Acetylcholine-induced endothelium-derived contracting factor in hypoxic pulmonary hypertensive rats

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Maruyama, J unko, Ayumu Yokochi, Kazuo Maruyama, and Shoichiro Nosaka. Acetylcholine-induced endothelium-derived contracting factor in hypoxic pulmonary hypertensive rats. J. Appl. Physiol. 86(5): 1687–1695, 1999.—We determined the role of an endothelium-derived contracting factor in the impaired relaxation response to ACh of conduit pulmonary arteries (PAs) isolated from rats with hypoxic pulmonary hypertension (PH). A PGH2/thromboxane A2 (TxA2)-receptor antagonist (ONO-3708) partially restored the impairment of ACh-induced relaxation, whereas TxA2 synthase inhibitors (OKY-046 and CV-4151) did not affect the impaired relaxation in phenylephrine-precontracted hypertensive PAs. Endothelium-denuded hypertensive PA rings showed no difference in the response to ACh between preparations with and without ONO-3708. In both endothelium-denuded control and hypertensive PAs, exogenous PGH2 induced contractions, and the magnitude of the contractions was greater in the control than in hypoxic PH preparations. An endothelin A-receptor antagonist (BQ-485), an endothelin B-receptor antagonist (BQ-788), and a superoxide anion scavenger (superoxide dismutase) did not restore the impaired response to ACh in hypertensive PAs. These findings suggest that PGH2 produced from the conduit PAs of rats with chronic hypoxic PH may be the endothelium-derived contracting factor responsible for the impairment of ACh-mediated vasorelaxation.

chronic hypoxia; prostaglandin H2; thromboxane A2; nitric oxide

ENDOTHELIAL CELLS play a significant role in the regulation of vascular tone through the release of endothelium-derived vasorelaxing and vasoconstricting substances (8, 14, 16, 26, 32). Nitric oxide (NO) and prostacyclin are endothelium-derived relaxing factors (EDRFs) (16, 26). Endothelium also produces endothelium-derived contracting factors (EDCFs) such as PGH2/thromboxane A2 (TxA2) (6, 19, 32), endothelin (ET) (14, 28), and superoxide anion (8, 29) under certain conditions. ACh-mediated vasconstriction is associated with an endothelium-derived contracting factor in hypoxic pulmonary hypertensive rats (16). This result might suggest involvement of ACh-induced impaired relaxation toward the normal level (16). This result might suggest involvement of contracting metabolites of the cyclooxygenase pathway. Vasoconstriction and vasoproliferation have both been implicated as important features in chronic hypoxic PH (17, 23). It is possible that the imbalance between EDRFs and EDCFs is involved in the pathogenesis of chronic hypoxic PH. Therefore, we conducted the present study to investigate the possible involvement of EDCFs in isolated PAs of rats exposed to hypobaric hypoxia (380 mmHg for 10 days). We used ONO-3708 as a PGH2/TxA2-receptor antagonist (12) and OKY-046(34) and CV-4151(30) as TxA2 synthase inhibitors. We also used BQ-485, an ETA-receptor antagonist (11), and BQ-788, an ETB-receptor antagonist (10), to assess the involvement of ET. In addition, we used superoxide dismutase, a superoxide anion scavenger, to determine the role of superoxide anion in the altered responses of hypertensive PAs to ACh.

MATERIALS AND METHODS

Induction of Hypoxic PH

Male Wistar rats (SLC, Shizuoka, Japan) weighing 170–220 g were randomly assigned to one of two groups: those exposed to chronic hypoxia in a hypobaric hypoxic chamber (air at 380 mmHg) for 10 days and age-matched control animals housed in room air at a normal atmospheric pressure. The pressure in the hypobaric chamber was reduced by using an electrically driven vacuum pump. Rats were housed under a 12:12-h light-dark cycle and were fed standard rat chow and water ad libitum. The chamber was opened for 15–30 min once a day so that the cage could be cleaned and food and water replenished. All rats were weighed at the start of the experiment and before they were killed for isolation of the PAs. Table 1 lists the number of rats and mean body weight of each group.

Preparation of PAs

After the 10-day hypoxia period, all of the control and experimental animals were anesthetized with intraperito-
Table 1. Body weights and right ventricular hypertrophy of control and experimental rats

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<thead>
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<th>Initial</th>
<th>10 Days of Hypoxia</th>
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<tr>
<td>Body weight, g</td>
<td>Control</td>
<td>208.1 ± 2.7 (34)</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>210.4 ± 1.8 (38)</td>
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<tr>
<td>RV/(LV + S)</td>
<td>Control</td>
<td>187.4 ± 2.0* (38)</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>187.4 ± 2.0* (38)</td>
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Values are means ± SE for no. of rats in parentheses. Experimental refers to the animals exposed to 10 days of hypoxia. RV, right ventricle; LV, left ventricle; S, septum. *P < 0.01 compared with control.

neal pentobarbital sodium (50 mg/kg). The lung and heart were removed en bloc and placed in modified Krebs-Henseleit solution at room temperature. The composition of this solution was the following (in mM): 115.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgCl2, 25.0 NaHCO3, 1.2 KH2PO4, and 10.0 dextrose. The right ventricle (RV) of the heart was dissected from the left ventricle plus septum (LV + S), and these cardiac portions were weighed separately. The value of RV/(LV + S) was then calculated to determine whether right ventricular hypertrophy had developed. Two pulmonary artery segments, a left extrapulmonary artery (EPA; 1.4–1.6 mm OD) and an intrapulmonary artery (IPA; 0.7–1.1 mm OD), were isolated and carefully cleaned of fat and connective tissue. Ring segments (2 mm) were cut (1–2 rings from the EPA and 2–4 rings from the IPA) and suspended vertically between hooks in an organ bath (20 ml) containing modified Krebs-Henseleit solution, maintained at 37°C, and bubbled with a mixture of 95% air-5% CO2. The optimal resting tension for the vasorelaxation studies was adjusted to 0.75 g for EPA rings and 0.5 g for IPA rings from the control rats, and 1.0 g and 0.75 g, respectively, for the experimental rat preparations, as previously described (16). At these resting tensions, the peak contraction was obtained in response to 70 mM KCl. In all preparations, the peak contraction induced by 70 mM KCl. After precontraction with phenylephrine, cumulative dose-tension curves were obtained for ACh (10^-6 – 10^-4 M). Finally, papaverine (10^-4 M) was added to produce maximal relaxation. Rings without ONO-3708 were tested at the same time.

In another set of rings from the other group of experimental rats, in the presence of an NO synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME; 10^-5 M), a cumulative concentration-response curve for ACh was obtained in the presence and absence of ONO-3708.

Blockade of TXA2 synthase, ETa receptor, ETb receptor, and superoxide. Similarly, the relaxation response to ACh in the phenylephrine-precontracted rings was also recorded in the presence of OKY-046 (a TXA2 synthase inhibitor; 10^-5 M or 10^-4 M), CV-4151 (a TXA2 synthase inhibitor; 10^-6 M), BQ-485 (an ETa-receptor antagonist; 10^-6 M), BQ-788 (an ETa-receptor antagonist; 10^-6 M), or SOD (a superoxide anion scavenger; 50 U/ml). Each of these drugs was examined in preparations of different rings from different experimental rats. In all experiments, rings from chronic hypoxic rats were examined in parallel with preparations from age-matched control rats.

Endothelium-Denuded PAs

Contractions to cumulative concentrations of ACh (10^-6 – 10^-4 M) were determined in hypertensive PA rings in the presence and absence of ONO-3708 (10^-5 M). To examine the smooth muscle sensitivity to PGH2, contractions to cumulative concentrations of PGH2 (10^-8 – 10^-6 M) in another set of rings of control rats and rats exposed to hypoxia for 10 days were determined in the presence and absence of ONO-3708.

Reagents

The following drugs were used: ACh hydrochloride, papaverine hydrochloride (Nacalai Tesque, Kyoto, Japan); phenylephrine (Kowa, Nagoya, Japan); ONO-3708, OKY-046 (Ono Pharmaceutical, Osaka, Japan); CV-4151 (Takeda Chemical Industries, Tokyo, Japan); PGB2, BQ-485, BQ-788 (Calbiochem, La Jolla, CA); SOD, and L-NAME (Sigma Chemical, St. Louis, MO). The concentrations of the drugs are expressed as the final molar concentrations in the organ bath. PGH2 was stored at -80°C and diluted with distilled water just before each experiment.

Data Analysis

Results are expressed as means ± SE. The significance of differences between control and experimental rats was determined by Student’s t-test. When more than two means were compared, a one-way analysis of variance was used. If a significant difference was found, Fisher’s least significant difference test was used to identify which groups were different. A P value of < 0.05 was considered to indicate statistical significance.

RESULTS

Body Weight and Right Ventricular Hypertrophy

The control rats gained weight throughout the experiment (Table 1). The hypoxic rats lost weight compared with the controls. The RV/(LV + S) ratios were increased in the experimental group after 10 days of hypoxia compared with control group, indicating the occurrence of right ventricular hypertrophy.
PA Ring Responses to 70 mM KCl and Phenylephrine

The endothelium-intact EPA rings from control rats and those from rats exposed to 10 days of hypoxia showed similar contractions in response to 70 mM KCl (Table 2). Phenylephrine induced greater contractions in endothelium-intact EPA and IPA rings from the hypoxic rats compared with those from control rats at 10⁻⁸, 10⁻⁶, 3 x 10⁻⁶, and 10⁻⁵ M in EPA and 3 x 10⁻⁸–10⁻⁶ M in IPA (Fig. 1). ONO-3708 attenuated phenylephrine-induced contraction in endothelium-intact rings from hypertensive rats at 3 x 10⁻⁶–3 x 10⁻⁸ M in EPA and at 10⁻⁸, 10⁻⁷, 3 x 10⁻⁷, 10⁻⁶, and 3 x 10⁻⁵ M in IPA (Fig. 2). Both endothelium-denuded EPA and IPA rings from hypoxic rats showed less contractility to phenylephrine compared with control rat preparations at 3 x 10⁻⁸–3 x 10⁻⁶ M in EPA and at all concentrations in IPA (Fig. 3).

Effects of ONO-3708 on ACh-Induced Relaxation in Endothelium-Intact PA Rings

In EPA and IPA rings from control rats precontracted with phenylephrine (10⁻⁵ M), ACh (10⁻⁸–10⁻⁴ M) caused relaxation in a concentration-dependent manner (Fig. 4). The relaxation induced by ACh was markedly reduced in both EPA and IPA rings from rats exposed to 10 days of hypoxia compared with the control rat rings (Fig. 4). There was an increase in tension at 10⁻⁶–10⁻⁴ M of ACh. ONO-3708 (10⁻⁵ M) did not affect the resting tone of endothelium-intact rings from either control or hypoxic rats. In control rat preparations, ONO-3708 did not significantly affect the relaxation caused by ACh (Fig. 4). In rings from rats exposed to 10 days of hypoxia, ONO-3708 did not affect the impaired relaxation at lower concentration of ACh (10⁻⁸–10⁻⁶ M), whereas it restored the impaired relaxation response at higher concentrations of ACh (10⁻⁵–10⁻⁴ M) toward those observed in the control rat preparations (Fig. 4). When EPA and IPA rings from experimental rats were precontracted with phenylephrine in the presence of L-NAME, ACh induced no relaxation but actually induced contraction at higher concentrations (Fig. 5).
ONO-3708 attenuated this contraction at higher concentrations of ACh in both EPA and IPA (Fig. 5).

Effects of OKY-046 and CV-4151 on ACh-Induced Relaxation in Endothelium-Intact PA Rings

OKY-046 (10^2–10^3 M) and CV-4151 (10^2–10^3 M) did not affect the resting tone of rings from either control or hypoxic rats. Both of these drugs did not restore the impaired relaxation of the endothelium-intact EPA and IPA rings in response to ACh (Figs. 6 and 7).

Effects of ONO-3708 on ACh-Induced Response and on Contraction to PGH2 in Endothelium-Denuded PA Rings

Phenylephrine-precontracted EPA and IPA rings from experimental rats showed similar response to ACh in the presence and absence of ONO-3708 (data not shown). In EPA and IPA rings from control rats and hypoxic rats, PGH2 caused contractions in a concentration-dependent manner (Fig. 8). ONO-3708 remarkably attenuated contractile responses to PGH2 at all concentrations in EPA and IPA from control rats and rats exposed to 10 days of hypoxia. The contraction induced by PGH2 was reduced in EPA rings from the hypoxic rats compared with the control rats at higher concentrations (10^-7–10^-6 M) (Fig. 8). In IPA, PGH2 induced greater contractions at 3 × 10^-8 and 10^-7 M in rings from the experimental rats compared with those from the control rats, whereas it induced similar contractions in rings from control and experimental rats at 3 × 10^-7 and 10^-6 M (Fig. 8).

Effects of BQ-485 and BQ-788 on ACh-Induced Relaxation of Endothelium-Intact PA Rings

BQ-485 (10^-6 M) and BQ-788 (10^-6 M) did not affect the resting tone of the rings from both control and experimental rats. BQ-485 (10^-6 M) partially reduced the response at concentrations of 10^-7 and 10^-6 M ACh in EPA rings of rats exposed to 10 days of hypoxia but not in those of control rats (Fig. 9). BQ-485 did not affect the impaired ACh-induced response in IPA from control and experimental rats. BQ-788 (10^-6 M) did not affect the impaired relaxation to ACh in phenylephrine-precontracted EPA and IPA rings from either control or experimental rats (Fig. 10).

Effects of SOD on ACh-Induced Relaxation of Endothelium-Intact PA Rings

SOD (50 U/ml) did not affect the resting tone of the rings from both control and experimental rats. SOD (50 U/ml) did not affect the impaired relaxation to ACh in phenylephrine-precontracted EPA and IPA rings from either control or experimental rats (Fig. 11).

DISCUSSION

ACh-induced relaxation was impaired in isolated conduit PAs from hypoxic PH in rats. Blockade of PGH2/TxA2 receptors restored the impaired relaxation at higher concentrations of ACh but not inhibition of TxA2 synthesis in both EPA and IPA. Contractions to PGH2 were reduced in endothelium-denuded rings in chronic hypoxic rats compared with those of normal rats. Blockade of ET and superoxide anion did not restore the impairment of ACh-induced relaxation. These results showed functional abnormalities in both...
endothelial cells and smooth muscle cells that might have modulated vascular tone in the hypertensive PAs. ACh-Induced Responses in Hypertensive Conduit PAs

We previously described that ACh-induced relaxation was impaired in isolated conduit PAs of rats with chronic hypoxic PH (16). The impairment of ACh-induced relaxation in isolated perfused rat lung with chronic PH (1) and in isolated PA rings from human lung with cor pulmonale (5) has been reported, consistent with the present results. However, recently there are contradictory reports concerning about the NO/cGMP mechanism of relaxation in chronic PH. Preserved or enhanced relaxation to endothelium-dependent relaxing substances was demonstrated in isolated perfused rat lung with chronic PH (9, 21). These findings might suggest that conduit PAs behave very differently from resistance PAs. Orton and co-workers (21) showed the difference in response to ACh between isolated conduit PAs and in vivo resistance PAs in calves with chronic PH.

Indomethacin, a cyclooxygenase inhibitor, partially restored the impairment of ACh-induced relaxation in hypertensive PAs (16). Blockade of the cyclooxygenase pathway may enhance the production of lipoxygenase metabolites. In fact, Vanderhoek and Bailey (33) demonstrated that cyclooxygenase blockade induced the release of lipoxygenase metabolites, leukotrienes, which are potent vasoconstrictors. If so, indomethacin would reduce ACh-induced relaxation. However, in a previous study, we reported that indomethacin enhanced ACh-induced relaxation in chronic hypoxic rats (16). We could not exclude the possibility that leukotrienes were increased in the impaired relaxation in hypoxic PH. However, we would suggest that constriction of the cyclooxygenase pathway is responsible at least in part. It is possible that one of main causes of the difference in response to ACh between conduit PAs and resistance PAs is the production and/or release of cyclooxygenase-derived EDCFs in conduit PAs.

PGH$_2$/TxA$_2$

In the present study, the impairment of endothelium-dependent ACh-induced relaxation was significantly attenuated by ONO-3708, a PGH$_2$/TxA$_2$-receptor antagonist (12), whereas OKY-046 (34) and CV-4151 (30), TxA$_2$ synthase inhibitors, failed to restore the ACh-induced response. Because ONO-3708 had no effect on the ACh-induced response in phenylephrine-precontracted endothelium-denuded rings from rats exposed to 10 days of hypoxia, the effects of ONO-3708 on ACh-induced responses in rings with endothelium seemed to take place through its effect on endothelial cells. These results suggested that the constricting substance was derived from endothelial cells in the PAs of hypoxic rats and that it might be a vasoconstrictor PG, most likely PGH$_2$, not TxA$_2$. Another candidate vasoconstrictor PG is PGF$_{2\alpha}$ (4). However, PGF$_{2\alpha}$ in unlikely because it has a low affinity to PGH$_2$/TxA$_2$ receptors (4).

In this study, the response to ACh in hypertensive rings was biphasic: impaired relaxation at lower concentrations of ACh and increased tension at higher concentrations. These results showed that the ACh-induced response might be dependent on its concentration in hypertensive PAs. ONO-3708 blocked the tension increase and caused relaxation at higher concentrations of ACh (toward those observed in the control rat preparations). L-NAME completely abolished relax-
Fig. 8. Contractile response induced by exogenous prostaglandin H$_2$ (PGH$_2$) in endothelium-denuded rings of EPA (A) and IPA (B) obtained from control rats and rats exposed to 10 days of hypoxia. Contraction produced by 70 mM KCl was taken as 100%. Values are means ± SE; nos. in parentheses are no. of rings from 8 control rats and 10 experimental rats at 10 days of hypoxia. ○, Experimental rats at 10 days of hypoxia without ONO-3708. **P < 0.01 compared with control rats with ONO-3708 (●). ††P < 0.01 compared with control rats without ONO-3708 (○). †P < 0.05, ‡P < 0.01 compared with experimental rats at 10 days of hypoxia with ONO-3708 (■).

Fig. 9. Effects of 10$^{-6}$ M BQ-485 on ACh-induced responses in precontracted (phenylephrine, 10$^{-5}$ M) endothelium-intact rings of EPA (A) and IPA (B) obtained from control rats and experimental rats exposed to 10 days of hypoxia. Relaxation produced by 10$^{-4}$ M papaverine was taken as 100%. Values are means ± SE; nos. in parentheses are no. of rings from 4 control and 7 experimental rats exposed to 10 days of hypoxia. ■, Experimental rats at 10 days of hypoxia with BQ-485. *P < 0.05, **P < 0.01 compared with control rats without BQ-485 (●). †P < 0.05, ‡P < 0.01 compared with experimental rats without BQ-485 (■). ††P < 0.01 compared with control rats with BQ-485 (k).

ACh-mediated vasoconstriction is associated with an endothelial PGH$_2$/TxA$_2$ release in the aorta (7), intrarenal artery (6), and cerebral arteriole (18) of spontaneously hypertensive rats (SHR). Ge et al. (7) described a greater expression of PGH synthase-1 and an increased release of PGH$_2$ in SHR aorta. In PH, indirect evidence of an increased production of TxA$_2$ has been described, although the origin of this vasoconstrictor has not been determined. The plasma TxB$_2$ (stable metabolite of TxA$_2$) concentration was found to be increased in patients with primary and secondary PH such as congenital heart disease with high pulmonary blood flow (3). Vasodilator PGs might also be produced. Shaul et al. (26) described that locally produced prostacyclin is increased in the rat main PAs after chronic hypoxia. The metabolism of prostanoids might thus be accelerated in PH. We suggested a relative increased production of PGH$_2$, not TxA$_2$, in rat chronic hypertensive PAs, which was consistent with the results in systemic hypertensive arteries.

We could not determine from our present results whether the existence of EDCF is a cause or a result of chronic PH. Chronic hypoxic PH is characterized by vascular changes, including the new muscularization of normally nonmuscular peripheral PAs and the medial hypertrophy of muscular arteries (17, 23). An elevation of pulmonary arterial pressure due to hypoxic pulmonary vasoconstriction (24) precedes the development of these vascular changes. Because prostacyclin and PGH$_2$/TxA$_2$ have opposite roles in the regulation of vasomotor tone and vascular smooth muscle cell differentiation and growth in the pulmonary circulation (20, 22), the imbalance between the release of these mediators may
be involved in the pathogenesis of the chronic PH. A previous study showed that indomethacin did not prevent the development of chronic hypoxic PH in rats (25). Indomethacin prevents the production of both vasodilator and vasoconstrictor PGs. Increased prostacyclin production might ameliorate chronic hypoxic PH. Rabinovitch et al. (25) have shown that continuous angiotensin II administration prevents chronic hypoxic PH and the associated structural vascular remodeling, most likely by inducing prostacyclin production. Further experiments are required to determine whether a chronic blockade of the production of PGH$_2$ can prevent chronic hypoxic PH.

ET

Recent studies of hypertensive renal vessels of rats have shown the increased release of ET-1 (13) in addition to PGH$_2$/TxA$_2$ (6). Moreover, ACh increased the local production of ET in coronary arteries of patients with atherosclerosis (14). It is possible that increases in several EDCFs exist in diseases characterized by an endothelial dysfunction, and ACh might augment the production and/or release of several EDCFs at the same time. Therefore, we also investigated the effect of ET and superoxide anion in the impaired relaxation response to ACh.

ET levels in lung tissue and plasma are increased in chronic hypoxic PH (15). ACh produces ET in certain diseases (14). In this study, BQ-485 and BQ-788 did not restore the impaired relaxation to ACh in EPA and IPA of rats with hypoxic PH. BQ-485 partially reduced the relaxation response at concentrations of $10^{-7}$ and $10^{-6}$ M ACh in EPA rings of rats exposed to 10 days of hypoxia. The reason for these results is unclear. If contracting substances coupled with ET receptors were released in hypertensive PAs, blockade of ET receptors would enhance ACh-induced relaxation. Therefore, these results at least suggested that one of main EDCFs associated with the impaired relaxation in response to ACh was not ET.

Superoxide Anion

In this study, pretreatment with SOD, an agent that scavenges superoxide anion, did not alter the relaxation of the EPA and IPA rings to ACh in both the control and hypoxic rats. The highly reactive free radical of oxygen has been proposed as an EDCF and is known as an inactivator of NO (8, 29). The EDCF(s) associated with the impaired relaxation to ACh in chronic hypoxic PH probably does not include a superoxide anion. In contrast, ACh produces superoxide anion in diabetic rat (8) and hyperlipidemic rabbit (29) aorta. A previous report described that PGH$_2$ not only directly induced contractions but also caused the formation of superoxide anions in isolated normal rabbit aortic rings, thereby suppressing EDRF/NO (31). The latter possibility is unlikely in chronic hypoxic PA, because SOD had no effect on the impaired ACh-induced responses.
In conclusion, the relaxation effect of ACh might be counteracted by the concomitant release of an EDCF, likely PGH$_2$ in PA isolated from rats with chronic hypoxic PH. ACh-induced ET or superoxide production was not detected in isolated hypertensive PAs. This study suggested that the impaired ACh-induced relaxation was due at least in part to the accelerated formation of PGH$_2$ in the PAs of rats with chronic hypoxia.

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