ROLE OF ENDOTHELIUM IN BIPHASIC HYPOXIC RESPONSE OF
THE ISOLATED PULMONARY ARTERY IN THE RAT

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We investigated the roles of the endothelium in the hypoxic responses of the
isolated main pulmonary artery (PA) in the rat. Hypoxia was induced by gass-
ing an organ chamber with 95% N\textsubscript{2}+5% CO\textsubscript{2} (PO\textsubscript{2}=34.6±3.1 Torr) instead of 16% O\textsubscript{2}+5% CO\textsubscript{2}+balance N\textsubscript{2} (PO\textsubscript{2}=92.8±3.0 Torr). Vascular rings were
precontracted with 2×10^{-8} M phenylephrine. A transient hypoxic contraction
and a subsequent relaxation were observed in the endothelium-intact rings. The
hypoxic contraction was reduced in the endothelium-denuded rings. In con-
trast, there were no significant differences between the hypoxic relaxation in
the endothelium-intact and endothelium-denuded rings. Inhibitors of endo-
thelium-derived relaxing factor (EDRF) activity, 2×10^{-6} M N\textsuperscript{0}-monomethyl-
L-arginine (L-NMMA) and 10^{-6} M methylene blue, produced 53% and 66% reductions in hypoxic contraction, respectively. Furthermore, the amount of
cyclic GMP in the endothelium-intact PA rings which had been precontracted
with phenylephrine decreased from 2.10±0.45 pmol/mg protein during normox-
ia to 0.90±0.18 pmol/mg protein during hypoxia. Indomethacin and OKY-046
did not influence hypoxic contraction or relaxation. These results suggest that
hypoxic contraction of the isolated pulmonary artery in the rat is partially
induced by inhibition of the release of EDRF. (Jpn Circ J 1993; 58: 228—236)

Hypoxic pulmonary vasoconstriction (HPV) is a regulatory mechanism
which matches local lung perfusion with ventilation; however, its precise mechanism
is unknown. The primary site of HPV is believed to be in the precapillary arteries
(diameter<300 \textmu m).\textsuperscript{2,3} In addition, several investigators have demonstrated that hypoxic
contraction is also observed in isolated large pulmonary artery.\textsuperscript{4,5} Recent reports have
shown that endothelium-derived relaxing factor (EDRF) plays an important role in the
regulation of vascular tone\textsuperscript{6} and that it is

nitric oxide (NO) or a similar nitrogen-based radical species? In earlier studies, De Mey
and Vanhoutte\textsuperscript{8} and Holden and McCall\textsuperscript{4} investigated the role of the pulmonary endo-
thelium in sensing hypoxia using a ring preparation of the pulmonary artery. They
demonstrated that hypoxic contraction depended upon the presence of functioning
endothelium. However, it is still unknown whether hypoxic contraction is due to the
interruption of the production of EDRF or to the generation of endothelium-derived
contracting factor (EDCF). Recently, Johns et al\textsuperscript{9} and Rodman et al\textsuperscript{10} indicated that inhibitors of EDRF activity (hemoglobin,
methylene blue and hydroquinone) atten-

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(a) Hypoxia Normoxia

(b) Hypoxia Normoxia
phenylephrine 2 ×10⁻⁶ M

(c) Hypoxia Normoxia
5-HT 5 ×10⁻⁶ M

(d) Hypoxia Normoxia
KCl 20 mM

(e) phenylephrine 2 ×10⁻⁶ M

Fig. 1. Typical traces of the response to hypoxia in the endothelium-intact PA rings. Resting ring received no agonist (a). Rings were precontracted with 2 ×10⁻⁶ M phenylephrine (b). 5 ×10⁻⁶ M 5-HT (c), or 20 mM KCl (d). Hypoxic contraction was not observed in the resting rings. However, a transient hypoxic contraction and a subsequent relaxation were observed in the precontracted rings. (e) shows the time course of the phenylephrine-induced contraction.

hanced hypoxic contraction

The purpose of the present experiments was to clarify the role of the endothelium in the hypoxic response of isolated rat pulmonary artery, by evaluating the effects of a specific EDRF inhibitor, L-NMMA, and by measuring the accumulation of cyclic GMP in the pulmonary arterial rings. We also investigated the role of eicosanoids in the hypoxic contraction and relaxation responses.

MATERIALS AND METHODS

The experiments were performed on the rings of the left main pulmonary arteries from male Sprague-Dawley rats (Charles River Japan Inc. Japan), 8–12 weeks old, weighing an average of 331 g. Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The chest were opened, the hearts and lungs were removed en bloc and placed in Krebs-Henseleit solution with the following composition: NaCl 118.1, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0, and glucose 11.0. The left main pulmonary arteries were then carefully excised. After the removal of connective tissue, these vessels were cut into ring segments 5 mm long. In some rings, the endothelium was denuded by inserting a teflon tube into the lumen. Endothelium-intact vessels were also used. The rings were then mounted in water-jacketed baths containing Krebs-Henseleit solution at 37°C, gassed with a mixture of 16% O₂+5% CO₂+ balance N₂ to produce a PO₂ of 92.8±3.0 Torr and a pH of 7.3–7.4. The upper end of the ring was connected to the lever of a force displacement transducer (TB611T, TB612T, Nihon Koden, Tokyo) by a silk thread. Resting tension for the rings was maintained at 1 g throughout the experiment. Isometric contraction was recorded on an XY recorder (D72-BP, SP-H3C, Riken Denshi, Tokyo). The rings were left to equilibrate for 2 h in the bath solution before the start of the experiments. The functional status of the endothelium was ascertained by measuring the relaxant effects of acetylcholine (ACh) after precontraction with 5 ×10⁻⁷ M phenylephrine. To produce hypoxia, the gas mixture was changed to 5% CO₂+95% N₂ to create a PO₂ of 34.6±3.1

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TABLE I THE HYPOXIC RESPONSES IN THE RAT PULMONARY ARTERIAL RINGS PRECONTRACTED WITH PHENYLEPHRINE (PHE), 5-HT OR KCL

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Precontraction (%)</th>
<th>Hypoxic contraction (mg)</th>
<th>Hypoxic relaxation (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phe 2×10⁻⁸ M</strong></td>
<td>9</td>
<td>144.6±15.7 mg (39.1%)</td>
<td>87.2±10.2 mg* (23.7%)</td>
<td>−85.7±35.7 mg</td>
</tr>
<tr>
<td><strong>5-HT 5×10⁻⁶ M</strong></td>
<td>6</td>
<td>141.7±24.7 mg (38.5%)</td>
<td>108.8±7.0 mg** (35.7%)</td>
<td>−91.7±18.2 mg</td>
</tr>
<tr>
<td><strong>KCl 20 mM</strong></td>
<td>7</td>
<td>151.0±25.3 mg (35.6%)</td>
<td>42.9±6.4 mg (10.1%)</td>
<td>−119.2±24.5 mg</td>
</tr>
<tr>
<td><strong>Phe 2×10⁻⁸ M</strong></td>
<td>7</td>
<td>110.3±18.3 mg (30.0%)</td>
<td>96.5±12.6 mg (26.2%)</td>
<td>−79.0±10.8 mg</td>
</tr>
<tr>
<td>+10⁻⁵ M indomethacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phe 2×10⁻⁸ M</strong></td>
<td>7</td>
<td>152.3±30.9 mg (41.3%)</td>
<td>89.7±18.4 mg (24.5%)</td>
<td>−92.0±15.6 mg</td>
</tr>
<tr>
<td>+10⁻⁵ M OKY-046</td>
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There was no significant difference between the precontraction or hypoxic relaxation among the 3 agonists. In contrast, the hypoxic contraction in the rings which had been precontracted with phenylephrine and 5-HT was greater than that in the rings which had been precontracted with KCl (*p<0.05, **p<0.01 compared with KCl). Indomethacin (10⁻⁵ M) and OKY-046 (10⁻⁵ M) did not affect the hypoxic response. Precontraction is expressed as a percentage of the respective maximum agonist-induced contraction, respectively. Endothelium was intact. n = Number of rings. Data are given as means±SE.

Torr and a pH of 7.3–7.4. The oxygen pressure decreased from 92.8±3.0 Torr to 34.6±3.1 Torr in about 60 sec. The oxygen tension was measured by a pH/Blood Gas Analyzer 813 (Instrumentation Laboratory).

**Hypoxic response protocol**
Resting rings and rings which had been precontracted with 2×10⁻⁸ M phenylephrine, 5×10⁻⁶ M 5-hydroxytryptamine (5-HT), or 20 mM KCl were tested for hypoxic response. After a steady-state was achieved, normoxic gas was exchanged for anoxic gas and the delta tension was measured. In the rings which had been precontracted with vasoconstrictive agents, the baseline level was the point at which the contraction became steady-state. The hypoxic challenges were repeated until the responses became constant (±5% difference from the previous response). The hypoxic responses were tested in the endothelium-intact and endothelium-denuded rings, and the effects of various inhibitors were observed. We used L-NMMA (2×10⁻⁶ M, 3×10⁻⁵ M) or methylene blue (10⁻⁶ M) to inhibit EDRF activity. The dose-response for L-NMMA in this preparation was also examined. The effects of indomethacin (cyclooxygenase inhibitor, 10⁻⁵ M) and OKY-046 (thromboxane synthase inhibitor, 10⁻⁵ M) on the hypoxic response were also tested. The specimens were incubated with the inhibitors for 30 min before the addition of phenylephrine. The ACh-induced vasorelaxation was evaluated by the EC₅₀ and as a percentage of the maximal contraction caused by 5×10⁻⁷ M phenylephrine.

**Hypoxia and Cyclic GMP**
The amount of cyclic GMP in the endothelium-intact rings which had been precontracted with 2×10⁻⁸ M phenylephrine was determined during normoxia and hypoxia. The maximal contraction was observed when the rings were exposed to anoxic gas for about 60–90 seconds. At the point of the maximal contraction, the organ baths were dropped and the rings were rapidly frozen by liquid nitrogen. Control normoxic rings were frozen during normoxic gas exposure at a similar point. Cyclic GMP was extracted by homogenizing the frozen rings in 0.5 ml of iced 6% trichloroacetic acid and centrifuging the homogenate at 3000 rpm for 15 min. Extraction was then performed in triplicate with 3 ml of diethyl ether. The ether was then removed by evaporation at 70°C. Measurements were made in duplicate on 100-μl aliquots with a radioimmunoassay kit (Yamasa-shoyu, Tokyo).

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Fig.2. Typical traces of the response to hypoxia in the endothelium-intact (a) and endothelium-denuded (b) PA rings. In the endothelium-denuded ring, ACh-induced vasorelaxation and hypoxic contraction were not observed.

Reagents
Standard reagents were obtained from Sigma Chemical Company (St. Louis, MO). L-NMMA was obtained from Calbiochem Corporation (La Jolla, CA). OKY-046 was a generous gift from Ono Pharmaceutical, Tokyo, Japan. All reagents, except for indomethacin, were dissolved with distilled and deionized water. Indomethacin was dissolved in alcohol and distilled water such that the maximum bath concentration was <0.01%.

Data Analysis
Results were expressed as means±SE. Statistical analyses were performed using a one way analysis of variance (ANOVA) followed by the Dunnett test. P values 0.05 were considered to be statistically significant.

RESULTS
PA ring hypoxic response
Neither endothelium-intact nor endothelium-denuded resting PA rings which had been exposed to hypoxic conditions developed tension. In the resting rings, acetylcholine did not produce endothelium-dependent relaxation. In contrast, a transient contraction and a subsequent relaxation were observed in the endothelium-intact rings which had been precontracted with
Fig. 4. Dose-response curve for L-NMMA in the isolated rat PA. Endothelium was intact. n=8, Data are given as means ± SE.

Fig. 5. The effects of L-NMMA on the hypoxic contraction of the isolated rat PA. Open bars indicate the 2×10⁻⁸ M phenylephrine-induced contraction, and closed bars indicate the developed hypoxic contraction. Although L-NMMA enhanced the phenylephrine-induced contraction, it attenuated the hypoxic contraction (*p<0.01). The hypoxic contraction was not reduced in the rings which had been precontracted with 10⁻⁷ M phenylephrine, which had a developed tension similar to that of rings which had been precontracted with 2×10⁻⁸ M phenylephrine plus 2×10⁻⁶ M L-NMMA. Endothelium was intact. n=9, Data are given as means ± SE.

The inhibitory effects of L-NMMA were tested in the endothelium-intact rings which had been precontracted with 2×10⁻⁸ M phenylephrine. L-NMMA produced dose-dependent contractile response (Fig. 4). Therefore, we used a relatively low dose of L-NMMA, 2×10⁻⁶ M, which did not significantly affect the basal tension. This dose of L-NMMA reduced the ACh-induced vasorelaxation: EC₅₀ went from 2.0×10⁻⁸
M to $1.2 \times 10^{-7}$ M, and the maximal relaxation fell from 74% to 39% ($p < 0.01$). Although the above dose of L-NMMA enhanced the phenylephrine-induced contraction, it reduced the hypoxic contraction from 78.1 ± 11.4 mg to 41.1 ± 13.2 mg (53%, $p < 0.01$). However, the hypoxic contraction may have been reduced outwardly because the phenylephrine-induced contraction was enhanced. To exclude this possibility, the hypoxic response was tested in rings which had been precontracted with $10^{-7}$ M phenylephrine and with a developed tension similar to that of rings which had been treated with $2 \times 10^{-8}$ M phenylephrine plus $2 \times 10^{-6}$ M L-NMMA. The hypoxic contraction of the rings which had been precontracted with $10^{-7}$ M phenylephrine was not reduced (Fig. 5). We also tested the effects of a relatively high dose of L-NMMA. L-NMMA at $3 \times 10^{-5}$ M produced contraction (54.3 ± 13.1 mg, $n = 9$) in this vessel. In the presence of $3 \times 10^{-5}$ M L-

NMMA, the phenylephrine-induced contraction increased from 125.8 ± 15.7 mg to 293.8 ± 21.2 mg, and the hypoxic contraction fell from 94.2 ± 13.9 mg to 64.0 ± 10.4 mg (77.5%, $p < 0.01$). Methylene blue, $10^{-6}$ M, also reduced the hypoxic contraction from 113.0 ± 63.0 mg to 57.0 ± 47.0 mg (Fig. 6). The effects of $10^{-5}$ M indomethacin or $10^{-5}$ M OKY-046 were tested in 7 paired endothelium-intact rings. There was no significant difference between the hypoxic contraction and hypoxic relaxation in the control and the indomethacin or OKY-046-treated rings (Table I).

**Cyclic GMP and hypoxia**

Hypoxia reduced the amount of cyclic GMP in the endothelium-intact rings from 2.10 ± 0.46 pmol/mg protein to 0.90 ± 0.18 pmol/mg protein (Fig. 7).

**DISCUSSION**

The primary site of hypoxic pulmonary vasoconstriction (HPV) has been thought to be the precapillary vessels. However, some investigators have indicated that hypoxic pulmonary contraction has also been observed in isolated large pulmonary arteries. Therefore, we examined the mechanism of HPV by using isolated pulmonary arteries.
In the present study, hypoxic contraction was observed in endothelium-intact rings which had been precontracted with vasoconstrictors, but not in the unstimulated rings. Earlier reports indicated that hypoxic contraction was observed in resting rings of the cat\(^2\) and rat\(^5\); and both vessels showed a spontaneous contraction or a small amount of basal activation. In our study, ACh-induced endothelium-dependent relaxation was not observed in the resting ring. An increased basal level of smooth muscle activation may contribute to the hypoxic contraction in resting rings\(^5\).

Recent studies have shown that the endothelium plays an important role in the regulation of vascular tone\(^9\). In the PA from the pig\(^1\), rat\(^5\) and dog\(^8\), hypoxic contraction was reduced by removal of the endothelium. In the present study, the hypoxic contraction of the endothelium-denuded rings was also reduced markedly. These data suggest that the endothelium might play an important role in hypoxic contraction. An earlier report indicated that endothelium-dependent relaxation caused by ACh was eliminated under anoxic conditions\(^15\). This finding suggested that either the release of EDRF was reduced or the activity of EDRF was attenuated under hypoxic conditions and that these mechanisms were related to HPV.

In our study, we tested the effects of chemical EDRF (NO) inhibitors on the response to hypoxia. L-NMMA (a specific inhibitor of the production of NO) and methylene blue (a soluble guanylate cyclase inhibitor) were used. These inhibitors caused a dose-dependent contraction in the isolated rat PA\(^10,12\). In the present experiment, L-NMMA caused a dose-dependent contractile response. Therefore, we used a relatively low dose of the drugs which inhibited ACh-induced relaxation but did not affect basal tension, because an elevated tone induced by a high dose of L-NMMA was thought to affect the responses to hypoxia\(^5\). Both L-NMMA and methylene blue enhanced the phenylephrine-induced contraction, probably because these drugs inhibited the EDRF activity which was enhanced by the elevated tone\(^16\). The hypoxic contraction was reduced in the presence of the EDRF inhibitors. Therefore, the effect of the suppression of the EDRF activity, i.e., the hypoxic contraction, may have been attenuated because the EDRF activity had already been reduced. The reduction in hypoxic contraction could not be explained simply by the increase in normoxic force produced by chemical inhibition of EDRF because the hypoxic contraction associated with the increase in force produced by \(10^{-7}\) M phenylephrine, which had a developed tension similar to that of rings which had been precontracted with \(2 \times 10^{-8}\) M phenylephrine plus \(2 \times 10^{-6}\) M L-NMMA, was not similarly attenuated. The magnitude of the hypoxic contraction in the rings which had been precontracted with phenylephrine or 5-HT was significantly greater than that in the rings which had been precontracted with KCl. The reason for this difference is unknown, however, phenylephrine and 5-HT have been reported to stimulate the release of EDRF by activating the \(\alpha_1\)-adrenoceptor\(^17\) and the 5-HT\(_1\)-like receptor\(^18\) respectively, in endothelial cells. The production of EDRF in rings which have been precontracted with phenylephrine or 5-HT may be higher than that in rings which have been precontracted with KCl. If so, the effect of suppression of EDRF activity would be greater in rings which have been precontracted with phenylephrine or 5-HT than in those precontracted with KCl. Furthermore, Ca\(^{2+}\) release from intracellular storage sites is reportedly involved in the mechanism of hypoxic contraction in isolated human pulmonary artery\(^19\). Hypoxic contraction in rings which have been precontracted with receptor agonists is thought to be greater than that in those which have been precontracted with KCl.

Cyclic GMP is the second messenger of the EDRF/NO dependent relaxation, and its concentration reflects the activity of EDRF. In our study, the accumulation of cyclic GMP in the endothelium-intact ring was reduced by hypoxic stimulation. These results agreed with earlier reports\(^8,10\) and were consistent with the hypothesis that the attenuation of EDRF activity is one of the mechanisms of hypoxic contraction. The precise mechanism of the hypoxia-induced inhibition of EDRF activity is not clear. High oxygen tension reportedly destroys EDRF as a result of the generation of the superoxide radical, however, hypoxia could
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not be due to an effect on EDRF once produced\textsuperscript{20} Recently, Rengasamy and Johns reported that hypoxia inhibited EDRF/NO synthase activity\textsuperscript{21}

On the other hand, some investigators have indicated that hypoxic contraction was generally enhanced by the inhibition of EDRF in experiments using perfused rat lung\textsuperscript{11-13} These different effects of EDRF inhibitors on hypoxic pulmonary contraction can not be explained by the differences between the species studied. The size of the vessel and the experimental methods may be important factors. Furthermore, NO synthesis may be enhanced secondarily by the elevated tone.

Other mechanisms of hypoxic contraction which involved the endothelium were also considered. Wadsworth et al. indicated that a decrease in prostacyclin enhanced hypoxic contraction in sheep coronary artery\textsuperscript{22} Biaggioni et al. reported that adenosine, the tissue levels of which increase during hypoxia, produced pulmonary vasocostriction in sheep by the generation of a thromboxane/endoperoxide product\textsuperscript{23} However, the effects of a cyclooxygenase inhibitor or thromboxane synthase inhibitor on hypoxic contraction or relaxation were not observed in the present study. The hypoxic response in isolated rat PA does not appear to be mediated by the production of progstanoids. Another possible mechanism is the release of endothelium-derived contracting factor (EDCF). Rubanyi and Vanhoutte indicated that hypoxia and anoxia cause a release of diffusible vasoconstrictor substances from endothelial cells in isolated canine coronary artery\textsuperscript{24} The EDCF produced by hypoxia has been referred to as EDCF\textsubscript{25} A vasoconstricting peptide, endothelin, has recently been isolated from cultured porcine endothelial cells\textsuperscript{26} However, endothelin is not believed to be a mediator of hypoxic vasoconstriction because endothelin release is not elevated by hypoxia, and because its effects are long-lasting, in contrast to the rapid onset of, and recovery from hypoxic contraction\textsuperscript{27-29}

Hypoxic relaxation did not depend on the endothelium in the present study. Relaxation may be due to an inhibitory effect of hypoxia on the contractile response. Recently, Daut et al. reported that the hypoxic dilatation of guinea pig coronary artery was mediated by ATP-sensitive potassium channels\textsuperscript{20}

In summary, our data indicate that hypoxic contraction of the isolated rat PA is in part induced by a decrease in the release of EDRF. Although these results were acquired from isolated large pulmonary arteries which were precontracted with vasoconstrictor agents, Ignarro et al. indicated that the production of EDRF in the smaller vessels was more than that in the large vessels\textsuperscript{6} This mechanism may be present under hypoxic conditions in vivo.

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