>
<td>(1.45±0.64)×10^{-6} M (10)</td>
<td>(8.55±0.78)×10^{-9} M (4)</td>
</tr>
<tr>
<td>3×10^{-6} M Clonidine</td>
<td></td>
<td></td>
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<tr>
<td>Endothelium (+)</td>
<td>(1.83±0.68)×10^{-5} M (4)</td>
<td>(5.58±0.46)×10^{-9} M (4)</td>
</tr>
<tr>
<td>+10^{-5} M Methylene blue</td>
<td>(9.50±2.40)×10^{-6} M (5)</td>
<td>(8.00±3.37)×10^{-9} M (6)</td>
</tr>
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rat aorta with endothelium, similar results were obtained, as shown in Table 4.

The 3×10^{-6} M clonidine-induced contraction in the aorta without endothelium was inhibited by prazosin with an IC50 of 8.00×10^{-9} M and also by higher concentrations of yohimbine with an IC50 of 9.5×10^{-6} M. Similar results were obtained in the aorta treated with methylene blue (Table 4).

E. Effects of clonidine on norepinephrine- and angiotensin II-induced contractions: As shown in Fig. 3, cumulative addition of clonidine during the 10^{-7} M norepinephrine-induced contraction inhibited the muscle tension in the rat aorta with endothelium (IC50=2.05×10^{-7} M). Either removal of endothelium or pretreatment with methylene blue enhanced the norepinephrine-induced contraction and decreased the inhibitory effect of clonidine (IC50=2.26×10^{-6} M and 1.82×10^{-6} M, respectively). However, 10^{-7} M yohimbine did not change the inhibitory effect of clonidine (Fig. 3 and Table 5).

In rat aorta with endothelium, 12 mM K induced a negligible contraction and 10^{-6} M angiotensin II induced a transient contraction followed by a small sustained contraction (6.2±0.3%, n=5, of the 60 mM K-induced contraction). Addition of 3×10^{-6} M clonidine in the presence of 12 mM K or 10^{-6} M angiotensin II induced large contractions (106.2±7.8%, n=4 and 37.7±6.4%, n=5, respectively, of the 60 mM K-induced contraction) without any preceding relax-
These contractions were inhibited by $10^{-6}$ M carbachol.

**Discussion**

Furchgott (17) has proposed that EDRF released by acetylcholine is a substance produced from arachidonic acid or some other unsaturated fatty acid because quinacrine, a phospholipase A₂ inhibitor, inhibited the relaxation induced by acetylcholine. Further, EDRF seems to stimulate guanylate cyclase and to increase cyclic GMP in smooth muscle cells (13-15). It has also been reported that EDRF is released spontaneously without any stimulation and thus decreases the contractile activity of stimulants (18). In the present experiments, we found that removal of endothelium increased the magnitude of norepinephrine-induced contraction and inhibited the carbachol-induced relaxation in rat and rabbit aortae. Quinacrine, methylene blue (an inhibitor of guanylate cyclase) and TEA (an inhibitor of K permeability) inhibited the
relaxation induced by carbachol and also increased the magnitude of norepinephrine-induced contraction only in the presence of endothelium. These results indicate that the weaker contractile effect of norepinephrine in the presence of endothelium is due to EDRF.

Toda (16) has suggested that endothelium-dependent relaxation in dog renal and mesenteric arteries induced by angiotensin II and histamine might be due to endogenous prostaglandin I₂. In rat and rabbit aortae, however, this possibility is not likely because indomethacin affected neither carbachol-induced relaxation nor norepinephrine-induced contraction, suggesting the existence of species and/or tissue difference in the role of EDRF and prostaglandins in vascular smooth muscle.

The clonidine-induced contraction was greatly potentiated when endothelium was removed, as reported by Eglème et al. (10). The clonidine-induced contraction was also potentiated by methylene blue-treatment. These results suggest that the contractile effect of clonidine is inhibited by EDRF. It has been known that clonidine is an agonist of α₂-adrenoceptor and also a partial agonist of α₁-adrenoceptor. Clonidine inhibited the norepinephrine-induced contraction, as expected of a partial agonist (19). However, the result that the clonidine-induced inhibition was less in the absence of endothelium or in the methylene blue-treated aorta suggests that the clonidine-induced relaxation is not solely due to a competition at the α₁-adrenoceptor on the smooth muscle cells. These results suggest the possibility that clonidine releases EDRF from endothelium which may be mediated by the α₂-adrenoceptors on the endothelium, as suggested by Eglème et al. (10).

To confirm this possibility, we used a relatively specific inhibitor of α₂-adrenoceptor yohimbine (20), and an inhibitor of α₁-adrenoceptor, prazosin. In the rat aorta without endothelium, the contractions induced by either norepinephrine or clonidine were similarly inhibited by prazosin and by higher concentrations of yohimbine, suggesting that both norepinephrine and clonidine are acting on the same adrenoceptor possibly the α₁-subtype, on the smooth muscle cells (21). Assuming that the adrenoceptors on the endothelium are the α₂-subtype, yohimbine is expected to inhibit the release of EDRF, but not the smooth muscle contraction induced by norepinephrine or clonidine and thus potentiates the contraction. However, this was not the case. Further, yohimbine did not affect the clonidine-induced relaxation in the aorta with endothelium. From these results, it does not seem likely that norepinephrine and clonidine act on the α₂-adrenoceptors on the endothelium to release EDRF.

The fact that clonidine did not inhibit but rather potentiated the contraction induced by angiotensin II indicates that clonidine does not release EDRF. The reason why the inhibitory effect of clonidine on norepinephrine-induced contraction was weaker in the aorta without endothelium or in the methylene blue-treated aorta may be that the inhibitory effect of clonidine is partially overcome by its own contractile effect in the absence of spontaneous release of EDRF. Further, the inhibitory effect of EDRF on clonidine-induced contraction was attenuated by angiotensin II and also by the membrane depolarization with 12 mM K or TEA. Removal of endothelium depolarizes the membrane of rat aorta by about 10 mV (Nishimura, A., Takewaki, Y. and Ohashi, H., personal communication) suggesting that the inhibitory effect of EDRF is at least partially mediated by a membrane hyperpolarization.

From these results, it seems likely that the contractile effects of norepinephrine and clonidine are inhibited by the spontaneously released EDRF and that clonidine does not seem to have effects to stimulate the release of EDRF.

References
3 Imaizumi, Y., Baba, M., Imaizumi, Y. and


