E-4010, a selective phosphodiesterase 5 inhibitor, attenuates hypoxic pulmonary hypertension in rats

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Hanasato, Norihisa, Masahiko Oka, Masashi Muramatsu, Mayu Nishino, Hideyuki Adachi, and Yoshinosuke Fukuchi. E-4010, a selective phosphodiesterase 5 inhibitor, attenuates hypoxic pulmonary hypertension in rats. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L225-L232, 1999.—The purpose of this study was to determine whether E-4010, a newly synthesized potent and selective orally active phosphodiesterase (PDE) 5 inhibitor, would prevent the development of chronic hypoxia-induced pulmonary hypertension in rats. In conscious, pulmonary hypertensive rats, a single oral administration of E-4010 (1.0 mg/kg) caused an acute, long-lasting reduction in mean pulmonary arterial pressure (PAP), with no significant effects on systemic arterial pressure, cardiac output, and heart rate. In rats that received food containing 0.01 or 0.1% E-4010 during the 3-wk exposure to hypoxia, mean PAP was significantly decreased (mean PAP 24.0 ± 0.9, 16.2 ± 0.8, and 12.8 ± 0.5 mmHg in rats treated with 0.0, 0.01, and 0.1% E-4010-containing food, respectively), whereas mean systemic arterial pressure was unchanged and cardiac output was slightly increased compared with chronically hypoxic control rats. Right ventricular hypertrophy, medial wall thickness in pulmonary arteries corresponding to the respiratory and terminal bronchioles, and the degree of muscularization of more distal arteries were less severe in E-4010-treated rats. Long-term treatment with E-4010 caused an increase in cGMP levels in lung tissue and plasma but not in aortic tissue and no significant change in cAMP levels in either lung, aorta, or plasma. These results suggest that long-term oral treatment with E-4010 reduced the increase in PAP, right ventricular hypertrophy, and pulmonary arterial remodeling induced by exposure to chronic hypoxia, probably through increasing cGMP levels in the pulmonary vascular smooth muscle.

Pulmonary hypertension (PH) is a hemodynamic abnormality caused by a variety of disease states, including primary PH and chronic obstructive pulmonary disease. PH is characterized by increased pulmonary vascular resistance that frequently results in an inability of the right ventricle to sustain its output and leads to right ventricular failure and death. Although the pathogenesis of PH differs among those disease states, it is likely that abnormal vasoconstriction contributes significantly to the vascular process of all forms of PH.

Although a wide variety of vasodilator agents have been used in the treatment of PH, their effects are generally limited because of the lack of pulmonary selectivity.

Inhaled nitric oxide (NO) has been established as a selective pulmonary vasodilator (10, 24). However, although this therapy has been reported to be very effective in some types of PH, such as persistent PH of the newborn and heart and/or lung perioperative PH in which the PH is reversible in a relatively short term, there are problems associated with long-term use of NO inhalation, including its potential toxicity and difficulty in ambulatory inhalation (33, 36). Although the exact mechanism of its efficacy is not fully elucidated, promising results have been accumulated regarding long-term treatment of primary PH patients with continuous intravenous prostacyclin (3, 19). However, this modality also has limitations because of its high cost and complexity of delivery system. To date, unfortunately, no ideal agent for the therapy of PH has yet been identified.

Recently, cGMP-specific phosphodiesterase (PDE) 5 inhibitors have been demonstrated to be potent, acute, selective pulmonary vasodilators (5, 6, 35). Branner et al. (5) have shown that M & B 22948 (zaprinast) produces selective dose-dependent pulmonary vasodilation during PH induced by U-46619 or by alveolar hypoxia in intact newborn lambs. Cohen et al. (6) have reported that E-4021 is a pulmonary vasodilator as selective and effective as inhaled NO in conscious pulmonary hypertensive rats. The mechanism responsible for the pulmonary selectivity of these PDE5 inhibitors is not fully understood, but it is likely due to abundant distribution of PDE5 in the lung tissue (6, 21, 32, 35).

Thus we hypothesized that long-term treatment with a selective PDE5 inhibitor would increase cGMP levels in lung tissue and prevent the development of chronic hypoxia-induced PH without systemic hypotension. To test this hypothesis, we used in this study a newly synthesized compound, E-4010 [4-(3-chloro-4-methoxybenzyl)amino-1-(4-hydroxypiperidino)-6-phthalazinecarbonitrile monohydrochloride], as a selective PDE5 inhibitor because this orally effective agent has been shown to be highly selective for PDE5 compared with other PDE isoenzymes (PDE1–4) (16). We first examined the acute hemodynamic effects of oral administration of E-4010 in conscious chronic hypoxia-induced pulmonary hypertensive rats. We then investigated the effects of long-term oral administration of E-4010 on the development of chronic hypoxia-induced PH in rats.

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and measured cGMP levels in lung and aortic tissues and plasma of these rats.

MATERIALS AND METHODS

Acute Hemodynamic Effects of E-4010 on Chronically Hypoxic Rats

Exposure to chronic hypoxia. Male adult Sprague-Dawley rats (260–320 g at the start of the experiment) were exposed to reduced barometric pressure (380 mmHg) in a hypobaric chamber for 3 wk to induce hypoxic PH (23). The chamber was maintained at room temperature and continuously flushed with room air to prevent accumulation of CO₂, NH₃, and water vapor. The chamber was recompressed briefly every 2 days to clean the cages and add fresh food and water. Normoxic control rats were kept in room air.

Conscious catheterized rats. After a 3 wk-exposure to hypoxia or normoxia, the rats were anesthetized with intramuscular ketamine (90 mg/kg) and pentobarbital sodium (15 mg/kg) for catheterization of the pulmonary and right carotid arteries and right jugular vein as previously described (23). The intravascular location of the catheter tips was determined by the blood pressure tracings, and the catheters were secured, filled with heparinized saline, sealed, and tunneled subcutaneously to the back of the neck where they were exteriorized and enclosed in a small plastic container. The cutdowns were repaired, and the rats were allowed to recover for 48 h in room air. After recovery, the conscious rat was placed in a ventilated (room air) plastic box for hemodynamic measurements and measured under normoxic conditions (the rats were breathing 21% O₂). After blood samples (0.1 ml) were taken from the carotid artery for measurement of hematocrit, baseline PAP, SAP, CO, and HR were measured. Then, either 0.1, 0.3, or 1.0 mg/kg of E-4010 or its vehicle (0.5% methylcel-
lulose) was given by gavage to conscious catheterized CH rats. PAP, SAP, and HR were monitored for 480 min, and CO was measured at 15 and 480 min after oral administration of E-4010. At the end of each hemodynamic study, the rat was killed by an overdose of pentobarbital sodium, and the heart was removed for measurement of right ventricle (RV) and left ventricle plus septum (LV+S) weights and for calculation of RV-to-LV+S weight ratio [RV/(LV+S)] as an index of right ventricular hypertrophy.

Prevention of Chronic Hypoxic PH

Experimental groups. Adult Sprague-Dawley rats (250–300 g at the start of the experiment) were randomly divided into four groups. Three groups of rats were exposed to chronic hypobaric hypoxia (barometric pressure 380 mmHg) for 3 wk (CH groups). One day before hypoxic exposure, two CH groups of rats were started on either a high (0.1%) or low (0.01%) rat food (CH₀.¹ group) or a low (0.01% rats received a calculated dose of 3 mg·kg⁻¹·day⁻¹; CH₀.₀₁ group) concentration of E-4010-containing food that was given throughout the study. One CH group of rats was fed with normal rat food (CH₀.₀ group). The fourth group of rats was maintained in room air (normoxic control rats; N group) and fed with normal rat food. After 3 wk of exposure to hypoxia or normoxia, all rats were catheterized (the pulmonary and right carotid arteries and right jugular vein) as described in Conscious catheterized rats and allowed to recover in room air for 24 h. The rats were then returned to the conditions under which they had been before catheterization for another 24 h.

Measurements. Hemodynamic measurements were made 48 h after catheterization under normoxic conditions. After blood samples (0.1 ml) were taken for measurement of hematocrit, the conscious rat was placed in a ventilated (room air) plastic box, and PAP, SAP, CO, and HR were measured. Blood samples (6 ml) were again collected for measurement of plasma cyclic nucleotide (cAMP and cGMP) levels. They were immediately centrifuged at 3,000 rpm for 10 min, and the supernatants were kept at −20°C until cyclic nucleotide levels were determined as described below. At the end of each study, the rat was killed by an overdose of pentobarbital sodium, and the heart [for calculation of RV/(LV+S)] and the lungs and thoracic aorta (for measurement of tissue cyclic nucleotide levels) were removed. The lung and aortic tissues were rapidly frozen in liquid N₂ and stored at −80°C. The concentration of cyclic nucleotides was measured with a radioimmunoassay kit (Amersham, Little Chalfont, UK). For measurement of cyclic nucleotide levels in plasma, 400 µl of ice-cold ethanol were added to 100 µl of plasma and centrifuged at 4°C at 11,000 rpm for 20 min. Then the supernatant was evaporated under a stream of N₂ gas and used for cyclic nucleotide measurement. For the measurement of intracellular cyclic nucleotide levels in the lung and aorta, each preparation was homogenized in 1 ml of 1 N HCl. The homogenate was centrifuged at 4°C at 3,000 rpm for 10 min, and the supernatant was evaporated under a stream of N₂ gas. The residue was subjected to radioimmunoassay. The protein content of the pellet was determined with bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Tissue cyclic nucleotide levels were normalized by the amount of the protein.

Histological analysis. In subsets of N, CH₀.₀, and CH₀.₁ groups of rats, histological changes were quantified by morphometry as previously described (4, 14). The rats were killed with an overdose of pentobarbital sodium, and the heart and lungs were removed en bloc. The pulmonary artery was cannulated and perfused with phosphate-buffered saline (37°C, 20 cmH₂O pressure) to remove residual blood and then injected with a barium-gelatin mixture (60°C) at 74 mmHg pressure for 3 min. The trachea was cannulated, and the lung was dissected with 10% Formalin in 36 cmH₂O pressure and fixed in an inflated state for 3 days. A block of tissue (1.0 × 0.7 × 0.2 cm) was taken from the midportion of the left lung parallel to the hilum. Sections were stained with elastic Van Gieson stain and assessed microscopically for the degree of arterial muscularization and wall thickness. In each tissue section, at least 50 consecutive barium-filled arteries (>15-µm external diameter) were analyzed at ×400 magnification. Each artery was classified by the structure of the accompanying airway as terminal bronchiole, respiratory bronchiole, alveolar duct, or alveolar wall. For each completely muscular artery corresponding to a respiratory or terminal bronchiole, we measured the medial thickness at two places of each artery. Medial wall thickness is expressed as the summation of the two points of (medial thickness/external diameter) × 100 (in percent).
The effect of the drug on the abnormal extension of smooth muscle into usually nonmuscular alveolar duct and wall vessels, which occurs during chronic hypoxia (14), was assessed as follows: each barium-containing vessel identified was viewed at ×400 magnification, and the structure of each vessel was then noted as either completely muscular, partially muscular, or nonmuscular.

Statistical Analysis

Data are presented as means ± SE. Statistical analysis was done by ANOVA with Scheffe’s post hoc test for multiple comparisons or repeated-measures ANOVA followed by Dunn-type multiple comparison. The Mann-Whitney U-test was used for comparison of muscularization of resistance vessels corresponding to the alveolar duct and alveolar wall. Differences were considered significant at \( P < 0.05 \).

RESULTS

Acute Hemodynamic Effects of E-4010

In normoxic rats (\( n = 7 \)), mean PAP, hematocrit, and RV/(LV+S) were 10.7 ± 0.5 mmHg, 43 ± 1%, and 23 ± 1%, respectively. All these parameters were significantly increased in CH rats (\( n = 26 \)); mean PAP 23.1 ± 0.4 mmHg; hematocrit 61 ± 0%; RV/LV+S 52 ± 1%), indicating the development of chronic hypoxia-induced PH. Oral administration of E-4010 (1.0 mg/kg) had little or no effect on mean PAP in normoxic rats (data not shown).

The time course of the acute effects of oral administration of E-4010 on mean PAP and SAP is shown in Fig. 1. E-4010 at the highest dose tested in this study (1.0 mg/kg) significantly reduced mean PAP, although its lower doses did not change mean PAP. The PAP-lowering effect of E-4010 was rapid and reached its maximum 60 min after administration of E-4010 and lasted over 360 min. In contrast, E-4010 did not significantly affect mean SAP even at the highest dose tested. The hypotensive effect of E-4010 on PAP was accompanied by no change in either HR or CO (Table 1).

Prevention of Chronic Hypoxic PH

At the end of the third week of hypobaric hypoxia, the body weight of CH Con rats was lower than that of N rats but similar to that of CH E0.01 and CH E0.1 rats. The hematocrit was increased in CH Con rats compared with that in N rats, and E-4010 treatment lowered it significantly.

Table 2. Body weight and hematocrit of rats exposed to normoxia or hypoxia with and without E-4010 treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N (n = 8)</th>
<th>CH Con (n = 21)</th>
<th>CH E0.01 (n = 20)</th>
<th>CH E0.1 (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>316 ± 4</td>
<td>267 ± 5*</td>
<td>266 ± 5*</td>
<td>270 ± 6*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>42 ± 1</td>
<td>59 ± 1*</td>
<td>53 ± 1†</td>
<td>51 ± 1†</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \), no. of animals/group. N, normoxic control rats; CH Con, chronically hypoxic control rats; CH E0.01, chronically hypoxic rats with low-dose E-4010 (0.01%) treatment; CH E0.1, chronically hypoxic rats with high-dose E-4010 (0.1%) treatment. Significant difference (\( P < 0.05 \)) from: *N; †CH Con (by ANOVA).
cantly (Table 2). Hemodynamic measurement data of conscious catheterized rats after exposure to 3 wk of normoxia or hypoxia with and without E-4010 treatment are shown in Fig. 2. Three weeks of hypobaric hypoxia resulted in a marked increase in mean PAP and a slight decrease in CO, with no change in either mean SAP or HR in CH_{Con} rats. Calculated TPR was markedly and TSR was slightly increased in these rats. Treatment with E-4010 caused a dose-dependent decrease in mean PAP and TPR but no change in mean SAP in CH_{E0.01} and CH_{E0.1} rats. TSR was decreased significantly in CH_{E0.1} rats, which probably reflects the increase in CO. Right ventricular hypertrophy as assessed by RV/(LV+S) was dose dependently prevented by E-4010 (Fig. 3).

As shown in Table 3, although cGMP levels in plasma and tissue (both lung and aorta) were not affected by chronic hypoxic exposure, E-4010 increased cGMP levels significantly in plasma and lung tissue but not in aortic tissue after chronic exposure to hypoxia. cAMP
levels were decreased in lung tissue and unchanged in aortic tissue but tended to increase in plasma after chronic exposure to hypoxia. These levels were unaffected by E-4010.

Tables 4 and 5 show the effect of E-4010 treatment on the development of hypoxia-induced pulmonary vascular remodeling. In the muscular pulmonary arteries corresponding to the respiratory and terminal bronchioles (vessel diameter ranging from 50 to 150 µm), there was a significant increase in medial wall thickness in CHCon rats compared with that in N rats. Treatment with E-4010 resulted in a significant reduction in the chronic hypoxia-induced increased medial wall thickness (Table 4, Fig. 4). In addition to a reduction in medial wall thickness in E-4010-treated rats, there was also a reduction in the degree of muscularization in the smaller resistance vessels corresponding to the alveolar duct and alveolar wall (Table 5).

**DISCUSSION**

This study has demonstrated that long-term oral administration of a selective PDE5 inhibitor, E-4010, markedly reduced the development of chronic hypoxia-induced PH in rats. Long-term treatment with E-4010 significantly attenuated the chronic hypoxia-induced increase in mean PAP and TPR. In addition, compared with chronically hypoxic control rats, the hypoxia-induced right ventricular hypertrophy and pulmonary vascular remodeling were less severe in E-4010-treated rats. These effects of E-4010 were associated with the increase in cGMP (but not cAMP) levels in lung but not in aortic tissue.

It is well known that cGMP plays an essential role in the regulation of vascular smooth muscle tone, including the pulmonary vascular bed. Several endogenous vasodilators such as NO and atrial natriuretic peptide cause vascular smooth muscle cell relaxation by increasing the intracellular concentration of cGMP ([cGMP]i) via activation of soluble or particulate guanylate cyclase (GC) and consequent activation of protein kinase

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**Table 4.** Medial wall thickness of pulmonary arteries accompanying terminal and respiratory bronchioles in rats exposed to normoxia or hypoxia with and without E-4010 treatment

<table>
<thead>
<tr>
<th>Diameter of Vessels</th>
<th>N</th>
<th>CHCon</th>
<th>CH0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>50–100 µm</td>
<td>3.7 ± 0.3</td>
<td>6.6 ± 0.2*</td>
<td>5.1 ± 0.2†</td>
</tr>
<tr>
<td>101–150 µm</td>
<td>3.6 ± 0.4</td>
<td>6.0 ± 0.2*</td>
<td>4.4 ± 0.2†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 animals/group. Significant difference (P < 0.05) from: *N; †CHCon (by ANOVA).

**Table 5.** Distribution of muscularization of vessels in rats exposed to normoxia or hypoxia with and without E-4010 treatment

<table>
<thead>
<tr>
<th>Number of Vessels</th>
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<tr>
<td></td>
</tr>
<tr>
<td>Alveolar duct</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Alveolar wall</td>
</tr>
</tbody>
</table>

Values are no. of vessels counted/group according to accompanying airway structure and degree of muscularization; n = 5 animals/group. Vessels were classified as nonmuscular (NM), partially muscular (PM), or completely muscular (CM). Comparison of muscularization was done separately at alveolar duct and alveolar wall levels by a nonparametric Mann-Whitney U-test. Significant difference (P < 0.05) from: *N; †CHCon (by Mann-Whitney U-test).
Long-term treatment with E-4010 during chronic exposure of rats to hypoxia dose dependently reduced PAP without affecting SAP, suggesting that this PDE5 inhibitor had a sustained and selective PAP-lowering effect. Similarly, chronic hypoxia-induced right ventricular hypertrophy, which is thought to be the result of increased pulmonary vascular tone, was alleviated by E-4010. Furthermore, E-4010 (higher concentration) increased the impaired CO after exposure to chronic hypoxia, which is also probably due to increased PAP. These results indicate that this selective PDE5 inhibitor provides a beneficial response (28) in the treatment of PH hemodynamically.

E-4010 also reduced the increased medial wall thickness in pulmonary arteries corresponding to the respiratory and terminal bronchioles and the degree of muscularization of more distal arteries induced by exposure to chronic hypoxia. Although the mechanism(s) responsible for the development of chronic hypoxia-induced vascular remodeling has not been fully elucidated, chronic vasoconstriction with resultant increases in pressure and shear stress is believed to play a major role in this process (8, 26). Therefore, the pulmonary vasodilating effect of E-4010 is considered to contribute, at least in part, to the prevention of vascular remodeling. Furthermore, because it has been reported that cGMP mediates the antiproliferative effects of NO (11, 30) apart from its vasodilating effect, E-4010 may have directly inhibited pulmonary smooth muscle cell proliferation via increasing cGMP levels in the smooth muscle.

In addition to reversible vasoconstriction and structural remodeling of pulmonary arteries, one more factor that is thought to contribute to the development and maintenance of chronic hypoxia-induced PH is hyperviscosity resulting from polycythemia (13, 27). It has been shown that chronic hypoxia-induced polycythemia is attributable to increased erythropoietin (EPO) activity that is directly stimulated by hypoxia (9, 12). Theoretically, therefore, even if the degree of PH is attenuated by some therapeutic agents, chronic hypoxia-induced polycythemia might not be affected. Indeed, previous studies (20, 34) have shown that although a variety of agents reduced the development of PH induced by chronic hypoxia, most of them had little effect on hematocrit. In this study, however, long-term treatment with E-4010 during chronic exposure to hypoxia...
of rats significantly decreased hematocrit. Although it is not clear why E-4010 suppressed the erythropoiesis induced by chronic hypoxia, it has been suggested that cGMP plays a modulatory role in the regulation of EPO activity (18). Thus a possible explanation may be that increased cGMP levels in the EPO-secreting cells of rats treated with E-4010 during exposure to chronic hypoxia may have influenced the EPO activity. This issue needs to be studied further. Although this effect of E-4010 on hematocrit is favorable for prevention of the development of PH, it may decrease systemic oxygen transport to organs of patients with chronic obstructive pulmonary disease. However, this problem can be overcome practically by long-term oxygen therapy concomitant with E-4010 treatment. Furthermore, long-term administration of E-4010 had no effects on hematocrit in rats during normoxic conditions (Oka, unpublished observations).

We measured cyclic nucleotide levels in this study and found that long-term treatment with E-4010 increased cGMP levels in lung but not in aortic tissue and did not change cAMP levels in these tissues. These results suggest that E-4010 is more effective in lung than in aortic tissue and is specific to cGMP, supporting our hypothesis that chronic treatment with a selective PDE5 inhibitor would preferentially decrease cGMP degradation rate and increase [cGMP] in lung tissue due to the predominant distribution of this isoenzyme in the lung. This may be the reason why selective PDE5 inhibitors have pulmonary selectivity.

Although we investigated in the present study the chronic effects of E-4010 on the development of hypoxia-induced PH only, one previous study (31) has demonstrated the protective effects of another selective PDE5 inhibitor, E-4021, on the development of right ventricular overload and medial thickening of pulmonary arteries in a different rat model of PH, i.e., monocrotaline-induced PH. In addition, several laboratory and clinical studies (15, 17, 22) have shown that PDE5 inhibitors potentiate the vasodilator effects of inhaled NO. Taken together, these reports suggest the possibility that selective PDE5 inhibitors may be useful in the treatment of PH.

In summary, data of this study have shown that an orally active selective PDE5 inhibitor, E-4010, caused selective pulmonary vasodilation and attenuated the increase in PAP, right ventricular hypertrophy, and pulmonary arterial remodeling induced by chronic hypoxia. These hemodynamic effects of E-4010 were associated with an increase in cGMP levels in lung but not in aortic tissue. These results suggest that E-4010 prevented the development of chronic hypoxia-induced PH, probably through increasing cGMP levels in the pulmonary vascular smooth muscle. We conclude that selective PDE5 inhibitors, including E-4010, may provide a new strategy for the treatment of PH.

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