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# DA-8159, a Potent cGMP Phosphodiesterase Inhibitor, Attenuates Monocrotaline-Induced Pulmonary Hypertension in Rats

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In this study, we evaluated the effects of oral administration of DA-8159, a selective phosphodiesterase-5 inhibitor, on the development of pulmonary hypertension (PH) induced by monocrotaline (MCT). Rats were administered either MCT (60 mg/kg) or saline. MCT-treated rats were divided into three groups and received orally administered vehicle, or 1 mg/kg or 5 mg/kg of DA-8159, twice a day for twenty-one days. The MCT group demonstrated increased right ventricular weights, medial wall thickening in the pulmonary arteries, myocardial fibrosis and the level of plasma cyclic guanosine monophosphate (cGMP), along with decreased body weight gains. However, DA-8159 markedly and dose-dependently reduced the development of right ventricular hypertrophy and medial wall thickening. DA-8159 also amplified the increase in plasma cGMP level and significantly increased the level of lung cGMP, compared with the MCT group. Although the body weight gain was still lower from the saline-treated control group, DA-8159 demonstrated a significant increase in body weight gains, in both 1 mg/kg and 5 mg/kg groups, when compared with the MCT group. In myocardial morphology, MCT-induced myocardial fibrosis was markedly prevented by DA-8159. These results suggest that DA-8159 may be a useful oral treatment option for PH.

**Key words:** Pulmonary hypertension (PH), Monocrotaline (MCT), Phosphodiesterase (PDE) inhibitor, DA-8159

## INTRODUCTION

Pulmonary hypertension (PH) is a disease with a median survival of 2.8 years from the time of diagnosis (Rich *et al.*, 1987) and is associated with progressive elevation of pulmonary arterial pressure, ultimately inducing heart failure and death. PH is characterized by increased pulmonary vascular resistance results in an inability of the right ventricle to sustain its output and leads to right ventricular failure and death (Hanasato *et al.*, 1999). Various vasodilators, such as prostacyclin and calcium antagonist, have been used in the treatment of PH. The continuous i.v. infusion of prostacyclin to patients with PH has been shown to reduce mortality rates by decreasing the pulmonary arterial pressure (Barst *et al.*, 1996). However, the application of systemic prostacyclin is limited by catheter infection, systemic hypotension, tachyphylaxis and a lack of selectivity for the pulmonary vasculature. Inhaled nitric oxide (NO) has been established as an effective therapy of selective pulmonary vasodilator (Frostell *et al.*, 1991; Pepke-Zaba *et al.*, 1991). However, there are problems associated with long-term use of NO inhalation, including its potential toxicity and difficulty in ambulatory inhalation (Troncy *et al.*, 1997). Therefore, the development of an orally effective and long-lasting agent for PH with few side effects would be very valuable, because no selective pulmonary vasodilator is available at present.

NO and natriuretic peptides are important factors in the regulation of pulmonary arterial vascular tone. Their vasorelaxant action is mediated by cyclic guanosine monophosphate (cGMP). Pulmonary vascular cGMP levels can be elevated by inhibiting the phosphodiesterases (PDEs) responsible for cGMP hydrolysis in the lung. PDE5 is the major cGMP-dependent PDE subtype in the pulmonary vasculature and is more abundant in the lung than other tissues (Thomas *et al.*, 1990). This offers the possibility that inhibitors of the cGMP-hydrolyzing PDE5 induce pulmonary vasodilation and can be useful for PH. Recently, cGMP specific PDE5 inhibitors have been de-

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monstrated to be potent, acute, selective pulmonary vasodilators (Hanasato *et al.*, 1999; Kodama and Adachi, 1999 Schermuly *et al.*, 2001a, b; Weimann *et al.*, 2000; Wilkens *et al.*, 2001; Zhao *et al.*, 2001). However, until now, there has been no approved PDE5 inhibitor as a treatment of PH for clinical use, mainly because of the nonspecific effects on other PDEs and on adenosine metabolism (Ziegler *et al.*, 1998). More recently, sildenafil, alone or combined with iloprost, showed a reduction of pulmonary artery pressure and an improvement of excise capacity in PH patients, and it is now being evaluated in clinical trials (Abrams *et al.*, 2000; Prasad *et al.*, 2000). This evidence suggests that PDE5 is a potential pharmacological target for pulmonary vasodilation.

DA-8159 is a selective and potent PDE5 inhibitor which is being developed by Dong-A Pharmaceutical Company (Kyunggi, Korea) as an oral drug for the treatment of erectile dysfunction. According to the results obtained from preliminary studies, DA-8159 has superior isozyme selectivity and a longer half-life than sildenafil (Shim *et al.*, 2001). Furthermore, it enhances and prolongs the action of cGMP, a primary mediator of vasodilation, by selectively inhibiting the cGMP-specific PDE5 isozyme. In this study, we examined the effect of long-term oral administration of DA-8159 on rats with PH induced by MCT

## MATERIALS AND METHODS

### Chemicals

DA-8159, a pyrazolopyrimidinone derivative with a molecular weight of 516.66, was synthesized by Dong-A Pharmaceutical Co. (Kyunggi, Korea) to >99.8% purity as determined by both HPLC and potentiometric titrations in glacial acetic acid. DA-8159 was stable for at least 12 weeks both at the ambient temperature and the accelerated condition (20°C, 75% RH). Monocrotaline (MCT) and sodium nitroprusside (SNP) were obtained from Sigma Chemical Co. (St. Louis, MO). DA-8159 was dissolved in Titrisol® buffer solution (citrate sodium hydroxide buffer, pH 5.0, Merck) for oral administration.

### Animals

The studies were performed in accordance with the institutional *Standard Procedure for Animal Care and Experiments* (SOP-ANC) of the Dong-A Pharmaceutical Company and with the "Guide for the Care and Use of Laboratory Animals" from the National Institute of Health. Male Sprague-Dawley rats (230~250 g; Charles River Japan, Japan) were used in this study. During the experiments, the animals were kept under standard laboratory conditions and allowed free access to food and UV-sterilized tap water.

### Experimental protocol

PH was induced by the administration of MCT according to the previously described method (Chen *et al.*, 2001). Adult, male Sprague-Dawley rats were maintained in a conditioned room (temperature 23±2°C, humidity range 40~70%, 12 h light/dark cycle with lighting from 7:00 to 19:00) for 1 week prior to the experiment. After acclimatization, the animals were randomized to receive subcutaneously administered MCT (60 mg/kg body weight) or an equal volume of vehicle (saline). MCT was prepared by dissolution in 1 N HCl. The pH was neutralized with 0.5N NaOH, and the volume of the solution was adjusted with phosphate-buffered saline (pH 7.40) to achieve a concentration of 30 mg/mL. After a single injection of MCT or vehicle, rats were immediately divided into four groups and subsequently maintained for a further 21 days on their respective treatment. DA-8159 or vehicle was administered orally twice a day (8:00 AM and 8:00 PM) in the following experimental groups; (1) Control (n=5):Rats treated with vehicle, (2) MCT control (n=20) :Rats treated with MCT and vehicle, (3) DA-8159 1 mg/kg (n=20):Rats treated with MCT and DA-8159 1 mg/kg, and (4) DA-8159 5 mg/kg (n=20):Rats treated with MCT and DA-8159 5 mg/kg.

At the end of the experiment (day 21), the rats were anesthetized with pentobarbital, and the chest and abdominal cavities were quickly opened. A blood sample was collected from the abdominal aorta into a plastic tube containing 3.8% sodium citrate, and then centrifuged at 3,000 rpm for 5 min at 4°C to separate the plasma. The plasma was stored at 20°C until used for the measurement of plasma concentrations of cGMP and NO.

### Measurement of organ weight

The heart and lungs were dissected, any excess blood was removed, and the organs were weighed. The ratios of various organ weights to body weight (BW) were calculated. The right ventricle free wall was separated from the left ventricle and the septum to determine the wet weight. The ratios of right ventricle weight to body weight (RV/BW), left ventricle plus septum weight to body weight (LV + S/BW) and cardiac weight (RV + LV + S/BW) were also calculated. As an index of right ventricular hypertrophy, the ratio of the right ventricle weight to the left ventricle plus septum weight (RV/LV + S) was calculated. After measuring the weight, the lung was frozen in liquid nitrogen and stored at 70°C until used for the measurement of the tissue concentrations of cGMP.

### Measurement of cyclic GMP levels in plasma and lung

The concentration of cGMP was measured with an

enzyme immunoassay (EIA) kit (Cayman Chemical, USA) according to the method of Dundore *et al.* (1993) and the kit manufacturers instructions. To determine the cGMP levels in the plasma, 2 mL of ice-cold ethanol was added to 500 μL of plasma and centrifuged at 1500 g for 10 min at 4°C. The supernatant was then evaporated by vacuum centrifugation (speed vacuum) and was used for cGMP measurement. For the measurement of intracellular cGMP levels in the lung, each tissue sample was homogenized in 1 mL of 0.1 N HCl. The homogenate was centrifuged at 3,000 g at 4 °C for 10 min, and the supernatant was evaporated. The concentration of cGMP in the residue was determined using an EIA kit (Cayman Chemical, USA). The protein content of the pellet was determined using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., USA). The cGMP level in the lung was normalized to the amount of protein.

### Measurement of plasma NO levels

Plasma NO level was determined using the method reported by Kodama *et al.* (1999). An aliquot of 100 μL of plasma was filtered through a 10 or 30 kDa molecular weight cut-off filter (Amicon, Beverly, MA, USA), and centrifuged at 11,000 g for 10 min at 4°C. The concentration of NO was measured with a Nitrate/Nitrite colorimetric assay kit (LDH method, Cayman Chemical, Ann Arbor, MI, USA).

### Measurement of pulmonary artery wall thickness

The medial wall thickness was measured according to the method described by Takahashi *et al.* (1996) and Chen *et al.* (2001). In brief, the left lung of each animal was collected, fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin for light microscopy. The thickness of the medial arterial layer and the external diameter were measured in 10 randomly selected vessels of each rat. The percent medial wall thickness, an index of medial hypertrophy, was calculated using the method of Cassis *et al.* (1992) as follows: {(2 × medial thickness)/external diameter} × 100.

### Light microscopy

Myocardial and lung tissues were fixed by 10% buffered formalin and embedded in paraffin for morphological analyses. Five-micrometer sections were stained with hematoxylin-eosin or Masson's trichrome stain.

### Statistics

Data are expressed as the mean±standard deviation (S.D.). Data were analyzed by one-way analysis of variance (ANOVA). A p value of <0.5 was considered to be statistically significant.

## RESULTS

### Body weight changes

Animals treated with MCT showed significantly decreased body weight gains over the 3-week follow-up period. However, although body weight gain was still lower than the saline-treated control group, DA-8159 treated animals demonstrated a significant increase in body weight gains over the 3-week period compared with the MCT treated animals (Table I).

### Effect of DA-8159 on right ventricle tissue weight

The MCT group developed right ventricular and cardiac hypertrophy, compared with control (saline), and demonstrated significant increases in the ratios of RV/BW, RV/LV+S, and RV+LV+S/BW (Table II). These changes were inhibited by DA-8159 in a dose-dependent manner. Right ventricular hypertrophy and cardiac hypertrophy were improved in both DA-8159 1 mg/kg and 5 mg/kg treated groups, as shown by decreases in RV/LV+S and RV+LV+S/BW ratios (Table II).

### Cyclic GMP levels in plasma and lung

In the MCT group, plasma cGMP level was significantly greater than that of control (Fig. 1A, $p<0.05$). On the contrary, MCT did not influence the lung cGMP level (Fig. 1B). DA-8159 elevated plasma cGMP and lung cGMP levels in a dose-dependent manner. The increase in plasma

**Table I.** Body weight changes in control and DA-8159 treated animals

| Parameter | Control | | DA-8159 | |
|---|---|---|---|---|
| | Saline | MCT | 1 mg/kg | 5 mg/kg |
| Baseline BW (g) | 287±10.6 | 285.7±8.3 | 286.8±9.6 | 288.0±9.6 |
| BW (g) | 92.2±9.2 | 41.3±12.2[a] | 70.2±13.1[a,b] | 78.6±16.0[b] |

Baseline BW, body weights immediately prior to MCT administration; BW, increase in body weight over the 3-week follow-up period.
[a,b] significantly different from the saline-control and MCT group, respectively ($p<0.05$).

**Table II.** Effects of DA-8159 on heart weights in MCT-induced PH animals

| Parameter | RV/BW (mg/g) | LV + S/BW (mg/g) | RV + LV + S/BW (mg/g) | RV/LV+S (%) |
|---|---|---|---|---|
| Control | 0.49±0.04 | 2.00±0.24 | 2.49±0.27 | 24.4±1.74 |
| MCT | 1.61±0.12[a] | 2.34±0.26[a] | 3.95±0.26[a] | 68.8±4.72[a] |
| DA-8159 1 mg/kg | 1.26±0.03[a,b] | 2.18±0.12 | 3.44±0.14[a,b] | 57.9±2.66[a,b] |
| DA-8159 5 mg/kg | 1.16±0.08[a,b] | 2.11±0.24 | 3.27±0.31[a,b] | 54.7±3.46[a,b] |

RV/BW, right ventricle weight to body weight; LV + S/BW, left ventricle plus septum weight to body weight; RV + LV + S/BW, right ventricle, left ventricle plus septum weight to body weight; RV/LV + S ratio, the ratio of the right ventricle weight to left ventricle plus septum weight. [a]$p<0.05$, [b]$p<0.05$ from control and MCT, respectively.

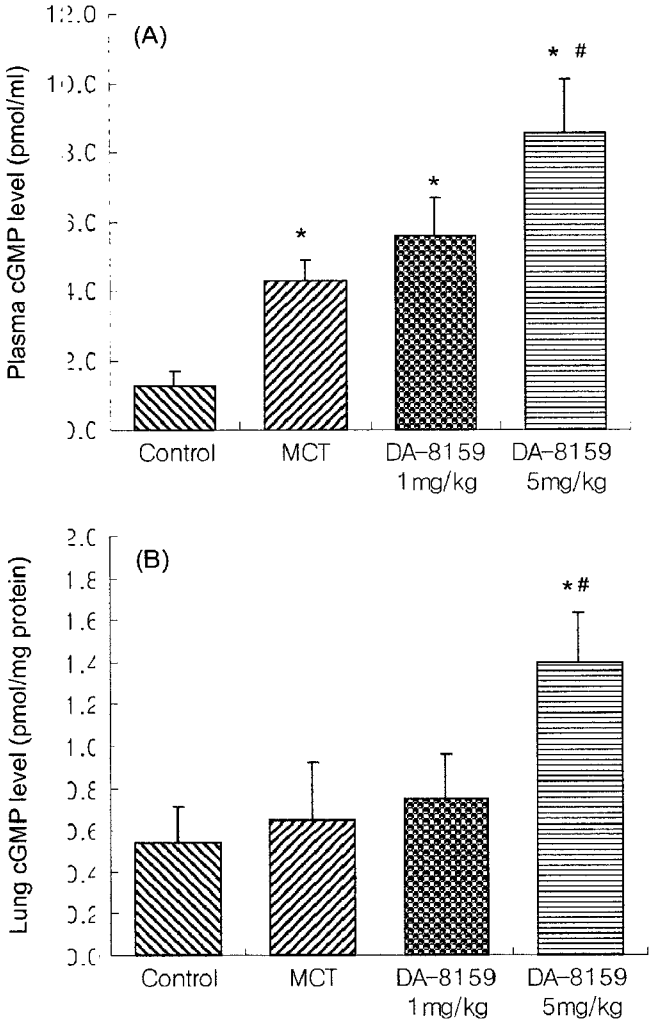


**Fig. 1** Effects of DA-8159 on plasma cGMP (A) and lung cGMP levels (B) in rats with pulmonary hypertension induced by MCT. Values are expressed as mean±S.D. *; Significantly different from the control ($p<0.05$). #; Significantly different from the MCT group ($p<0.05$).

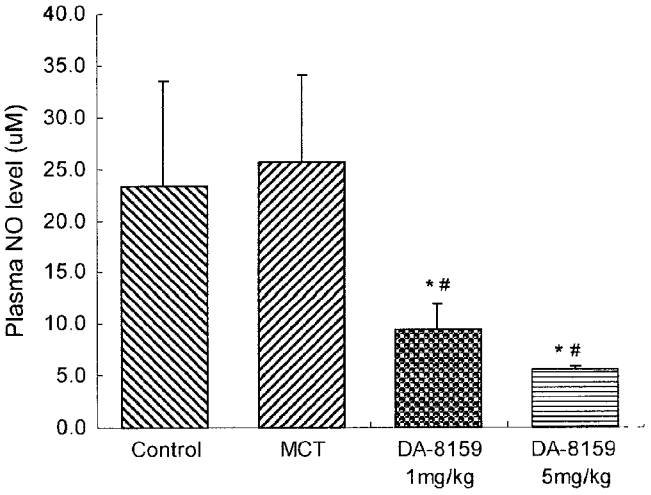


**Fig. 2.** Effects of DA-8159 on plasma NO level in rats with pulmonary hypertension induced by MCT. Values are expressed as mean±S.D. *; Significantly different from the control ($p<0.05$). #; Significantly different from the MCT group ($p<0.05$).

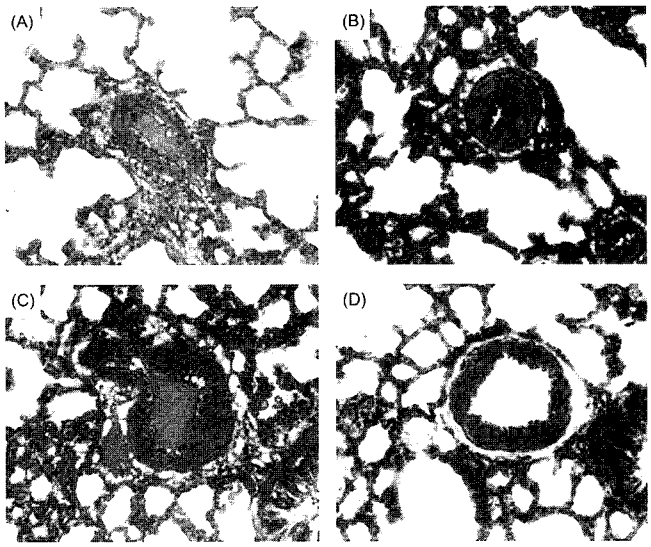


**Fig. 3.** Representative micrographs of pulmonary artery vessels after vehicle (A), or MCT administration without (B) or with (C) DA-8159 (1 mg/kg; D, 5 mg/kg). Massons trichrome stain : Magnification, ×400.

cGMP levels was amplified significantly in the groups treated with DA-8159 1 mg/kg and 5 mg/kg (Fig. 1A, $p<0.05$) compared with the MCT group. Lung cGMP level was also increased significantly in the group treated with DA-8159 5 mg/kg, compared with control or MCT (Fig. 1B, $p<0.05$).

### Plasma NO levels

Treatment with MCT did not affect plasma NO levels. However, DA-8159 decreased the plasma NO levels significantly, both in low- and high-dose groups, in a dose-dependent manner (Fig. 2).

### Medial wall thickness of pulmonary artery

Fig. 3 and Fig. 4 show the effect of DA-8159 on the development of MCT-induced pulmonary vascular wall thickness. MCT produced a significant increase in medial wall thickening. However, DA-8159 treated rats showed a significant reduction in medial wall thickness, which was comparable to that of the control group.

### Myocardial morphology

No histopathological abnormalities were observed in the hearts of animals treated with DA-8159. On the contrary, right ventricle samples from MCT-treated animals showed the presence of focal fibrosis. Representative micrographs of fibrosis are shown in Fig. 5.

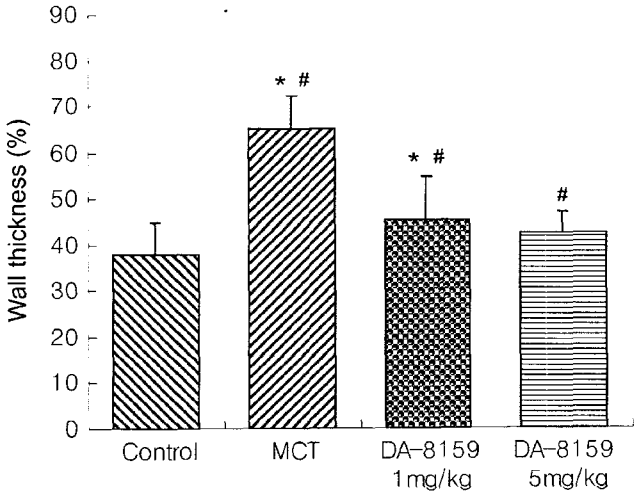


**Fig. 4.** Effects of DA-8159 on the development of medial wall thickness of pulmonary arteries induced by MCT. Values are expressed as mean±S.D. *; Significantly different from the control ($p<0.05$). #; Significantly different from the MCT group ($p<0.05$).

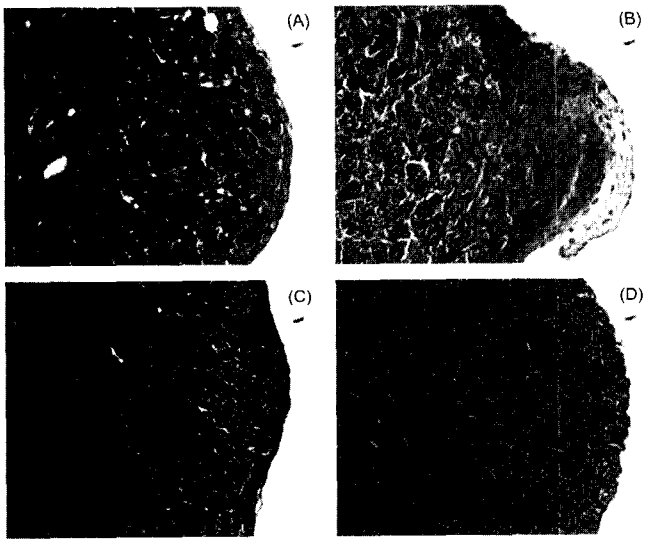


**Fig. 5.** Representative micrographs from the right ventricular myocardium of vehicle treated rat (A), MCT treated rat (B), DA-8159 1 mg/kg (C), and DA-8159 5 mg/kg treated rat (D). Massons trichrome stain : Magnification, ×400.

## DISCUSSION

The present study demonstrated that long-term oral administration of a selective PDE5 inhibitor, DA-8159, markedly and dose-dependently attenuated the development of right ventricular hypertrophy in rats with MCT-induced PH. These favorable effects of DA-8159 might be associated with the increase in cGMP levels, both in plasma and lung.

The MCT-induced PH model is one of the most widely studied animal models. Pyrrolizidine alkaloid MCT produces its effects primarily by injuring the pulmonary vessel endothelium, resulting in reduced vasodilating responses, increased vascular resistance, compensatory right ventricular hypertrophy and heart failure (Schultze and Roth, 1998; Rosenberg and Rabinovitch, 1998; Nakazawa *et al.*, 1999; Pichardo *et al.*, 1999). This experimental model is well characterized and resembles clinical pulmonary vascular disease. In addition, this model is advantageous in the sense that the myocardial involvement occurs secondarily to initiating factors that are of non-cardiac origin, thus allowing the possibility of closer mechanistic assessment of therapeutic strategies.

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 via activation of soluble guanylate cyclase (GC) and consequent activation of protein kinase G (Dinerman *et al.*, 1993). In addition to GC activity, the intracellular concentration of cGMP is determined by PDE activity that rapidly inactivates cGMP to GMP (Polson and Strada, 1996). In other words, the intracellular concentration of cGMP and smooth muscle cell relaxation is governed by the balance between GC and PDE activities (Hanasato *et al.*, 1999).

Among PDE isoenzymes, PDE5, which is an isoenzyme that specifically hydrolyzes cGMP, has been reported to be distributed abundantly in lung tissue. It is also reported that PDE5 activity is much higher in lung than in aortic tissue of chronically hypoxic PH rats (Yamaguchi *et al.*, 1998). Thus, inhibition of this enzyme by selective inhibitors is likely to increase lung tissue cGMP levels and cause pulmonary vasodilation. Indeed, previous studies have demonstrated that the selective PDE5 inhibitors produce pulmonary vasodilation and attenuate the development of PH in animal models (Hanasato *et al.*, 1999; Kodama and Adachi, 1999; Zhao *et al.*, 2001). Dipyridamole and zaprinast attenuated hypoxia-induced pulmonary vasoconstriction and remodeling (Rosenkrantz *et al.*, 1972; McMahon *et al.*, 1993; Braner *et al.*, 1993), but their clinical use is limited by their lack of selectivity. E4021 and E4010, selective PDE5 inhibitors, have been reported to reduce pulmonary artery pressure (PAP) in animal models of PH but are not available for use in humans (Cohen *et al.*, 1996; Dukarm *et al.*, 1999; Hanasato *et al.*, 1999; Kodama and Adachi, 1999). More recently, sildenafil, an orally active inhibitor of PDE5 for the treatment of erectile dysfunction, has been reported to produce dose dependent reductions of PAP in animal models and in clinical trials (Weimann *et al.*, 2000; Zhao *et al.*, 2001), but is not approved for clinical use.

In the present study, we used a novel selective PDE5

inhibitor, DA-8159, which is being developed by Dong-A Pharmaceutical company (Kyunggi, Korea) for the treatment of erectile dysfunction. The $IC_{50}$ value of DA-8159 for PDE5 is 5 ng/mL, which is comparable to that of sildenafil. A phase I clinical study showed that the half-life of DA-8159 in humans is about 10 h, which is longer than that of sildenafil. Accordingly, the primary goal of this study was to evaluate the potential contribution of DA-8159 to compensatory myocardial hypertrophy and subsequent responses that occur after MCT-induced PH.

Weight gain, which has been shown to be greatly reduced after MCT administration (Kodama and Adachi, 1999), was significantly higher in the DA-8159 groups than in MCT alone. These results demonstrate that animals on DA-8159 treatment fared better than their untreated controls, suggesting a marked reduction in mortality in these animals.

DA-8159 markedly and dose-dependently attenuated the MCT-induced right ventricular hypertrophy, which is thought to be the result of increased pulmonary vascular tone. In addition, the medial wall thickness in pulmonary arteries was reduced in DA-8159 treated animals, and the plasma NO level was also decreased significantly. Although the mechanism responsible for the effects of DA-8159 is not clearly elucidated, the pulmonary vasodilating effect of DA-8159 via increasing cGMP levels in the smooth muscle is considered to contribute, at least in part, to the prevention of right ventricular hypertrophy and medial wall thickening.

In this study, we measured cGMP levels and found that DA-8159 increased cGMP levels in plasma and lung. Considering the specific inhibition activity of DA-8159 on PDE5, these results suggest that DA-8159 is effective in both lung and plasma, supporting the hypothesis that chronic treatment with selective PDE5 inhibitor would preferentially decrease the cGMP degradation rate and increase intracellular cGMP concentration in lung tissue due to the predominant distribution of this isoenzyme in the lung. Therefore, we considered that the amelioration effects of DA-8159 on PH occurred as a result of augmentation of the pulmonary arterial relaxation induced by elevated cGMP levels. These results were comparable to those of other PDE5 inhibitors such as E-4010 and sildenafil (Hanasato *et al.*, 1999; Zhao *et al.*, 2001).

In myocardial morphology, right ventricle samples from MCT-treated animals showed the presence of focal myocyte degeneration (data not shown) and fibrosis. On the contrary, there were no histopathological abnormalities, including fibrotic changes, in the group treated with 5 mg/kg of DA-8159. These results suggest that the beneficial effects of DA-8159 were also observed in respects to the morphological abnormalities.

In summary, our results demonstrated that DA-8159, an orally active, selective PDE5 inhibitor, markedly and dose-dependently attenuated the development of MCT-induced PH. The beneficial effects were clearly observed with respect to a variety of indices, including reduced RV hypertrophy, medial wall thickness in pulmonary arteries, body weight gains and abrogation of morphological changes. These effects were probably associated with an increase in cGMP levels in pulmonary vascular smooth muscle and plasma. From these results, we concluded that DA-8159, a selective PDE5 inhibitor, may be useful as an orally active treatment for PH, with the proviso that the safety and efficacy of administration need to be studied in humans.

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