Pulmonary hypertension is a disease associated with progressive elevation of pulmonary arterial pressure, ultimately inducing heart failure and death. Various vasodilators, such as prostacyclin and Ca\(^{2+}\) blockers, have been used as a bridge to heart-lung transplantation (Rubin, 1992). The goal of a vasodilating agent in the treatment of pulmonary hypertension is to reduce pulmonary arterial pressure without inducing systemic hypotension and to prolong life expectancy. Long-term i.v. infusion of prostacyclin to patients with pulmonary hypertension has been shown to improve the survival rate in association with a decrease in pulmonary arterial pressure (Barst et al., 1996). However, because no selective pulmonary vasodilator is available at present, the development of an orally effective and long-lasting agent for pulmonary hypertension with little effect on systemic arterial pressure would be very valuable.

Pulmonary arterial vascular tone is regulated by cGMP. Nitric oxide (NO) and natriuretic peptides dilate the pulmonary artery through activation of guanylate cyclase, which synthesizes cGMP. Increased cGMP is then hydrolyzed by cGMP-specific phosphodiesterase (PDE), classified as PDE5, which is abundant in pulmonary artery (Rabe et al., 1994).

As PDE5 plays an important role in the regulation of pulmonary vascular tone, inhibition of PDE5 induces pulmonary vasodilation and could be useful for pulmonary hypertension. We reported previously that E4021, a selective and potent PDE5 inhibitor, reduced pulmonary arterial pressure in conscious pigs (Saeki et al., 1995; Adachi and Nishino, 1998). In addition, E4021 lowered the increased pulmonary arterial pressure without effect on systemic arterial pressure in pulmonary hypertensive rats (Yamaguchi et al., 1998). Recently, we have found that E4010, 4-(3-chloro-4-methoxybenzyl)amino-1-(4-hydroxypiperidino)-6-phthalazinecarbonitrile monohydrochloride (Fig. 1), selectively and potently inhibited PDE5 isolated from porcine platelet (Ishihara et al., 1998). Furthermore, E4010 markedly reduced the increased pulmonary arterial pressure with a slight effect on systemic arterial pressure in the porcine model of heart failure (Adachi et al., 1998) similar to the action of E4021. Therefore, we expect that E4010 would be useful for the treatment of patients with pulmonary hypertension.

Although PDE5 inhibitors selectively lower elevated pulmonary arterial pressure, it is not yet clear whether these agents improve the decreased survival rate induced by pulmonary hypertension. Among the experimental pulmonary hypertensive models, monocrotaline (MCT)-induced rats...
model (Ghodsi and Will, 1981) is suitable for evaluating the survival rate of pulmonary hypertension due to the short life span of the animal (Takahashi et al., 1996a). Accordingly, the purpose of this study is to investigate the effect of long-term oral administration of E4010 on the survival rate of rats with pulmonary hypertension induced by MCT.

Materials and Methods

Animals. All experiments were carried out in accordance with our company’s guidelines for animal experimentation (Eisai Research Laboratories, Ibaraki, Japan).

Experimental Protocol. Male Wistar rats (Charles River, Kanagawa, Japan) of 4 weeks of age were used. MCT (Sigma, St. Louis, MO) was dissolved in 1 N HCl at a concentration of 100 mg/ml, neutralized with 1 N NaOH, and diluted with distilled water to 4 mg/ml. MCT at a dose of 40 mg/kg was injected s.c. into rats at a volume of 1 ml/100 g, and control-aged matched rats were injected with the same volume of vehicle. After injection of MCT or vehicle, rats were immediately divided into four groups and fed diets as follows.

Experimental schedule is expressed in Fig. 2.

1. Control (n = 8): Rats were treated with vehicle and fed a commercial diet [mouse flat (MF); Oriental Yeast Co., Tokyo, Japan].
2. MCT (n = 32): Rats were treated with MCT and fed MF.
3. E4010 0.01% (n = 32): Rats were treated with MCT and fed MF containing E4010 0.01%.
4. E4010 0.1% (n = 32): Rats were treated with MCT and fed MF containing E4010 0.1%.

The animals were housed in a temperature- (23 ± 1°C) and moisture-controlled (55 ± 10%) room with a 12-h light/dark cycle (lights on at 7:00 AM and lights off at 7:00 PM). The survival rates of all groups were monitored until the survival rate of the MCT-treated rats reached less than 30%, and were expressed as the percentage of survivors per group. At the end of the experiment (day 23), the rats were anesthetized with pentobarbital, and the chest and abdominal cavities were quickly opened. A blood sample was drawn from the abdominal artery and collected into a plastic tube containing EDTA, then centrifuged at 1300g for 20 min at 4°C to isolate the plasma. The plasma was stored at −20°C until used for the measurement of plasma concentrations of cGMP, cAMP, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and NO.

Measurement of Organ Weight. The heart and lungs were dissected and weighed. The ratio of the organ weight to the body weight (BW) was calculated. The right ventricle free wall was separated from the left ventricle and the septum to determine the wet weight. The right ventricle weight to body weight (RV/BW), left ventricle plus septum weight to body weight (LV + S/BW), cardiac weight (RV + LV + S/BW), lung weight to body weight (LU/BW) ratios were also calculated. As an index of right ventricular hypertrophy, the ratio of the right ventricle weight to left ventricle plus septum weight (RV/LV + S) was calculated.

After measuring the weight, the lung was frozen in liquid nitrogen and stored at −80°C until used for the measurement of the concentrations of cGMP and cAMP in the lung.

Measurement of Cyclic Nucleotide Levels in Plasma and Lung. The concentration of cyclic nucleotides was measured with a radioimmunoassay kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). To determine the cyclic nucleotide levels in the plasma, 400 μl of ice-cold ethanol was added to 100 μl of plasma, and was centrifuged at 11,000g for 20 min at 4°C. The supernatant was then evaporated under a stream of N2 gas, and was used for cyclic nucleotide measurement.

For the measurement of intracellular cyclic nucleotide levels in lung, each tissue sample was homogenized in 1 ml of 0.1 N HCl. The homogenate was centrifuged at 3000g for 10 min at 4°C 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 using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL). The cyclic nucleotide levels in lung were normalized to the amount of protein.

Measurement of Plasma Natriuretic Peptide Levels. For measurement of plasma natriuretic peptide levels, 500 μl of buffer A (1% trifluoroacetic acid) was added to 500 μl of plasma, and was centrifuged at 11,000g for 20 min at 4°C. The supernatant was then loaded into a Sep-Pak Vac C18 column (Waters, Milford, MA) that had been previously washed with 1 ml of buffer B (acetonitrile:H2O: trifluoroacetic acid = 60:39:1, v/v/v) followed by 3 ml of buffer A (three times). The column was washed with 3 ml of buffer A (three times), and eluted with 3 ml of buffer B. The eluate was then evaporated under a stream of N2 gas to eliminate the acetonitrile. After evaporation, the residue was subjected to radioimmunoassay (Peninsula Laboratories, Inc., Belmont, CA).

Measurement of Plasma NO Levels. An aliquot of 100 μl of plasma was filtered through a microconcentrator (Amicon, Beverly, MA), and centrifuged at 11,000g for 10 min at 4°C. The concentration of NO was measured with an NO assay kit (Cayman Chemical, Ann Arbor, MI).

Drugs. E4010 was synthesized at Eisai Chemicals (Kashima, Ibaraki, Japan). MF diets containing E4010 were ordered from Oriental Yeast Company (Ibaraki, Japan).

Statistical Analysis. Data are expressed as mean ± S.E.M. The differences between the control and MCT groups were examined with the unpaired t test. Then the data of the MCT, E4010 0.01%, and E4010 0.1% treatment groups were further analyzed by Dunnett’s multiple range test. The survival rates were presented as Kaplan-Meier curves. The survival curves of individual groups were compared by the log-rank test. Differences between control and MCT were determined by log-rank test. If significant, statistical analyses between MCT and E4010 0.01% or E4010 0.01% were performed.
using log-rank test with Bonferroni adjustment. P values of less than .05 were considered significant.

**Results**

**Body Weight Changes.** Changes in body weight are summarized in Table 1. As compared with control, the increases in body weight were inhibited from day 14 to 23 in the MCT, E4010 0.01%, and E4010 0.1% groups.

In both E4010-treated groups, the food intake per rat was 23.4 g/head/day at day 7 and 14, but decreased to 8.6 g/head/day at day 21.

**Effect of E4010 on Organ Weight.** The MCT group developed right ventricular (P < .01) and cardiac hypertrophy (P < .01), compared with control, and an increased RV/LV + S ratio (P < .01, Table 2). Right ventricular hypertrophy was improved in both E4010 0.01%- (P < .01) and E4010 0.1%-treated groups (P < .01) as shown by decreases in RV/LV + S. MCT also induced lung weight increases (P < .01), which is an index of lung edema. E4010 did not suppress the increase in lung weight (Table 2).

**Effects of E4010 on Cyclic Nucleotide Levels in Plasma and Lung.** In the MCT group, both plasma cGMP (19.3 ± 4.2 pmol/ml versus 7.3 ± 1.1 pmol/ml, P < .05, Fig. 3A) and cAMP (33.3 ± 4.0 pmol/ml versus 16.1 ± 1.8 pmol/ml, P < .01, Fig. 3B) levels were increased compared with control. Although plasma cGMP levels were dose-dependently amplified for both E4010 0.01% (86.6 ± 13.5 pmol/ml, P < .01) and E4010 0.1% (135.8 ± 7.8 pmol/ml, P < .01) treatment groups, plasma cAMP levels were not significantly affected.

MCT did not influence the cGMP levels in the lung (494.3 ± 40.3 pmol/mg protein versus 569.5 ± 55.6 pmol/mg protein) compared with control, whereas E4010 elevated cGMP levels at both doses, that is, 0.01% (984.2 ± 99.9 pmol/mg protein, P < .01) and 0.1% (1078.2 ± 70.6 pmol/mg protein, P < .01) compared with MCT (Fig. 4A). On the contrary, there were no significant differences in the lung cAMP levels among the four groups (Fig. 4B).

**Effects of E4010 on Plasma Natriuretic Peptide Levels.** In the MCT group, both plasma ANP (217.3 ± 45.2 pg/ml versus 28.5 ± 4.4 pg/ml, P < .01, Fig. 5A) and BNP (91.1 ± 10.6 pg/ml versus 21.1 ± 2.0 pg/ml, P < .01, Fig. 5B) levels were elevated compared with control. However, the E4010-treated groups did not show an effect on either plasma ANP or BNP levels.

**Effect of E4010 on Plasma NO Levels.** There were no significant differences in the plasma NO levels among the four groups (Fig. 6).

**Effects of E4010 on Mortality.** All rats in the control group survived for the entire experimental period. The survival rate of the MCT group decreased gradually to 28.1% at day 23 (P < .01 versus control). Although E4010 0.01% did not significantly decrease the mortality (50.0%), E4010 0.1% markedly improved the survival rate: 84.4% (P < .01 versus MCT, Fig. 7).

**Discussion**

The major finding of this study was that long-term treatment with a selective and potent PDE5 inhibitor, E4010, improved the mortality in rats with pulmonary hypertension induced by MCT. This favorable effect of E4010 was associated with an amelioration of right ventricular hypertrophy, and increased cGMP levels in both plasma and lung.

A single MCT injection causes vascular endothelial damage, medial thickening of the muscularization of pulmonary arterioles, increase in pulmonary arterial pressure, and development of right ventricular hypertrophy (Ghods and Will, 1981). This hypertrophic heart shows a decreased inotropic response that is similar to that of human failing myocardium (Parker et al., 1991; Brown et al., 1998). In addition, this increased wall stretch led by both cardiac hypertrophy and pulmonary hypertension induced the elevation of plasma ANP (Hirata et al., 1992; Comini et al., 1995) and BNP (Hill et al., 1994) levels, and then resulted in an increase in plasma cGMP level (Hirata et al., 1992). In our study, we observed that right ventricular hypertrophy was accompanied by the elevation of plasma ANP, BNP, and cGMP levels, similar to previous reports, as well as the elevation of plasma cAMP level. In MCT-treated pulmonary hypertensive rats, it has been shown that adrenomedullin, which activates adenylate cyclase, was increased (Shimokubo et al., 1995). Thus, elevated plasma cAMP might be the reflection of increased adrenomedullin in MCT-treated rats. Clinically, in addition to the increased plasma ANP, BNP, and cGMP levels (Hirata et al., 1987; Yasue et al., 1994), a rise in plasma adrenomedullin level has also been reported in patients with heart failure (Jougasaki et al., 1996). Taken together, these characteristic changes in right ventricular hypertrophy accompanied by decreased cardiac contraction and plasma biochemical parameters suggest that the cause of the increased mortality in MCT-treated rats might be induced by pulmonary hypertension followed by right heart failure.

Zaprinast, a classical PDE5 inhibitor, dilates the isolated human pulmonary arteries (Rabe et al., 1994) and selectively decreases pulmonary arterial pressure in pulmonary hypertensive dogs induced by U46619 (Braner et al., 1993). Similarly, E4021 also has been reported as a selective pulmonary vasodilator (Cohen et al., 1996; Takahashi et al., 1996b; Yamaguchi et al., 1998). Considering that the novel PDE5 inhibitor E4010, which we have recently found is more potent

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**TABLE 1**

BW changes

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 23</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>93 ± 1 (n = 8)</td>
<td>156 ± 1 (n = 8)</td>
<td>210 ± 5 (n = 8)</td>
<td>267 ± 4 (n = 8)</td>
<td>282 ± 5 (n = 8)</td>
</tr>
<tr>
<td>MCT</td>
<td>95 ± 1 (n = 32)</td>
<td>147 ± 1 (n = 32)</td>
<td>190 ± 5 (n = 32)</td>
<td>196 ± 5 (n = 18)</td>
<td>188 ± 9 (n = 9)</td>
</tr>
<tr>
<td>E4010 0.01%</td>
<td>93 ± 1 (n = 32)</td>
<td>145 ± 1 (n = 32)</td>
<td>197 ± 5 (n = 32)</td>
<td>204 ± 3 (n = 26)</td>
<td>203 ± 5 (n = 16)</td>
</tr>
<tr>
<td>E4010 0.1%</td>
<td>95 ± 1 (n = 32)</td>
<td>144 ± 2 (n = 32)</td>
<td>198 ± 2 (n = 32)</td>
<td>212 ± 4 (n = 31)</td>
<td>206 ± 5 (n = 27)</td>
</tr>
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</table>
IC$_{50}$ = 5.66 nM; Ishihara et al., 1998) than E4021 (IC$_{50}$ = 3.9 nM; Saeki et al., 1995), we expect that E4010 would also be valuable for the treatment of pulmonary hypertension.

Both a prostacyclin analog (Terao, 1997) and a Ca$^{2+}$ blocker (Takahashi et al., 1996a) have been shown to improve MCT-induced mortality, which is associated with a decrease in elevated pulmonary arterial pressure. Thus, the amelioration of pulmonary hypertension is the key to improving the survival rate in MCT-induced pulmonary hypertensive rats. In the present study, E4010 improved the MCT-induced morbidity associated with an amelioration of right ventricular hypertrophy. Because MCT-induced right ventricular progression is in parallel with the increase in pulmonary arterial pressure (Miyauchi et al., 1993), we can use the degree of the right ventricular weight as an index of pulmonary arterial pressure. Accordingly, on the basis of the suppressive effect of E4010 on right ventricular hypertrophy, one possible explanation for the favorable effect of E4010 on the mortality in MCT-treated rats may be related to the decrease in the elevated pulmonary arterial pressure. We observed in other studies that long-term administration with diet containing E4010 0.1% decreased pulmonary arterial pressure in conscious rats with pulmonary hypertension induced by chronic hypoxia (Hanasato et al., submitted). Because E4010 amplified vasorelaxation induced by ANP in isolated porcine pulmonary arteries (Ishihara et al., 1998), we believe that the ameliorative effect of E4010 on pulmonary hypertension is

<table>
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<th>TABLE 2</th>
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<tr>
<td>Organ weight</td>
</tr>
<tr>
<td>Values are mean ± S.E.M. Number of animals is shown in parentheses. The measurements of organ weight were performed day 23 after the injection of MCT.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>RV/BW (mg/g)</th>
<th>LV/S/BW (mg/g)</th>
<th>RV+LV/S/BW (mg/g)</th>
<th>RV/LV+S/BW (%)</th>
<th>LU/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.49 ± 0.01</td>
<td>2.22 ± 0.02</td>
<td>2.72 ± 0.02</td>
<td>22.2 ± 0.4</td>
<td>3.85 ± 0.03</td>
</tr>
<tr>
<td>MCT (n = 9)</td>
<td>1.77 ± 0.07*</td>
<td>3.52 ± 0.14</td>
<td>4.29 ± 0.15*</td>
<td>72.6 ± 6.0*</td>
<td>7.41 ± 0.43</td>
</tr>
<tr>
<td>E4010 0.01% (n = 16)</td>
<td>1.56 ± 0.06*</td>
<td>2.63 ± 0.07</td>
<td>4.19 ± 0.12</td>
<td>59.4 ± 1.8*</td>
<td>7.69 ± 0.43</td>
</tr>
<tr>
<td>E4010 0.1% (n = 27)</td>
<td>1.57 ± 0.04*</td>
<td>2.72 ± 0.07</td>
<td>4.30 ± 0.10</td>
<td>58.2 ± 1.3*</td>
<td>8.06 ± 0.32</td>
</tr>
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* P < .01 versus control.
† P < .05.
‡ P < .01 versus MCT.

![Fig. 3. Effects of long-term E4010 treatment on plasma cGMP (A) and cAMP (B) levels in rats with pulmonary hypertension induced by MCT. Values are mean ± S.E.M. Control (vehicle-treated rats fed MF, n = 8), MCT (MCT-treated rats fed MF, n = 9), E4010 0.01% (MCT-treated rats fed MF containing E4010 0.01%, n = 16), E4010 0.1% (MCT-treated rats fed MF containing E4010 0.1%, n = 27). * P < .05, † P < .01 versus control; **P < .01 versus MCT.](image)

![Fig. 4. Effects of long-term E4010 treatment on lung cGMP (A) and cAMP (B) levels in rats with pulmonary hypertension induced by MCT. Values are mean ± S.E.M. **P < .01 versus MCT.](image)

![Fig. 5. Effect of long-term E4010 treatment on plasma ANP (A) and BNP (B) levels in rats with pulmonary hypertension induced by MCT. Values are mean ± S.E.M. **P < .01 versus control.](image)

![Fig. 6. Effect of long-term E4010 treatment on plasma NO level in rats with pulmonary hypertension induced by MCT. Values are mean ± S.E.M. ## P < .01 versus MCT.](image)

![Fig. 7. Survival curves of rats with pulmonary hypertension induced by MCT. Survival rates were monitored during the treatment period from day 0 to 23. Day 0 indicates the time of MCT injection and the beginning of drug treatment. •, vehicle-treated rats fed MF; ○, MCT-treated rats fed MF; ●, MCT-treated rats fed MF containing E4010 0.01%; ▲, MCT-treated rats fed MF containing E4010 0.1%. **P < .01 versus vehicle-treated rats fed MF; ***P < .01 versus MCT-treated rats fed MF.](image)
brought about by the augmentation of pulmonary arterial relaxation induced by elevated ANP and BNP. Furthermore, the elevation of both plasma and lung cGMP levels without effect on cAMP levels in the E4010-treated groups suggests that E4010 should selectively inhibit PDE5 in an in vivo model.

Owing to its small effect on systemic blood pressure, inhalation of NO is at present being applied as a clinical remedy for patients with pulmonary hypertension (Adnot et al., 1993). On the other hand, the NO inhalation therapy has some disadvantages, such as short-lasting vasodilator activity and no proof of safety with long-term inhalation. Zapristin has been shown to prolong the pulmonary vasodilating effect induced by NO inhalation in lambs (Ichinose et al., 1995). Thus, in this report we suggest that the combined use of a PDE5 inhibitor and NO inhalation for the treatment of pulmonary hypertension would be desirable from the point of view of the long-lasting pulmonary hypotensive effect and the decline in the demand for NO. Moreover, in pulmonary hypertensive rats, the activity of PDE5 in the pulmonary artery was elevated and might cause an increased pulmonary vascular resistance (Macleain et al., 1997). These results further support the clinical usefulness of a PDE5 inhibitor for the treatment of pulmonary hypertension.

In conclusion, long-term E4010 administration to MCT-induced pulmonary hypertensive rats improved mortality. These results suggest that orally effective E4010, a selective and potent PDE5 inhibitor, could be potentially useful for the treatment of the patients with pulmonary hypertension and might prolong their life expectancy.

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References

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