Attenuation of Pulmonary Hypertension Secondary to Left Ventricular Dysfunction in the Rat by Rho-Kinase Inhibitor Fasudil

Zen-Kong Dai, MD, PhD,1,2 Bin-Nan Wu, PhD,3 I-Chen Chen, MD,1 Chee-Yin Chai, MD, PhD,4 Jiunn-Ren Wu, MD,1 Shah-Hwa Chou, MD,5 Jwu-Lai Yeh, PhD,3 Ing-Jun Chen, PhD,3 and Mian-Shin Tan, PhD6*

Summary. Pulmonary hypertension (PH) in left ventricular dysfunction is attributable not only to backward failure of the left ventricle, but also to increased pulmonary vascular resistance (PVR) in some patients. Recently, Rho-kinase has been known as a potent growth stimulator and mediator of vasoconstriction, and Rho-kinase inhibitors could ameliorate PVR, little is known about the role of Rho-kinase in left ventricular dysfunction-induced PH. We utilized the ascending aortic-banded rat and assessed the effect of Rho-kinase inhibitor fasudil on the development of PH secondary to left ventricular dysfunction. Subsequently, in rats subjected to aortic banding for 6 weeks, there were increases in mean pulmonary arterial pressure, pulmonary arteriolar medial thickness, active RhoA, Rho-kinase II, Rho-kinase activity, endothelial nitric oxide synthase (eNOS) and endothelin-1(ET-1) concomitant with decreased levels in NO and cGMP in the lung. Treatment with fasudil at a dose of 30 mg/kg/day from days 1 to 28 or from days 29 to 42 decreased the mean pulmonary arterial pressure by 57% and 56%, right ventricular hypertrophy by 31% and 30%, pulmonary arteriolar medial thickness by 50% and 50%, and pulmonary expression of Rho-kinase II by 41% and 28%, respectively, as well as augmented pulmonary expression of eNOS by 16% and 31% and NO by 50% and 76%, respectively, when compared with the vehicle controls. In conclusion, these results suggest that inhibition of Rho-kinase may provide therapeutic potential for preventing and attenuating the development of PH in left ventricular dysfunction. Further translational study in human is needed to substantiate the findings. Pediatr Pulmonol. 2011; 46:45–59.

© 2010 Wiley-Liss, Inc.

Key words: pulmonary vascular remodeling; RhoA; Rho kinase; ROCK; pulmonary hypertension; endothelial nitric oxide synthase; nitric oxide; left ventricular dysfunction.

Funding source: National Science Council of the Republic of China, Number NSC-96-2314-037-033-MY2; Cardiac Children’s Foundation of the Republic of China, Number CCFT0804; Kaohsiung Medical University Hospital, Number KMUH98-8R13.

INTRODUCTION

Pulmonary hypertension (PH) is a severely progressive disease and, if untreated, will ultimately lead to high mortality due to right ventricular failure. Although various complex and multifactorial processes have been realized, PH is still not identified through diagnosis. Rather, it remains a physiological description in which the mean...
pulmonary arterial pressure is more than 25 mm Hg.\(^1\) It has been shown that pulmonary arterial pressure is regulated by pulmonary vascular tone and pulmonary vascular remodeling. Accordingly, PH patients with different etiologies are often similarly characterized by pulmonary vascular remodeling and pulmonary endothelial dysfunction.\(^2\) Impaired endothelial function results from an imbalance in vasodilatation and vasoconstriction, and it plays a critical role in the development of PH.\(^3\) For example, impaired expression of prostaglandin\(^4\) and endothelial nitric oxide synthase (eNOS), which is responsible for the generation of the vasodilator nitric oxide (NO)/cGMP, and increased expression of the vasoconstrictor endothelin-1 (ET-1) have been found in different PH patients. As a result, many non-conventional vasodilators such as nitric oxide (NO) and prostaglandin analogues as well as phosphodiesterase inhibitors (PDEIs) and ET-1 receptor antagonists (ERAs) have been evaluated clinically for the treatment of patients with PH, and beneficial effects are proven.

Recently, on the Dana Point clinical classification of PH in 2008,\(^5\) PH due to left heart disease was classified as group 2, and has been noted in more than two-thirds of patients with left ventricular dysfunction.\(^6\) These patients are frequently associated with pulmonary endothelial dysfunction in addition to backward failure of the left ventricle.\(^7,8\) Although, in left ventricular dysfunction patients, small number of clinical trials with vasodilators had been reported,\(^9–13\) some experts insisted that no studies using medications approved for PH with left heart disease have been performed in recent years,\(^5\) and some major adverse effects have been frequently observed because of the opening of preapillary sphincter\(^14\) and left ventricular volume overloading.\(^15\) Additionally, it has been further demonstrated that some inotropic agents might increase mortality,\(^16\) and the effects of some vasodilator might be limited and treatments could be time-dependent in left ventricular dysfunction, that is, early intervention with vasodilators could exaggerate left ventricular hypertrophy.\(^17\) It is worth noting that current medical therapies for severe PH patients only improve the quality of life but does not prevent the progression to death due to right heart failure. Hence, more effective and non-invasive therapy for PH should be developed, specially, in left ventricular dysfunction.

In the 1990s, Rho-kinase was identified as one of the main downstream effectors of the small G protein RhoA, with a molecular mass of 160 kDa.\(^18,19\) At least two isoforms of Rho-kinase, that is, ROCK I and ROCK II, have been identified. Recent reports show that Rho-kinase is a major regulator of vascular tone,\(^20,21\) and it plays key roles in many cellular functions,\(^22\) including proliferation, migration and contraction of vascular smooth muscle cell (VSMC),\(^20,23,24\) cell adhesion and motility, actin cytoskeleton organization, cytokinesis, and gene expression. After the G protein-coupled receptor is activated by vasoconstrictors, RhoA in the cytoplasm (cytosolic RhoA) is translocated to the peripheral membrane (membrane RhoA) and up-regulates Rho-kinase protein and its activity therein. Lines of evidence have shown that RhoA/Rho-kinase is involved in the pathogenesis of various cardiovascular diseases, especially arteriosclerosis,\(^25,26\) coronary artery disease, and PH.\(^27\) In addition, up-regulated expression of RhoA and Rho-kinase has been noted in hypoxia-\(^28\) and monocrotaline (MCT)-induced PH models\(^29,30\) and in patients with idiopathic PAH.\(^31\) Several inhibitors of Rho-kinase have been shown to acutely attenuate hypoxic pulmonary vasoconstriction\(^28,32\) and ameliorate the development of PH in animal models.\(^28\) Recently, it had been reported that long-term administration of ROCK inhibitor could improve LV geometry and LV dysfunction in aortic-banded rats,\(^33\) improve cardiac contractility in hypertension-sensitive rats,\(^34\) and attenuate cardiac hypertrophy and fibrosis in myocardial infarcted mice.\(^35\) However, few clinical trials with inhibitors of RhoA/Rho-kinase has been reported in PH associated with left ventricular dysfunction. Remarkably, chronic left ventricular pressure overload, such as systemic hypertension and congenital heart disease with left ventricular outflow tract obstruction (LVOTO) is a major contributor to left ventricular dysfunction. Therefore, the aim of the present study was to examine the expression of RhoA/Rho-kinase in the lung and evaluate the therapeutic effects of Rho-kinase inhibitor on the establishment of PH, and the pulmonary expressions of eNOS/NO/cGMP and ET-1, in left ventricular dysfunction due to pressure overload.

**OBJECTIVE**

In our recent series of studies, reversible alterations of eNOS and ET-1 expression in lung tissues, associated with the progression of PH, were noted in the lung of aortic-banded rats, suggesting that pulmonary endothelial dysfunction and pulmonary vascular remodeling were involved in the pathogenesis of PH secondary to left ventricular dysfunction in this animal model.\(^36–38\) Thus, we hypothesized that activation of RhoA/Rho-kinase in the lung could contribute to the development of PH, and inhibition of Rho-kinase might prevent and ameliorate the development of PH in left ventricular dysfunction, consistently with changes in eNOS/NO/cGMP and ET-1 expression. Therefore, in this present study, we first examined the expression of active RhoA, Rho-kinase II, and Rho-kinase activity in the lung of aortic-banded rats, and further tested whether chronic administration of the Rho-kinase inhibitor fasudil could prevent and attenuate the development of PH, with altered expressions of eNOS/NO/cGMP and ET-1, in left ventricular dysfunction due to pressure overload.
MATERIALS AND METHODS

Animal Model of PH

All protocols were approved by the animal research committee of Kaohsiung Medical University. Male Wistar rats (6-week-old, weighing approximately 220 g) were randomly assigned to aortic-banded or sham-operated groups. As previously described, a left parasternal thoracotomy in the 4th intercostal space was performed after animals were anesthetized with sodium pentobarbital (20 mg/kg, i.p.) and ketamine (60 mg/kg, i.m.) and orotracheally ventilated using rodent respirators (Harvard, South Natick, MA). A blunt, sheathed hypodermic needle (19-gauge) was placed along the axis of the ascending aorta, and a length of 3-0 nylon suture was tied around the aorta. The sheathed hypodermic needle was then removed, leaving a stenosis in the ascending aorta approximately 1 cm distal to the aortic valve. The procedure took less than 30 min for each rat. Sham-operated animals underwent the same operation, except that the aorta was not banded. The day of aortic banding was designated as day 0. An effective aortic banding was confirmed by a pressure gradient of around 40 mm Hg, determined by transthoracic echocardiography (Hewlett-Packard, 5-MHz transducer) on day 1. All animals were individually housed in a 12-h dark/light cycle-controlled room and fed regular rat diet. Sham-operated rats of similar body weight served as controls.

It had been previously demonstrated that an ascending aortic banding could result in pulmonary vascular dysfunction, increased mean pulmonary arterial pressure, and up-regulated expression of eNOS and ET-1 in the lung. In the present study, fasudil (Eril; Asahi Kasei Pharma Corp, Tokyo, Japan) was used as a pharmacological inhibitor of Rho-kinase in the early treatment and late treatment protocols described previously. As shown in Figure 1A, in the early treatment protocol, the rats were randomized to undergo sham-operation or ascending aortic banding, and the aortic-banded rats were further randomized for subcutaneous treatment with saline...
(AOB28) or fasudil at a dose of 30 mg/kg/day (AOB28/Fas1–28) from days 1 to 28. Additionally, in the survival studies, one group of sham-operated rats received fasudil from days 1 to 28 after banding (Sham28/Fas1–28). In the late treatment protocol, the rats were randomized to undergo sham-operation or ascending aortic banding, then, surviving aortic-banded rats were further randomized to receive saline (AOB42) or fasudil at 30 mg/kg/day (AOB42/Fas39–42) for 14 days starting from day 29. On days 28 and 42 in the early and late treatment protocols, respectively, all rats were again ventilated and sacrificed after checking hemodynamic data. The lung and heart were rapidly perfused with normal saline under a pressure of 100 cm H2O prior to removal. The banded heart were rapidly perfused with normal saline under a microscope at a magnification of 400× to confirm banding effectiveness.

Measurement of Systemic and Pulmonary Arterial Pressures, and Left Atrial Pressures

After the animal was ventilated, a PE-50 catheter (Becton-Dickinson, Sparks, MD) was inserted into the femoral artery using a cut-down procedure for recording pressures. Thereafter, a left parasternal thoracotomy was performed, and a catheter was inserted via the left auricle into the left atrium followed by insertion of a second catheter into the main pulmonary artery via the right ventricular outflow tract. The catheters were connected to pressure transducers. Subsequently, the system was filled and flushed with less than 2 ml of heparin solution (1,000 IU/ml). Pulmonary and femoral artery pressures were recorded simultaneously by a polygraph system. Blood samples (2 ml) were aspirated from the pulmonary arterial cannulae, collected in chilled syringes, and transferred to polypropylene tubes containing EDTA (1 mg/ml of blood) and aprotinin (500 KIU/ml of blood) at 4°C. The samples were centrifuged at 2,000g for 15 min at 4°C. Plasma was stored at −70°C until assay for ET-1 level.

Tissue Preparation and Morphometric Analysis of Pulmonary Arterioles

After the rats were sacrificed, three pieces of lung tissues from different lobes were excised and immersed in 10% formalin for 24 hr. Hematoxylin-Eosin (H-E) staining was subsequently performed, and the areas around 100 μm in diameter of the pulmonary arterioles were evaluated for measurement of medial wall thickness under a microscope at a magnification of 400×. For each arteriole, the median wall thickness was expressed as follows:

\[
\text{percent wall thickness} = \frac{\text{medial thickness} \times 2}{\text{external diameter}} \times 100.
\]

One-half of the remaining lung tissue was homogenized for protein extraction, and the other half was frozen in liquid nitrogen and stored at −70°C for Western blot analysis. The right ventricle (RV) was isolated by dissection along its septal insertion. The weights of RV, left ventricle (LV), and the interventricular septum (IVS) were measured to determine the extent of hypertrophy in RV (RVH) and LV (LVH) by the ratios of RV/Body Weight (BW) and (LV + IVS)/BW, respectively. Left ventricular dysfunction was evident in banded rats as biventricular cardiac hypertrophy and elevated left atrial pressure (LAP)40 were noted.

Organ Chamber Experiments

Small intrapulmonary arteries (300–400 μm) from the sham-operated rat, aortic-banded rat (AOB28), and aortic-banded rat treated with fasudil (AOB28/Fas1–28) in the early treatment protocol were carefully isolated and cleaned of any connective tissue in physiological salt solution (PSS).44 The rings from each pulmonary artery (2 mm in length) were fitted with two stainless steel wires (40 μm) and mounted in a dual-channel Mulvany-Halpern myograph (Model 410A, DMT A/S, Aarhus, Denmark) for measurement of isometric tension in a bath filled with PSS at 37°C. Each preparation was equilibrated with a resting tension of 2 mN for 90 min, and the bath solution was replaced every 15 min. Intrapulmonary artery reactivity was examined in the presence of 80 mM KCl to obtain maximal contraction.

Western Blot Analysis for RhoA, Rho-kinase II, eNOS

Lung tissues (100 mg) were homogenized in 1 ml RIPA buffer (1% Triton X-100, 15 mM HEPES-NaOH, pH 7.5, 0.15 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM EDTA, and 1% protease inhibitor cocktail (Sigma, St. Louis, MO)) and centrifuged at 15,000g for 20 min at 4°C. The protein (100 μg) was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a PVDF membrane (Pall Life Sciences, Ann Arbor, MI, USA). The membrane was blocked with 5% non-fat dry milk in tris-buffered solution (TBS) and probed with anti-ROCK I, anti-ROCK II (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-eNOS (1:1,000, Millipore, Billerica, MA, USA) or anti-actin (1:10,000, Millipore) antibodies. Afterwards, it was then incubated with horseradish peroxidase-conjugated secondary antibody, and the signal was detected with the Western Lighting Chemiluminescent kit (Millipore) according to manufacturer’s specifications.
Measurement of Pulmonary RhoA/Rho-Kinase Activity

Activation of RhoA was determined by membrane translocation of the protein. Lung tissue (100 mg) was homogenized in 1 ml lysis buffer (10 mM HEPES, 2 mM EDTA, 1 mM MgCl₂) containing protease inhibitors (Sigma). The homogenate was centrifuged at 40,000 g for 30 min, and the supernatant was collected as the cytosolic fraction. The pellet was resuspended in lysis buffer and centrifuged again (40,000 g for 15 min) to obtain the membrane fraction. RhoA protein in the membrane and cytosolic fractions were determined by Western blot analysis using anti-RhoA antibodies (1:250 dilutions, Santa Cruz Biotechnology).

Rho-kinase activity was quantified by Western blot analysis for the total and phosphorylated ERM (ezrin, radixin, and moesin) family, a substrate for Rho-kinase using antibodies raised against the total ERM (t-ERM) and the phosphorylated ERM (p-ERM) (1:500, Santa Cruz Biotechnology). The level of p-ERM was normalized by that of total ERM.

NOx Assay

The lung tissue (100 mg) was homogenized in 1 ml 0.1 M phosphate-buffered saline (pH 7.4) and centrifuged at 15,000 g for 20 min at 4°C. The supernatant was loaded onto a 30 kDa cut-off filter column (Millipore) and centrifuged at 15,000 g for 20 min. The nitrite/nitrate (NOx) concentration, recognized as NO production, in the filtrate was determined by Griess reaction (Cayman Chemical, Ann Arbor, MI, USA).

Cyclic GMP (cGMP) Measurement

Frozen lung tissues were homogenized in cold trichloroacetic acid (TCA, 5% w/v) and centrifuged for 10 min at 1,500 g and the supernatant was collected. TCA was extracted from the supernatant with four washes of water-saturated ether. The residual ether was removed from the aqueous layer by heating the samples to 70°C for 5 min. The cGMP assay was performed using commercial kits (Cyclic GMP EIA kit, Cayman Chemical) according to manufacturer’s instructions.

Measurement of Pulmonary ET-1 Concentration

The lung tissues (100 mg) was homogenized in 1 ml 0.1 M phosphate-buffered saline (pH 7.4) containing 1% Triton X-100 and centrifuged at 15,000 g for 20 min at 4°C. The ET-1 level of the supernatant was determined by rat ET-1 ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA).

Immunohistochemical Staining for ET-1 and eNOS

Immunohistochemical staining for ET-1 and eNOS was performed on 5 μm-thick tissue sections fixed in formalin and embedded in paraffin. Slides were deparaffinized, hydrated and treated with 0.3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. They were then washed with PBS and incubated with anti-ET-1 or anti-eNOS (1:100) antibody (Oncogene, Boston, MA) for 1 hr at room temperature. Afterwards, slides were washed for 30 min in PBS and incubated with biotinylated link antibody (DAKO, Glostrup, Denmark). Specimens were again washed with PBS, incubated with peroxidase-labeled streptavidin (DAKO) for 30 min, and examined under a light microscope after incubation with diaminobenzidine (Sigma) and counterstained with Mayer’s hematoxylin.

Statistical Analysis

The results obtained from Western blots were analyzed by densitometry and expressed as mean ± standard error of mean. All data from the three groups in each treatment protocol were analyzed statistically using one-way ANOVA followed by Tukey’s test. Comparison of beneficial efficacy of fasudil on all data between the early treatment and the late treatment protocols were analyzed statistically by two-way ANOVA. Cumulative survival rate was analyzed by Kaplan–Meier life table and examined by log-rank test. A P value of <0.05 was considered statistically significant.

RESULTS

In the early treatment protocol, six animals were used in each of the three groups: sham-operated (sham28), aortic-banded rats for 28 days (AOB28), and aortic-banded rats administrated with fasudil from days 1 to 28 (AOB28/Fas1–28). Likewise, in the late treatment protocol, the same number of rats was included in the following three groups: sham-operated (sham42), aortic-banded for 42 days (AOB42), and aortic-banded plus fasudil treatment from days 29 to 42 (AOB42/Fas29–42). In addition, six sham-operated rats were used in the sham28/Fas1–28 rats.

Survival Study

As shown in Figure 1B, in the early treatment protocol, the Kaplan–Meier survival rate of aortic-banded rats treated with fasudil was 58.3% at days 14 and 28, and it was not different from that of the vehicle-treated rats subjected to aortic banding (58.3%). In contrast, in the late treatment protocol, no mortality was noted in fasudil-treated and vehicle-treated rats subjected to aortic banding for more than 28 days. Also, all of sham28 rats, sham42 rats and sham28/Fas1–28 rats survived over the study period.
Fasudil Reduced Pulmonary Arterial Pressure, Left Atrial Pressures, RV Hypertrophy, and Pulmonary Vascular Remodeling, But Not Systemic Arterial Pressure

Compared with sham-operated rats (sham28, sham42), there was a significant increase in the mean pulmonary arterial pressure in both the AOB28 and AOB42 groups, as shown in Tables 1A and 1B. Treatment with fasudil significantly decreased the mean pulmonary arterial pressure in AOB28/Fas1–28 rats and AOB42/Fas29–42 rats with similar efficacy, when compared with the respective vehicle-treated aortic-banded animals.

Compared with sham-operated rats (sham28 or sham42), there was a significant increase in the left atrial pressure in both the AOB28 and AOB42 groups, as shown in Tables 1A and 1B. Treatment with fasudil significantly decreased the left atrial pressure in AOB28/Fas1–28 rats and AOB42/Fas29–42 rats with similar efficacy, when compared with the respective vehicle-treated aortic-banded animals.

However, there were no significant differences in the mean systemic arterial pressure among the three groups of rats under either the early treatment or the late treatment protocol. Compared with the respective sham-operated rats, aortic banding resulted in significant increases in the RVW/BW ratios in both AOB28 and AOB42 rats. Fasudil treatment significantly decreased the ratios of RVW/BW in AOB28/Fas1–28 rats and AOB42/Fas29–42 rats with similar efficacy, when compared with the respective vehicle-treated rats subjected to aortic banding.

In H-E staining, the medial wall thickness of pulmonary arteriole of 50–100 μm in diameter were increased in both AOB28 and AOB42 rats compared with the respective sham-operated rats (Fig. 2). Treatment with fasudil significantly attenuated the medial wall thickness of pulmonary arteriole 50–100 μm in diameter in AOB28/Fas1–28 rats and AOB42/Fas29–42 rats with similar efficacy, when compared with the respective vehicle-treated rats subjected to aortic banding.

Fasudil Reduced the Increase in the Ratio of Membrane-Associated RhoA to Cytosolic RhoA

Upon activation, RhoA was found to translocate from the cytosolic compartment to become membrane-associated, and the results were quantified by Western Blot analysis. As shown in Figure 3, there were significant increases in the ratios of membrane-bound RhoA/cytosolic RhoA in the lung of AOB28 and AOB42 rats when compared with matched sham-operated rats. These results suggest that there was an increased activation of RhoA in the lung of aortic-banded rats. This activation of

### TABLE 1A—Comparison of Hemodynamics and Heart Weight, Lung Weights Among the Sham28, AOB28, and AOB28/Fas1–28 Groups in the Experimental Protocol

<table>
<thead>
<tr>
<th></th>
<th>Sham28</th>
<th>AOB28</th>
<th>AOB28/Fas1–28</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPAP (mm Hg)</td>
<td>13 ± 1</td>
<td>28 ± 3**</td>
<td>12 ± 1 ++</td>
</tr>
<tr>
<td>mSAP (mm Hg)</td>
<td>124 ± 3</td>
<td>127 ± 3</td>
<td>124 ± 4</td>
</tr>
<tr>
<td>LAP (mm Hg)</td>
<td>2.2 ± 0.3</td>
<td>7.3 ± 0.9</td>
<td>2.8 ± 0.3 #</td>
</tr>
<tr>
<td>LungW/BW (mg/g)</td>
<td>2.78 ± 0.23</td>
<td>4.70 ± 0.17**</td>
<td>4.27 ± 0.09¥</td>
</tr>
<tr>
<td>RVW/BW (mg/g)</td>
<td>0.68 ± 0.11</td>
<td>1.19 ± 0.08**</td>
<td>0.82 ± 0.02 ++</td>
</tr>
</tbody>
</table>

BW, body weight; LungW, lung weight; mPAP, mean pulmonary arterial pressure; mSAP, mean systemic arterial pressure; LAP, left atrial pressure; RVW, right ventricle weight. AOB28, aorta-banded rat treated with vehicle for 28 days; AOB28/Fas1–28, aorta-banded rat given fasudil from days 1 to 28.

**P < 0.01 compared with sham-operated rat (sham28).

††P < 0.01 with AOB28.

### TABLE 1B—Comparison of Hemodynamics and Heart Weight, Lung Weights Among the Sham42, AOB42, and AOB42/Fas29–42 Groups in the Experimental Protocol

<table>
<thead>
<tr>
<th></th>
<th>Sham42</th>
<th>AOB42</th>
<th>AOB42/Fas29–42</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPAP (mm Hg)</td>
<td>13 ± 1</td>
<td>25 ± 2**</td>
<td>11 ± 1 ++</td>
</tr>
<tr>
<td>mSAP (mm Hg)</td>
<td>126 ± 4</td>
<td>127 ± 4</td>
<td>131 ± 4</td>
</tr>
<tr>
<td>LAP (mm Hg)</td>
<td>2.4 ± 0.4</td>
<td>7.0 ± 0.9</td>
<td>2.0 ± 0.5 #</td>
</tr>
<tr>
<td>LungW/BW (mg/g)</td>
<td>3.05 ± 0.31</td>
<td>4.82 ± 0.18**</td>
<td>4.15 ± 0.06¥</td>
</tr>
<tr>
<td>RVW/BW (mg/g)</td>
<td>0.61 ± 0.08</td>
<td>1.32 ± 0.04**</td>
<td>0.92 ± 0.04 ++</td>
</tr>
</tbody>
</table>

AOB42, aorta-banded rat treated with vehicle for 42 days; AOB42/Fas29–42, aorta-banded rats given fasudil from days 29 to 42.

**P < 0.01 compared with sham-operated rat (sham42).

††P < 0.05 with AOB42.

††P < 0.01 with AOB42.
RhoA was reversed by treatment with fasudil; the membrane-associated RhoA/cytosolic RhoA ratios were significantly decreased with similar efficacy, when compared with the respective vehicle-treated rats subjected to aortic banding, in the lung of AOB28/Fas1–28 and AOB42/Fas29–42 rats.

**Fasudil Decreased Rho-Kinase Activity and the Expression of Rho-Kinase II, But Not Rho-Kinase I**

The expressions of Rho-kinase I (ROCK I) and Rho-kinase II (ROCK II) protein were evaluated by Western blot analysis (Fig. 4). Compared with matched sham-operated rats, the expressions of ROCK II, but not ROCK I, in the lung of AOB28 and AOB42 rats were significantly increased by 117% and 103%, respectively. Treatment with fasudil resulted in significant decreases in ROCK II, but not ROCK I, in the lung of AOB28/Fas1–28 and AOB42/Fas29–42 rats with similar efficacy, when compared with respective vehicle-treated aortic-banded rats.

The activity of Rho-kinase was determined using the ratio of phosphorylated ERM (p-ERM) to total ERM. Compared with matched sham-operated rats, the ratios of p-ERM/total ERM in the lung of AOB28 and AOB42 rats were increased, respectively (Fig. 5). Similar to the results obtained in Rho-kinase protein expression, significant decreases of AOB28/Fas1–28 and AOB42/Fas29–42 rats were seen, when compared with respective vehicle-treated aortic-banded rats, with equal efficacy.

**Fasudil Up-Regulated the Expression of eNOS, NOx, and cGMP**

As shown in Figure 6, in the early treatment protocol, aortic banding did not alter the expression of pulmonary eNOS when compared with matched sham-operated rats. In contrast, when compared with matched sham-operated rats in the late treatment protocol, the expression of pulmonary eNOS in AOB42 rats was increased. Treatment with fasudil significantly enhanced the expression of eNOS in the lung of AOB28/Fas1–28 rats and in AOB42/Fas29–42 rats with similar efficacy, when compared with the respective vehicle-treated aortic-banded rats.

The production of NO was measured as nitrite/nitrate (NOx). The levels of NOx in the lung of AOB28 and AOB42 rats were decreased, respectively, compared with matched sham-operated rats (Fig. 7). Upon fasudil treatment, the levels of pulmonary NOx were significantly augmented in AOB28/Fas1–28 rats and in AOB42/Fas29–42 rats with similar efficacy, when compared with the respective vehicle-treated aortic-banded rats.
Fig. 3. Expression of cytosolic RhoA and membrane-associated RhoA in lung tissues. The upper panels show Western blots of membrane-associated and cytosolic RhoA in the early treatment (left) and late treatment (right) protocols. The lower panels depict the ratios of membrane-associated RhoA/cytosolic RhoA as quantified by densitometry. There was a significant increase in the ratios of membrane-associated RhoA/cytosolic RhoA in aortic-banded rats on days 28 (AOB28) and 42 (AOB42), compared with matched sham-operated rats (sham28, sham42). Fasudil treatment from days 1 to 28 (AOB28/Fas1–28) or from days 29 to 42 (AOB42/Fas29–42) significantly decreased the ratio in the banded rats in the early and late treatment protocols, respectively. Values represent mean ± SEM. Abbreviation: mRhoA, membrane-associated RhoA; cyto RhoA, cytosolic RhoA. **P < 0.01; *P < 0.05.

Fig. 4. Expression of Rho-kinase I (ROCK I) and Rho-kinase II (ROCK II) in lung tissues. The upper panels show Western blots of ROCK I and ROCK II in the early treatment (left) and late treatment (right) protocols, while the lower panels depict normalized ratios of ROCK I and ROCK II RhoA/actin relative to the sham-operated controls. There was an increased expression of ROCK II, but not ROCK I, in the lung of aortic-banded rats at day 28 (AOB28) and day 42 (AOB42), compared with matched sham-operated rats (sham28, sham42). Administration of fasudil from days 1 to 28 (AOB28/Fas1–28) or from days 29 to 42 (AOB42/Fas29–42) decreased the expression of ROCK II, but not ROCK I, in the lung of aortic-banded rats in both the early treatment and late treatment protocols. **P < 0.01; Values represent mean ± SEM.
similar efficacy (Fig. 7A), when compared with the respective vehicle-treated aortic-banded rats.

The levels of cGMP in the lung of AOB28 and AOB42 rats were decreased when compared with matched sham-operated rats (Fig. 7B). Upon fasudil treatment, the levels of pulmonary cGMP were significantly augmented in both the AOB28/Fas1–28 and AOB42/Fas29–42 rats with similar efficacy (Fig. 7B), when compared with the respective vehicle-treated aortic-banded rats.

**Fasudil Reduced the Levels of Pulmonary ET-1**

Compared with sham-operated rats, the levels of pulmonary ET-1 were significantly increased in AOB28 and AOB42 rats (Fig. 8). Interestingly, ET-1 contents in the lung of AOB42/Fas29–42 rats, but not of AOB28/Fas1–28 rats, was significantly decreased when compared with the respective vehicle-treated AOB rats.

**Fasudil Increased the Contractile Sensitivity to KCl in Intralobar Pulmonary Artery**

Compared with sham-operated rats, the contractile response to 80 mM KCl in intralobar pulmonary artery of the AOB28 rats was significantly decreased (Fig. 9). Treatment with fasudil normalized the sensitivity to KCl in the intralobar pulmonary artery of the AOB28/Fas1–28 rats.

**Immunohistochemical Staining of eNOS and ET-1 in the Lung**

In lung tissues stained for eNOS, there was increased immunostaining in the subendothelial area of pulmonary arterioles of around 100 μm in diameter in AOB42 rats, but not in AOB28 rats, compared with matched sham-operated rats (Fig. 6B). The eNOS immunostaining in the same area was enhanced in AOB28/Fas1–28 and AOB42/Fas29–42 rats (Fig. 6B). In contrast, the ET-1 immunostaining in this area was increased in both the AOB28 and AOB42 rats, when compared with the respective sham-operated rats (Fig. 8B). Unlike the results of the eNOS immunostaining, fasudil decreased the ET-1 immunostaining in the subendothelial area of pulmonary arterioles in both the AOB28/Fas1–28 and AOB42/Fas29–42 rats, when compared with the AOB28 and AOB42 rats, respectively (Fig. 8B).

**DISCUSSION**

PH is frequently found in patients with chronic left ventricular dysfunction, and its severity correlates with mortality. In group 2 of the Dana Point clinical classification, PH associated with left ventricular dysfunction is not only caused by passive increases in pulmonary vascular pressures, but also is frequently associated with reactive increases in pulmonary vascular resistance (PVR) which leads to an elevated transpulmonary pressure gradient that is in-fact superimposed on the pulmonary venous pressure. Indeed, increased PVR is also seen in patients with PH, without left heart failure, classified as group 1 in same classification, and, it is closely related to pulmonary vascular remodeling and pulmonary endothelial dysfunction in the pulmonary vasculature. In this study, we have measured expressions of eNOS and ET-1, and levels of NOx and cGMP in lung tissues, as indices of pulmonary endothelial dysfunction. However, pulmonary endothelial dysfunction is also needed to be confirmed by isolated pulmonary artery...
relaxation in response to acetylcholine or another eNOS activator. We attempted to compensate the lack of such information by examining pulmonary levels of NO/cGMP, which are relevant to endothelium-dependent vasorelaxation, since endothelial dysfunction was broadly defined as an imbalance of vasodilators and vasoconstrictors produced in the endothelium. Thus, in patients with left ventricular dysfunction, a crucial therapeutic strategy is to revere the increased PVR and prevent the adverse effects by certain vasodilators, such as sildenafil and iloprost.

Very importantly, contrary to in vivo studies that the anti-PH vasodilator therapies could be associated with aggravated left ventricular hypertrophy in animal models of heart failure and worsened heart failure in patients with congestive heart failure, we demonstrated that fasudil prevented the development of PH in the early treatment protocol and induced regression of PH in the late treatment protocol. Furthermore, these treatments led to decreases in RhoA activity, ROCK II, and ET-1 expression as well as augmented eNOS expression and NO/cGMP production in the lung with similar efficacy. Consistent with earlier studies, fasudil was further found to ameliorate the development of ventricular remodeling, including perivascular fibrosis and interstitial fibrosis, and reduced the increase in LAP in both fasudil-treated rats, when compared with the respective vehicle-treated rats subjected to aortic banding with similar efficacy (not shown). Taken together, these similar beneficial efficacies of fasudil in both protocols could be explained by reversible alternations of elevated PVR and LV remodeling, within limited duration of aortic banding, as previously reported in our laboratory.

**Fig. 6. A:** Expression of eNOS in lung tissues. The upper panels show Western blots of eNOS in the early treatment (left) and late treatment (right) protocols. The lower panels depict normalized ratios of eNOS/actin relative to the sham-operated controls. There was an increased expression of eNOS in aortic-banded rats on day 42 (AOB42), but not on day 28 (AOB28), compared with sham-operated rats (sham28, sham42), respectively. Administration of fasudil from days 1 to 28 (AOB42/Fas1–28) or from days 29 to 42 (AOB42/Fas29–42) significantly up-regulated the expression of eNOS in aortic-banded rats in the early treatment and late treatment protocols, respectively. *P < 0.05. Values represent mean ± SEM. B: Immunohistochemical staining for eNOS in lung tissues in the early treatment (upper panels) and the late treatment protocols (lower panel) (magnification: 400×). There was increased eNOS staining in the subendothelial area of pulmonary arterioles with 50–100 μm in diameter in aortic-banded rats on day 42 (AOB42), compared with sham-operated rats (sham28). Treatment with fasudil from days 1 to 28 (AOB28/Fas1–28) or from days 29 to 42 (AOB42/Fas29–42) increased the eNOS immunostaining in aortic-banded rats in both the early treatment and late treatment protocols (arrows), respectively. eNOS, endothelial nitric oxide synthase.

**Pediatric Pulmonology**
In the early treatment protocol, fasudil was found not to confer a survival advantage in AOB28/Fas1–28 rats, when compared with respective vehicle-treated rats. Based on previous observations in our laboratory, it was shown that aortic banding in adult rats, not weanling rats, could result in acute left heart failure immediately, then led to PH associated with hypertrophic LV dysfunction, through the adapting stage from acute heart failure. Thus, it could be proposed that acute heart failure was difficultly prevented by fasudil, and related to the high mortality in fasudil-treated rats during the early post-banding period, when compared with other PH models, such as hypoxic and monocrotaline-induced PH, in which PH slowly developed without adapting from acute left heart failure. Moreover, no death noted in the sham-operated rats treated with fasudil could protect that the compound did not adversely influence the mortality in the PH model of left ventricular dysfunction. Rather, it prevented and attenuated the progression of PH.

Recently, it has been shown that activation of Rho A/Rho-kinase signaling is involved in acute hypoxic pulmonary vasoconstriction stimulated by various vasoconstrictors, such as ET-1, serotonin, and thromboxane A2. In preclinical studies, stimulated Rho-kinase signaling led to vasoconstriction in severely occlusive PH in rats, and activation of Rho-kinase was involved in hypoxic pulmonary vasoconstriction, hypoxia-induced PH, monocrotaline-induced PH as well as polystyrene microspheres-induced pulmonary embolism. Consistent with these findings, our results similarly demonstrated enhanced expressions of membrane-associated RhoA, Rho-kinase II and Rho-kinase activity in the lung of rats with left ventricular dysfunction. Recent studies reported that fasudil acutely attenuated pulmonary vascular resistance in the fawn-hooded PH rat, monocrotaline-induced PH, and hypoxia-induced PH rat, and hypoxia-induced PH mouse. Likewise, Y-27632, another Rho-kinase inhibitor, also reduced the pulmonary arterial pressure and total pulmonary resistance in monocrotaline-induced PH rat.

It has been shown that Rho-kinase is involved in pulmonary vascular remodeling in experimental PH animal models and that transglutaminase-mediated activation of RhoA by serotonin plays a role in the development of pulmonary vascular remodeling in chronic hypoxia-induced PH. In addition, treatment with Y-27632 decreased the muscularization of distal pulmonary vessels.
pulmonary arteries and up-regulated the expression of eNOS in chronic hypoxia-induced PH model.28 Furthermore, fasudil decreased the sustained vasoconstriction and pulmonary vascular remodeling in monocrotaline-induced PH rat.29 Consistent with the above findings, our results similarly showed that fasudil not only affected pulmonary vascular remodeling by slowing progression of PH, but also by inducing “reverse remodeling” of the small pulmonary arterioles in lungs of left ventricular dysfunction. Furthermore, taking into consideration our data, it could convincingly support the notion that activation of RhoA/Rho-kinase can play a central role in the establishment of pulmonary endothelial dysfunction and pulmonary vascular remodeling in PH in rats subjected to left ventricular dysfunction.

Interestingly, in the present study, the production of pulmonary NO/cGMP was similarly decreased in both the AOB28 and AOB42 rats, despite of variable expressions of eNOS in the lung of the respective groups. These findings are compatible with a report showing that a decreased NO production was attributable to the decreased biological activity of eNOS and impaired release of NO in the lung in left heart failure.8 Moreover, we found that the increased activity of RhoA in lung tissues was reduced by fasudil in the aortic-banded rats, when compared with the vehicle-treated animals, in the both treatment protocols. There are several explanations for these results. First, pulmonary cGMP was increased upon treatment with fasudil, and cGMP-dependent protein kinase (PKG) could, in turn, prevent the translocation of active GTP-bound RhoA to the membrane,59,60 through phosphorylating RhoA.61 Second, the inhibition of Rho-kinase could up-regulate the eNOS expression in cultured endothelial cells.52,63 Third, ET-1 can activate the Rho pathway.64 Therefore, it

Fig. 8. A: The levels of ET-1 in the lung tissues. There were increased pulmonary ET-1 contents in aortic-banded rats on day 28 (AOB28) (left panel) and day 42 (AOB42) (right panel), compared with the respective sham-operated rats (sham28, sham42). Fasudil decreased the ET-1 content in aortic-banded rats when administrated from days 29 to 42 (AOB42/Fas29–42), but not from days 1 to 28 (AOB28/Fas1–28). **P < 0.01; *P < 0.05. Values represent mean ± SEM. B: Immunohistochemical staining for ET-1 in lung tissues (magnification: 400×). The results obtained from the early treatment protocol are displayed in the upper panels, while those from the late treatment protocol are shown in the lower panels. There was increased ET-1 immunostaining in the subendothelial area of pulmonary arterioles 50–100 μm in diameter in aortic-banded rats at day 28 (AOB28) and day 42 (AOB42) (arrows), compared with the respective sham-operated rats (sham28, sham42). Administration with fasudil decreased the ET-1 immunostaining in aortic-banded rats administrated from days 29 to 42 (AOB42/Fas29–42) in the late treatment protocol, but not administrated from days 1 to 28 (AOB28/Fas1–28) in the early treatment protocol.
is possible that the decrease in RhoA activity in lung tissues of fasudil-treated rats may result from a combination of up-regulated NO/cGMP and reduced ET-1 expressions.

Lastly, Rho-kinase inhibitors have been demonstrated to significantly improve the impaired intrinsic contractile response to KCl in hypoxia- and monocrotaline-induced PH rats. Our results also showed that the contractile response to high concentration of KCl was decreased in the intralobar pulmonary artery of AOB28 rats, and this impairment was reversed by fasudil before the establishment of PH. However, the precise mechanism by which the contractile response to KCl was decreased in the intralobar pulmonary artery is unclear.

In conclusion, the present study suggested that activation of Rho-kinase pathway was importantly involved in the development of PH, and fasudil significantly prevented and attenuated the development of PH, associated with the augmentation of eNOS/NO/cGMP and down-regulation of ET-1 expression in the lung, in rats with left ventricular dysfunction. Future clinical studies are needed to determine if the present beneficial findings can be translated to patients with PH in left ventricular dysfunction.

ACKNOWLEDGMENTS

We thank National Science Council of the Republic of China (NSC-96-2314-037-033-MY2), Cardiac Children’s Foundation of the Republic of China (CCFT0804) and Kaohsiung Medical University Hospital (KMUH98-8R13) for grant support, Dr. Hiroyuki Meguro for pharmacological support, and Miss Hsiao-Ching Weng for technical support. Finally, we thank the help from the Statistical Analysis Laboratory, Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University.

REFERENCES


34. Satoh S, Ueda Y, Koyanagi M, Kadokami T, Sugano M, Yoshikawa Y, Makino N. Chronic inhibition of Rho kinase blunts the process of left ventricular hypertrophy leading to cardiac contractile dysfunction in hypertension-induced heart failure. J Mol Cell Cardiol 2003;35:59–70.


