Cilostazol Therapy Attenuates Monocrotaline-Induced Pulmonary Arterial Hypertension in Rat Model

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Background  Pulmonary arterial hypertension (PAH) is characterized by a progressive increase in pulmonary vascular resistance caused by a proliferation of vascular endothelial and smooth muscle cells, resulting in occlusion of the lumen of small pulmonary arteries. Cilostazol, with its antiproliferative effects on vascular endothelial and smooth muscle cells, may ameliorate monocrotaline (MCT)-induced PAH in rats.

Methods and Results  Male Sprague–Dawley rats (n=10/each group) were randomized to receive MCT (75 mg/kg) only (group 1), MCT plus cilostazol (20 mg·kg–1·day–1) (group 2) and saline injection only (group 3). Hemodynamic measurement on day 28 following MCT treatment indicated the development of significant PAH on MCT-treated groups (p<0.0001). Cilostazol was given to group 2 orally on days 28–90. By day 90 following MCT treatment, the right ventricular (RV) systolic blood pressure and RV hypertrophy were significantly higher in group 1 than in groups 2 and 3 (all values of p<0.01). Additionally, connexin43 and endothelial nitric oxide synthase gene expressions of lung and RV, and Bcl-2 protein expression of RV, were significantly lower in group 1 than in groups 2 and 3 (all values of p<0.01). Furthermore, the number of alveolar sac and small arterioles of the lung were significantly lower in group 1 than in groups 2 and 3 (all values of p<0.01).

Conclusion  Cilostazol therapy effectively attenuates of MCT-induced PAH. (Circ J 2008; 72: 825–831)

Key Words: Cilostazol therapy; Monocrotaline; Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is caused by various pathophysiological mechanisms and characterized by a progressive increase in pulmonary vascular resistance caused by vascular cell proliferation and obliteration of pulmonary microvasculature, leading to severe pulmonary hypertension, right side heart failure and death.1–7 Strategic managements for PAH include long-term oxygen or inhaled nitric oxide therapy, diuretic therapy, anticoagulant agents, vasodilators, calcium channel blocker agents, intravenous prostacyclin, phosphodiesterase inhibitors and endothelin antagonists.8–14 However, all of these therapies remain problematic due to either high cost, limited effectiveness or serious side effects.

Regarding the mechanistic basis of PAH, inhibiting the abnormal proliferation of vascular endothelial and smooth muscle cells may be an effective therapeutic strategy for preventing progressive PAH.15,16 Cilostazol, a phosphodiesterase III inhibitor approved by the US Food and Drug Administration (FDA) for treatment of intermittent claudication, reduces smooth muscle proliferation and intimal hyperplasia after endothelial injury and lowers restenosis after coronary artery stenting.17–20 However, whether cilostazol therapy can reverse PAH through inhibition of endothelial and smooth muscle cell proliferation has not been reported. Therefore, the present study tested the hypothesis that cilostazol therapy can effectively attenuate PAH after the administration of monocrotaline (MCT), which is well known to induce selective pulmonary endothelial injury in the rat.

Methods

Ethics, Experimental Procedures  All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at our hospital and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996).

All animal experimental procedures were done by investigators who blinded to the experimental conditions of all rats.

Animal Models of PAH and Study Sample  On day 0, pathogen-free, adult male Sprague-Dawley (SD) rats (n=20), weighing 300–320 g (Charles River Technology, BioLASCO Taiwan Co, Ltd, Taiwan) were given 1 subcutaneous injection of MCT (75 mg/kg; Sigma, St. Louis, MO, USA).

On day 28, after right ventricular systolic blood pressure (RVSBP) was measured in each rat, MCT-treated rats were assigned to 2 experimental groups: group 1 (MCT alone,
n=10) or group 2 (cilostazol treatment, n=10). Another 10 SD rats (group 3) received only subcutaneous injection of 2 cc saline and served as the control group.

The Rationale for Cilostazol Therapeutic Dose

Before the present study, we had tested the therapeutic doses of cilostazol of 10 mg·kg⁻¹·day⁻¹ and 20 mg·kg⁻¹·day⁻¹ orally on 6 rats (3 rats for each individual dose) from day 28 to day 60 after MCT-induced PAH. We found that the ability to inhibit the proliferation of lung parenchyma was better on a dose of 20 mg·kg⁻¹·day⁻¹ than on the dose of 10 mg·kg⁻¹·day⁻¹ (Fig 1). Thus, the group 2 rats were given 20 mg·kg⁻¹·day⁻¹ doses of cilostazol from day 28 to day 90 before they were killed on day 90.

Hemodynamic Studies and Tissue Sample Collection

Proximal pulmonary arterial blood flow (PABF) was measured using a commercially available echocardiographic system (UF-750XT) equipped with an 8-MHz linear-array transducer for animals (FUKUDA Denshi Co, Tokyo, Japan). The PABF was measured before MCT treatment and on days 35 and 90 following MCT treatment.

On days 28 and 90 after MCT-induced PAH, the animals were anesthetized using intraperitoneal injections of chloral hydrate (35 mg/kg). The chest was then shaved, and the rat was placed in a supine position on a warming pad at 37°C and intubated with positive-pressure ventilation (180 ml/min) with room air using a Small Animal Ventilator (SAR-830/A, CWE, Inc, Weston, WI, USA). Prior to opening the chest wall, local anesthesia with 2 cc of 2% xylocain (60 mg/kg) was injected into the third, fourth and fifth intercostal spaces. Under sterile conditions, the heart was exposed via a left thoracotomy. A sterile 20-gauge, soft-plastic coated needle was inserted into the right ventricle (RV) chamber of each rat. The needle was then pulled back, and the soft needle was connected to a hemodynamic monitor (Hewlett M1165A, Packard Model 56s) to measure the RVSBP. The muscle and skin were closed in layers and air was evacuated from the chest wall cavity using gentle compression of the chest wall with a needle holder prior to final closure of the muscle layer. On day 90, RVSBP was measured again before killing the animals and harvesting the heart and lungs. The ratio of RV to left ventricular plus septum (RV/LV+S) weight was determined as an index of RV hypertrophy. The left lung was sectioned and embedded in paraffin. The right lung was dissected into pieces, frozen in liquid nitrogen and then stored at –80°C until use.

Western Blot Analysis for Lung and RV

To evaluate the anti-apoptotic effect of cilostazol, equal amounts (30 μg) of protein extracts from RV were analyzed using Western blot for Bcl-2. In addition, to investigate the impact of cilostazol on prevention of down regulation of connexin43 (Cx43), protein aliquots (30 μg) of RV and lung were Western blotted for Cx43 according to manufacturer instructions. Protein bands were quantified using a densitometer.

Real-Time Polymerase Chain Reaction (RT-PCR) for Endothelial Nitric Oxide Synthase Expression in Lung and RV

A RT-PCR was conducted using a LightCycler TaqMan Master (Roche, Germany) in a single capillary tube according to the manufacturer’s guidelines. Forward and reverse primers (Table 1) were each designed in a different exon of the target gene sequence, eliminating the possibility for amplifying genomic DNA.

Immunolabeling of Cx43 and Quantitative Image Data Analysis

Six serial sections of RV tissue (3 longitudinal and 3 transverse) and 3 serial sections of lung tissue were prepared at 4-μm thickness using Cryostat (Leica CM3050S) for Cx43 immunolabeling. To colocalize troponin I and Cx43 or smooth-α actin and Cx43 in the same sample, the tissue sections were first incubated with a mixture of the polyclonal anti-Cx43 (1:200) plus anti-Troponin I (1:200) [for lung tissue, plus anti-smooth-α actin (1:200)] for 24 h at 4°C, followed by incubation with anti-mouse fluorescein isothiocyanate-conjugated (1:200) and anti-rabbit rhodamine (1:200) for 30 min at room temperature, respectively.

Calculation of the integrated area (μm²) of Cx43 spots in

Table 1 Primer Used for Real-Time Polymerase Chain Reaction (PCR) Amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>PCR product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>eNOS</td>
<td>NM021838-2</td>
<td>TGGAAAATTAAACGTGGCTTG</td>
<td>GCTTCCTGCTCATTTTCAA</td>
<td>112</td>
</tr>
<tr>
<td>α-actin</td>
<td>U19893-1</td>
<td>CTGGGCGCTGAGGAGTTCCG</td>
<td>CCCGTTGAATCAGCATCA</td>
<td>87</td>
</tr>
</tbody>
</table>

Fig 1. HE stain (×100) demonstrated that the lung parenchyma was notably crowded and the alveolar septum was thicker in rats with 10 mg (kg/day, orally) of cilostazol treatment (A) than in rats with 20 mg (kg/day, orally) of cilostazol treatment (B) and normal control rat (C) on day 60 after monocrotaline-induced pulmonary artery hypertension.
the slides was achieved using Image Tool 3 image analysis software (University of Texas Health Science Center in San Antonio, UTHSCSA, Image Tool for Windows, Version 3.0, USA). Three selected slides for each rat were quantified. Three randomly selected high-power fields (HPF) were analyzed in each slide. The number of pixels in each Cx43 spot per HPF was first determined, followed by summation of the pixel numbers obtained from the 3 HPF in each slide. The procedure was repeated in 2 other slides for each animal. The mean pixel number per HPF for each animal was then determined by summation of all pixel numbers divided by 9. The mean area of Cx43 per HPF was obtained by adopting a conversion factor of 19.24 (1μm² represented 19.24 pixels).

**Distribution of Alveolar Sac and Vessels in Lung Parenchyma**

HE stain and immunohistochemical staining of smooth-acting (Sigma) was performed to determine the number of alveolar space and arterioles according to manufacturer instructions. Three lung sections in each rat were evaluated to determine the number of alveolar spaces and arterioles. For quantification, 3 randomly selected HPF (×200) were analyzed in each slide. Thus, the mean number per HPF for each animal was then determined by summation of all number pixels divided by 9.

**Statistics Analysis**

Data are expressed as mean values (mean±SD). The differences in the data only between 2 groups were determined by t-test. The means among groups on Table 2 were compared using one-way ANOVA or repeated measures of ANOVA, followed by Tukey multiple comparison procedure. Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC, USA). A probability value <0.05 was considered statistically significant.

**Results**

**Body Weight on Day 0 and on Day 90 (Table 2)**

On day 0, the 3 groups did not significantly differ in weight or deceleration time (DT) (a time interval between pulmonary valve open and close) of PABF. Additional, the mean systolic blood pressure and the heart rate did not differ among 3 groups on day 90 following MCT treatment. However, the body weight was significantly higher in groups 2 (MCT plus cilostazol) and 3 (control) than in group 1 (MCT alone) and significantly higher in group 3 than in group 1 (all values of p<0.001) on day 90 following MCT treatment.

**Hemodynamic Results and the Ratio of RV to LV plus S Weight by Day 90 Following MCT Treatment (Table 2)**

The RVSBP was significantly higher in groups 1 and 2...
than in group 3 on day 28 following MCT treatment. Additionally, the RVSBP was significantly higher on day 90 than on day 28 in group 1 (p<0.0001). Moreover, the RVSBP was more significantly increased in group 1 than in groups 2 and 3 on day 90 following MCT-induced PAH (Fig 2). Although the RVSBP was still higher in group 2 than in group 3 on day 90 following MCT treatment, compared to day 28 following MCT treatment, the RVSBP was significantly lower on day 90 following cilostazol in group 2 rats (p<0.01).

By day 90, the ratio of RV/LV+S in group 1 was remarkably higher than in group 2 and group 3, and significantly higher in group 2 than in group 3.

The DT-PABF did not differ among the 3 groups before MCT treatment. However, the DT-PABF was more significantly decreased in groups 1 and 2 than in group 3 on days 35 and 90. Although the DT-PABF did not differ between group 1 and group 2 on day 35, the DT-PABF was more significantly decreased in group 1 than in group 2 on day 90 following MCT treatment. The DT-PABF was still significantly lower in group 2 than in group 3 by day 90 following cilostazol treatment; however, compared to day 35 following MCT treatment the DT-PABF did not further decreased on day 90 following cilostazol treatment in group 2 rats.
Mortality in 3 Groups

By day 90, 4 rats had died in total in group 1, 3 died in group 2 and none in group 3. Therefore, the mortality rate was significantly higher in groups 1 and 2 than in group 3 (p<0.0001).

Western Blot Results

The Cx43 protein expression of RV and lung was substantially lower in group 1 than in groups 2 and 3 on day 90 following MCT treatment (Figs 3A, B and C). Additionally, the Bcl-2 expression of RV was significantly lower in group 1 than in groups 2 and 3 by day 90 following MCT treatment (Fig 3D).

eNOS Gene Expression in Lung and RV

Changes in lung and RV eNOS mRNA expression were measured using RT-PCR. On day 90 following MCT treatment, the expressions of eNOS mRNA in both lungs (Fig 4A) and RV (Fig 4B) were significantly lower in group 1 than in groups 2 and 3 and significantly lower in group 2 than in group 3.

Cx43 Expression of RV

Fig 5A shows the results of quantification of an integrated area (μm²) of clustered Cx43 spots in each group of RV myocardium on day 90 following MCT treatment. The summation area of Cx43 was significantly lower in group 1 than in groups 2 and 3 as well as significantly lower in group 2 than in group 3. Fig 5B shows a relatively decreased number of Cx43-positive spots located between cardiomyocytes in group 1 compared to the groups 2 and 3. Additionally, compared with groups 2 and 3, the numbers of intact Cx43 gap junctions were notably reduced in group 1.

Quantitative Analysis of Number of Alveolar Sacs and Arteriolar Density of Lung Parenchyma

The number of arteriolar density of lung parenchyma was substantially lower in group 1 than in groups 2 and 3 on day 90 following MCT treatment (Fig 6A). Additionally, histopathological findings showed arteriolar wall thickness (measured an arterial wall from intima to adventitia) was notably increased in group 1 compared to groups 2 and 3 (Figs 6A, B). Furthermore, the number of alveolar sacs was remarkably lower in group 1 than in groups 2 and 3, and was notably lower in group 2 than in group 3 on day 90 following MCT treatment (Fig 6C).

Discussion

The major finding of the present study was that cilostazol therapy is effective at attenuating MCT-induced cardiovascular remodeling with vascular endothelial and smooth cell proliferation, obliteration of arterioles in the lung and increased RV blood pressure, a reflex of PAH, and RV hypertrophy in rats. Therefore, cilostazol therapy may offer an attractively alternative method for the treatment of PAH.

PAH involves histopathological changes of medial hypertrophy, intimal proliferation and occlusion of pulmonary microvascularity.7,15,16 For this reason, current studies demonstrate that PAH is a progressive disease with high morbidity and mortality.3,4,21,22 Despite the significant advances in the therapy for PAH in recent years, the prognosis remains poor.8–14

Cilostazol, a type 3 phosphodiesterase inhibitor, has been proven to have anti-platelet and anti-thromboembolic effects as well as inhibiting smooth muscle proliferation, obliteration of arterioles in the lung and increased RV blood pressure, a reflex of PAH, and RV hypertrophy in rats. Therefore, cilostazol therapy may offer an attractively alternative method for the treatment of PAH.

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study demonstrated that cilostazol therapy markedly enhanced the Bcl-2 expression in RV myocardium, an index of anti-apoptotic protein and improved Cx43 expression in lung and RV myocardium, an index of Cx43 gap junction integrity. The finding of increased Bcl-2 protein levels in RV myocardium following cilostazol treatment indicates reduced RV wall stress and RV hypertrophy, which can cause cellular apoptosis. In addition, the finding of preserved Cx43 expression in RV myocardium with cilostazol treatment suggests reduced RV wall stress and RV hypertrophy. This suggestion is supported by a recent study showing that Cx43 expression was substantially down-regulated by RV hypertrophy resulting from PAH in rats. Further, a previous study has demonstrated that DT of early diastolic transmitral flow inversely correlates to total pulmonary resistance, an indicator of the severity of PAH, and that the shortened DT in PAH is attributable to RV pressure overload, which causes geometric changes. In the present study, we directly measured the DT-PABF and the results showed that DT-PABF was significantly shortened in MCT-induced PAH rats. Therefore, our findings are supported by previous study. Accordingly, our experimental results explain why the RVSBP, an index of pulmonary arterial systolic blood pressure, was significantly decreased whereas the DT-PABF, an index of pulmonary artery resistance, was notably improved in MCT-induced PAH rats following cilostazol treatment. Given these considerations, it is reasonable to speculate that cilostazol therapy provides...
a broad spectrum of effects in preventing vascular remodeling, including inhibition of platelet aggregation in microvascularities, prevention of thrombus formation in vascular beds and maintaining the arterial smooth muscle cells in a contractile function rather than in a mitotic stage in MCT-induced PAH rats.

Nishimura et al previously demonstrated that simvastatin effectively attenuates MCT-induced PAH by increasing eNOS mRNA expression and inhibiting smooth muscle neointimal proliferation in a SD rat model. Given that cilostazol with and simvastatin without the additional effects of anti-platelet activation and anti-thromboembolism, and that both these drugs are inhibitors of smooth muscle proliferation, it is not surprising that the findings of this current study are consistent with the results from Nishimura et al. A previous study has demonstrated that cilostazol enhances NO production by vascular smooth muscle cells. Additionally, Hashimoto et al has recently reported that cilostazol induces NO production by eNOS activation via cyclic-AMP/protein kinase A and phosphatidylinositol 3-kinase/Akt-dependent pathways. Accordingly, our finding is further supported by the results from these studies.

**Study Limitations**

This study has limitations. First, although the reversal model of the present study provides striking implications, the findings are still not predictive of the response to therapy in PAH patients. Second, these results raise another important issue that has not been addressed: whether early administration of cilostazol can prevent the development of PAH in the same animal model.

**Conclusion**

Cilostazol therapy effectively reverses MCT-induced PAH. Our findings, therefore, raise the need of a prospective study to investigate whether cilostazol can be utilized to prevent PAH in genetically susceptible patients as well as for treatment of patients already suffering from PAH.

**Acknowledgements**

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**References**