Evidence for Rho-Kinase Activation in Patients With Pulmonary Arterial Hypertension

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**Background:** Direct evidence for Rho-kinase activation in patients with pulmonary hypertension (PH) is still lacking.

**Methods and Results:** Rho-kinase activity in circulating neutrophils was examined by determining the ratio of phosphorylated/total forms of myosin-binding subunit, a substrate of Rho-kinase, in 40 consecutive PH patients and 40 healthy controls. Next, Rho-kinase expression and activity was examined in isolated human lung tissues (5 patients with idiopathic pulmonary arterial hypertension [IPAH], 5 controls) and vascular reactivity of isolated small human pulmonary arteries in vitro (4 IPAH, 4 controls). Rho-kinase activity in circulating neutrophils was significantly increased in the PH patients overall compared with controls (P<0.0001). Significant correlations were noted between Rho-kinase activity and the severity and duration of PAH (all P<0.05). Rho-kinase expression and activity in isolated lung tissues also were significantly increased in the IPAH patients compared with the controls (both P<0.0001). Endothelium-dependent relaxation was markedly impaired and serotonin-induced contraction (in the absence of the endothelium) markedly enhanced in the PAH patients compared with the controls, and the hypercontraction to serotonin was abolished by hydroxyfasudil, a specific Rho-kinase inhibitor.

**Conclusions:** These results provide the first direct evidence for Rho-kinase activation in patients with PAH, suggesting the therapeutic importance of Rho-kinase in the disorder. (*Circ J* 2009; 73: 1731–1739)

**Key Words:** Endothelial function; Pulmonary hypertension; Rho-kinase; Signal transduction; Vascular smooth muscle

Pulmonary arterial hypertension (PAH) is a fatal disease caused by small pulmonary artery obstruction as a result of vascular proliferation and remodeling.1 PAH is characterized by markedly elevated pulmonary artery pressure and increased pulmonary vascular resistance, which frequently leads to right-sided heart failure and death.1 The pathological changes in the pulmonary arteries of PAH patients include endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells (VSMCs), and migration of inflammatory cells.23 Although anticoagulant agents, vasodilators and lung transplantation are currently used for the treatment of PAH, more effective treatments need to be developed.4

**Editorial p 1597**

In 1990s, Rho-kinase (ROK/ROCK) was identified as an effector of the small GTP-binding protein Rho,5,6 which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expressions.7–9 Indeed, we and others have previously demonstrated that long-term inhibition of Rho-kinase ameliorates monocrotaline (MCT)-induced PAH and hypoxia-induced PAH in animal models.10–13 In those studies, Rho-kinase activity in circulating neutrophils was enhanced, irrespective of the different etiologies,10–13 and long-term treatment with Rho-kinase inhibitors ameliorated endothelial dysfunction and suppressed hypercontraction and proliferation of VSMCs and migration of inflammatory cells.10 Subsequently, we and others have demonstrated that a Rho-kinase inhibitor, fasudil, acutely reduces pulmonary vascular resistance in patients with severe PAH.14,15 However, direct evidence for Rho-kinase activation in patients with PAH is still lacking and its discovery would validate the development of Rho-kinase inhibitors for the treatment of PAH. In the present study, we thus addressed this important issue in patients with PAH.

**Methods**

The Ethical Committees of Tohoku University Hospital approved the study protocol and all patients provided written informed consent. In the present study, we performed two...
Table 1. Characteristics of the Control Subjects and Patients With PH (Protocol 1)

<table>
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<th>PH</th>
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<td>40</td>
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<tr>
<td>Age (years)</td>
<td>43.8±6.9</td>
<td>44.4±7.1</td>
<td></td>
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<tr>
<td>Gender</td>
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<td></td>
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<tr>
<td>Male, N (%)</td>
<td>8 (20%)</td>
<td>7 (18%)</td>
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</tr>
<tr>
<td>Female, N (%)</td>
<td>32 (80%)</td>
<td>33 (82%)</td>
<td></td>
</tr>
<tr>
<td>Type of PH, N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPAH</td>
<td>–</td>
<td>18 (44%)</td>
<td></td>
</tr>
<tr>
<td>CTD-PAH</td>
<td>–</td>
<td>8 (20%)</td>
<td></td>
</tr>
<tr>
<td>CHD-PAH</td>
<td>–</td>
<td>7 (18%)</td>
<td></td>
</tr>
<tr>
<td>CTEPH</td>
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<td>7 (18%)</td>
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<tr>
<td>Treatment</td>
<td></td>
<td></td>
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<tr>
<td>Epoprostenol</td>
<td>–</td>
<td>17 (43%)</td>
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<tr>
<td>Beraprost</td>
<td>–</td>
<td>16 (40%)</td>
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<tr>
<td>Bosentan</td>
<td>–</td>
<td>18 (45%)</td>
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<td>Sildenafil</td>
<td>–</td>
<td>4 (10%)</td>
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<td>Oxygen therapy</td>
<td>–</td>
<td>38 (95%)</td>
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<tr>
<td>BNP (pg/ml)</td>
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<td>213±34</td>
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<tr>
<td>White blood cells (/μl)</td>
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<td>2,410±380</td>
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<tr>
<td>CRP (mg/dl)</td>
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<tr>
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<td>75±12</td>
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<tr>
<td>Mean PAP (mmHg)</td>
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<td>52±8</td>
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<tr>
<td>Mean PVR (dyne·s⁻¹·cm⁻⁵)</td>
<td>–</td>
<td>915±145</td>
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<td>SaO₂</td>
<td>–</td>
<td>94.3±2.1</td>
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Values are mean±SEM.

PH, pulmonary hypertension; Control, control subjects; IPAH, idiopathic pulmonary arterial hypertension; CTD, connective tissue disease; CHD, congenital heart disease; CTEPH, chronic thromboembolic pulmonary hypertension; BNP, brain natriuretic peptide; CRP, C-reactive protein; PAP, pulmonary arterial pressure; PVR, pulmonary vascular resistance; SaO₂, oxygen saturation.

Table 2. Clinical Characteristics of Controls and Patients With IPAH (Protocol 2)

<table>
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<tr>
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<tr>
<td>Female, N (%)</td>
<td>5 (100%)</td>
<td>3 (60%)</td>
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<tr>
<td>Laboratory data</td>
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<tr>
<td>White blood cells (/μl)</td>
<td>5,100±473</td>
<td>8,675±232</td>
<td>NS</td>
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<tr>
<td>CRP (mg/dl)</td>
<td>0.10±0.00</td>
<td>0.13±0.03</td>
<td>NS</td>
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<tr>
<td>SaO₂</td>
<td>–</td>
<td>97.4±0.8</td>
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Values are mean±SEM.

Abbreviations see in Table 1.

proteins.

Protocol 1

In this protocol, we examined whether Rho-kinase activity is locally upregulated in the lungs and pulmonary arteries isolated from patients with PAH.

Study Population

We prospectively examined lung tissues from 5 consecutive patients with IPAH who underwent lung transplantation, and normal lung tissues from the lung cancer tissues from 5 control subjects with cancer who underwent operation (Table 2). All 5 control subjects had lung cancer and thus did not receive any anti-cancer drugs before the surgery. Moreover, none of the IPAH patients or the lung cancer subjects received statins, which can reduce Rho-kinase activity.

Western Blot Analysis

Protein was extracted from human lung tissues, and the same amount of extracted protein (10–30 μg) was loaded for SDS-PAGE/immunoblot analysis. There are 2 isoforms of Rho-kinase, ROCK1 and ROCK2, and to quantify Rho-kinase expression in human lung tissue, we used anti-mouse ROCK1 antibody (BD Biosciences, Tokyo, Japan) and anti-mouse ROCK2 antibody (BD Biosciences). We also quantified protein kinase C and CPI-17 (protein kinase C potentiated inhibitor protein 17) expression in human lung tissue, by using anti-mouse PKCα (BD Biosciences) and/or anti-mouse PKCδ antibody (BD Biosciences), anti-rabbit CPI-17 antibody (Epitomics).

Immunohistological Analysis

All patients with IPAH were treated with intravenous epoprostenol with or without other oral vasodilators. Compared with the controls with lung cancer, the patients with IPAH who underwent lung transplantation were characterized by younger age and comparable levels of C-reactive protein (CRP; Table 2).

We carefully dissected under microscope apparently normal lung tissues from the lung resected from the patients with lung cancer. Immunostaining was performed using anti-mouse ROCK1 antibody (BD Biosciences), anti-mouse ROCK2 antibody (BD Biosciences), anti-rabbit phospho-MBS antibody (Upstate, Tokyo, Japan) and anti-mouse MBS antibody (BD Biosciences),20–22 and CPI-17 antibody (Epitomics). Diaminobezidine and hydrogen dioxide were applied to develop color. Immunostaining was performed by the biotin–avidin complex method (Histofine SAB-PO(M) Kit/SAB-PO(R) Kit). Slides were viewed with a standard light microscope (Olympus BX51, Olympus)
Optical, Tokyo, Japan) and analyzed by DP manager (ver.2.2.1.195) and DP controller (ver.2.2.1.227). The intensity of immunostaining was scored with a semi-quantitative method. Briefly, in the lung tissues, 131 lesions from the 5 control subjects and 131 lesions from the 5 IPAH patients were studied. The intensity of the specific immuno-positive pulmonary arterioles, smaller than 200 μm in diameter, was categorized visually into 3 levels (negative, slightly positive, and positive) by 2 independent well-trained persons in a blinded manner.23,24

Organ Chamber Experiments: Endothelial and VSMC functions of the pulmonary arteries were examined in 4 patients with IPAH and 4 controls. Because of the limited availability of blood vessels, these examinations were not performed in 1 patient with IPAH and 1 control. Under a microscope, small pulmonary arteries (400–600 μm in diameter) were carefully isolated and cleaned of any connective tissue in Krebs solution, as previously reported.10 Endothelium-dependent relaxation to acetylcholine (ACh, 10^{-5} to 10^{-7} mol/L) or bradykinin (BK, 10^{-11} to 10^{-6} mol/L) was examined in rings of artery with endothelium during a contraction evoked by prostaglandin F_{2α} (3×10^{-6} mol/L).10 Endothelium-independent contraction to serotonin (5HT, 10^{-9} to 10^{-5} mol/L) and endothelium-independent relaxation to sodium nitroprusside (SNP, 10^{-10} to 10^{-5} mol/L) were also examined in rings without endothelium.10 The acute inhibitory effect of hydroxyfasudil (10^{-5} mol/L for 30 min preincubation), a specific Rho-kinase inhibitor, on serotonin-induced VSMC contraction also was examined.10

Statistical Analysis: Results are expressed as the mean±SEM. Throughout the text, N is the number of subjects and n is the number of small pulmonary arteries in the lung tissue samples. Independent-sample t-test and Dunnett t-test were used to compare mean values of Rho-kinase activity between PAH and control subjects. Independent-sample t-test was used to compare mean values of Rho-kinase expression and CPI-17 expression between lung tissue of IPAH and control subjects. The Mann-Whitney test was used to compare the number of immuno-positive pulmonary arteries between control and IPAH lung tissues. Statistical analyses were performed using SPSS (SPSS Inc, Chicago, IL, USA) and P values less than 0.05 were considered to be statistically significant.

Results: Rho-Kinase Activity in Circulating Neutrophils: Rho-kinase activity, as evaluated by the p-MBS/t-MBS ratio,16 was significantly increased in patients with PH as compared with the controls (Figures 1A, B). Among the 4 subgroups of PH, Rho-kinase activity was significantly increased in patients with IPAH (IPAH, CTD-PAH, and CHD-PAH).
Correlation Between Rho-Kinase Activity and Severity/Duration of PAH

In patients with PAH, weak but significant correlations were noted between Rho-kinase activity in circulating neutrophils and mean pulmonary arterial pressure and duration of the disorder (Figures 2A, B). In contrast, there were no significant correlations between Rho-kinase activity and pulmonary vascular resistance, cardiac index, right atrial pressure or plasma levels of brain natriuretic peptide (BNP) (data not shown).

Rho-Kinase Expression and Activity in Human Lung Tissues

The expression of PKC (PKCα and PKCδ) was significantly increased in the lung tissues from the IPAH patients as compared with the control subjects (Figures 3A, B). The expression of ROCK1 and ROCK2 proteins in the whole lung tissues tended to be increased in the IPAH patients (Figures 3C, D). Furthermore, immunohistochemical analyses demonstrated that the expression of both ROCK1 and ROCK2 was significantly increased in the intima and media of pulmonary arteries from the IPAH patients compared with those from the controls (Figure 4). In order to examine Rho-kinase activity in the pulmonary arteries, we further performed immunohistochemical analyses for p-MBS and t-MBS. The endothelium of the pulmonary arteries in the control subjects was negative for p-MBS and positive for t-MBS, whereas VSMC in the media were negative for p-MBS and t-MBS. By contrast, the thickened intima and the media of the pulmonary arteries from the PAH patients were positive for both p-MBS and t-MBS (Figure 5). In contrast, the expression of PKC-potentiated inhibitor protein 17 (CPI-17), another potential inhibitor of myosin phosphatase, was comparable between the IPAH patient and the control subjects (Figure 6).

**Figure 2.** Correlation between Rho-kinase activity in circulating neutrophils and the severity or duration of PAH. In patients with PAH, weak but significant correlations were noted between Rho-kinase activity in circulating neutrophils and mPAP (A) and the duration of the disorder (B). mPAP, mean pulmonary arterial pressure. See Figure 1 for other abbreviations.

**Figure 3.** Western blot analysis. Representative western blotting and quantitative results of the protein expression of PKCα (A), PKCδ (B), ROCK1 (C), and ROCK2 (D) of the whole lung tissues from the control subjects and the IPAH patients. The expression of PKCα and PKCδ was significantly increased and that of ROCK1 and ROCK2 tended to be increased in patients with IPAH. Results are expressed as the mean±SEM. IPAH, idiopathic pulmonary arterial hypertension; PKC, protein kinase C; ROCK1, ROCK2, isoforms of Rho-kinase.
Figure 4. Rho-kinase expression is enhanced in pulmonary arteries of patients with IPAH. (A, C) Representative immunostaining for ROCK1 and ROCK2 expressions in lung tissue from a control subject and an IPAH patient. (B, D) Expressions of ROCK1 and ROCK2 were low in the small pulmonary arteries of the controls, whereas it was significantly higher in both the thickened intima and media of small pulmonary arteries of the IPAH patients. Results are expressed as mean±SEM. n=131 each from 5 patients and 5 controls. Scale bar, 50μm. See Figure 3 for abbreviations.

Figure 5. Rho-kinase activity is enhanced in small pulmonary arteries of patients with IPAH. (A, C) Representative immunostaining for p-MBS and t-MBS of lung tissue from a control subject and an IPAH patient. (B) Expression of p-MBS was low in the small pulmonary arteries of the controls, whereas it was significantly increased in both the thickened intima and the media of small pulmonary arteries in the IPAH patients. (D) t-MBS was immuno-positive in the endothelium but negative in the media of the small pulmonary arteries of the controls, whereas its expression was significantly higher in both the thickened intima and the media of small pulmonary arteries in the IPAH patients. Results are expressed as mean±SEM. n=131 each in 5 IPAH patients and 5 controls. Scale bar, 50μm. See Figures 1, 3 for abbreviations.
Vascular Reactivity of Isolated Pulmonary Arteries

Endothelium-dependent relaxation to both ACh and BK was significantly impaired in isolated small pulmonary arteries of the PAH patients compared with the controls (Figures 7A, B). In contrast, endothelium-independent relaxation to SNP was comparable between the 2 groups (Figure 7C). Serotonin-induced contractions of VSMC were significantly enhanced in the PAH patients as compared with the controls (Figure 7D). Although hydroxyfasudil, a specific Rho-kinase inhibitor, had no effect on...
basal tension, it abolished the hypercontraction to serotonin in the PAH patients, but did not affect the responses to serotonin in the controls (Figure 7D).

Discussion
The novel finding of the present study is that Rho-kinase is activated in both the circulating blood cells and the pulmonary arteries of patients with PAH, resulting in hypercontraction of the artery. The present findings support our previous findings in animal models of PAH, and during right-heart cardiac catheterization in patients with PAH.9,23–32 To the best of our knowledge, this is the first study to provide direct evidence for Rho-kinase activation in patients with PAH.

Role of Rho-Kinase Activation in the Pathogenesis of PAH
Increased pulmonary vascular resistance in PAH can be caused by both pulmonary vascular remodeling and sustained pulmonary vasoconstriction, in which endothelial dysfunction and VSMC hypercontraction may be involved.33–35 It has previously been demonstrated that expression of endothelial nitric oxide synthase (eNOS) is reduced and pulmonary VSMC are hyper-reactive in patients with PAH.33–35 Accumulating evidence indicates that Rho-kinase activation causes several important abnormalities, including downregulation of eNOS in endothelial cells,36 VSMC hypercontraction through inhibition of myosin phosphate, VSMC proliferation and migration, and inhibition of VSMC apoptosis.9,26,37,38 Indeed, previous findings from our own and other laboratories suggested the involvement of Rho-kinase activation in the pathogenesis of PAH in animals and humans.9–15 although direct evidence for Rho-kinase activation was still lacking. In the present study, we thus addressed this important issue in patients with PAH.

Systemic Activation of Rho-Kinase in Patients With PAH
It is an interesting though somewhat unexpected finding of the present study that Rho-kinase is systemically activated in patients with PAH, as evidenced in the increased Rho-kinase activity in circulating neutrophils. The present study also demonstrated a significant correlation between Rho-kinase activation and the severity (mean pulmonary arterial pressure) and duration of the disorder. However, no significant correlation was noted between Rho-kinase activity and the cardiac index, right atrial pressure or plasma levels of BNP. These results suggest that the systemic Rho-kinase activation is not a simple result of PAH, because activation was associated with progression of the disorder, but not with development of right-sided heart failure (eg, cardiac index, BNP). Interestingly, Rho-kinase was activated in patients with IPAH, CTD-PAH, and CHD-PAH, but not in those with CTEPH. Indeed, although CTEPH is regarded as a consequence of pulmonary thromboembolism (PTE) because of venous thromboembolism, the occurrence of CTEPH in patients with acute PTE is rare.39,40 Thus, it is conceivable that the pathophysiology of IPAH and CTEPH differs.

Upregulation and Activation of Rho-Kinase in the Pulmonary Arteries of Patients With PAH
The 2 isoforms of Rho-kinase, ROCK1 and ROCK2, are widely expressed in various organs.41 ROCK1 is ubiquitously expressed, except in the brain and skeletal muscle, whereas ROCK2 is expressed abundantly in brain, muscle, heart and lung.41 In the present study, western blot analyses showed that the levels of ROCK1 and ROCK2 proteins in the whole lung tissue tended to be increased in the IPAH patients compared with the control subjects. Furthermore, immunostaining showed that in normal lung tissues from patients with lung cancer low levels of ROCK1 and ROCK2 were expressed. In contrast, lung tissues from IPAH patients highly expressed both ROCK1 and ROCK2 in both the intima and the media of small pulmonary arteries. Furthermore, Rho-kinase activity (expression of p-MBS)20 was increased in both the intima and media of pulmonary arteries of IPAH patients compared with control subjects. Therefore, it is conceivable that myosin phosphatase is upregulated in the media of the pulmonary arteries of IPAH patients. These results suggest that Rho-kinase expression and activity are increased in the small pulmonary arteries of patients with IPAH.

Rho-Kinase-Mediated Pathway and Vascular Hypercontraction in PAH
Vascular smooth muscle tension is determined by the balance between myosin light chain kinase and myosin light chain phosphatase (MLCP) activities. Regarding the inhibition of MLCP activity, there are 2 major pathways involved: phosphorylation of MBS and that of CPI-17.9,26,37 In the present study, western blot analysis demonstrated that expression of PKC (PKCa and PKCD), but not that of CPI-17, was significantly increased in the lung tissues from the IPAH patient as compared with the control subjects. Although there was no significant increase in total CPI-17, it is also conceivable that CPI-17 phosphorylation is increased in the pulmonary arteries of IPAH patients, enhancing the pulmonary artery tone. The present finding in patients with PAH is consistent with our previous finding in a porcine model of coronary spasm that PKC (especially PKCδ) and Rho-kinase coexist in the intracellular signaling pathway, with PKC located upstream of RhoA/Rho-kinase, suggesting that a strategy to inhibit Rho-kinase rather than PKC would be more specific and useful for the treatment of the vasospastic disorders.28

Endothelial Dysfunction and VSMC Hypercontraction of Pulmonary Arteries From Patients With PAH
In the present study, we were able to directly demonstrate that endothelial vasodilator function was impaired and VSMC contraction enhanced in isolated pulmonary arteries from patients with PAH. These findings are consistent with our previous studies with MCT-induced PH in rats and hypoxia-induced PH in mice.10,11 and previous clinical studies of patients with PAH.23–35 Furthermore, we were able to demonstrate that inhibition of Rho-kinase abolished VSMC hypercontraction of pulmonary arteries from IPAH patients. This finding also is consistent with our previous clinical study that found acute inhibition of Rho-kinase improves pulmonary hemodynamics in PAH patients.14,15 Although we only tested the vasoconstrictor responses to serotonin in the present study, the vasoconstrictor responses also could be enhanced to other Rho-kinase-dependent agonists. We consider that Rho-kinase activation plays an important role because we have previously demonstrated that Rho-kinase activation downregulates eNOS56 and that long-term inhibition of Rho-kinase markedly ameliorates endothelial dysfunction in animal models of PH.10 These findings are the first to directly demonstrate endothelial dysfunction in patients with PAH.

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The effects of Rho-kinase activation in the pulmonary arteries from patients with PAH. It remains to be examined in future studies whether those functional abnormalities of the pulmonary arteries can be ameliorated by long-term treatment with a Rho-kinase inhibitor.37,38

Rho-Kinase Inhibitors for the Treatment of PAH

Despite the recent advances in the medical treatment of PAH, such as the use of prostacyclin and bosentan, none of the monotherapies is sufficient in many patients and combination therapy is frequently needed to treat this fatal disorder.12,13 We recently demonstrated that prostacyclin or its oral analog, beraprost sodium (BPS), lacked an inhibitory effect on Rho-kinase44 and that the combination of fasudil and BPS was more effective than each monotherapy for ameliorating PH in a rat model of MCT-induced PH.45 Indeed, we have demonstrated that IPAH patients administered intravenous prostacyclin therapy showed favorable acute responses to a Rho-kinase inhibitor.14 Thus, it is highly expected that combination therapy with prostacyclin and a Rho-kinase inhibitor would exert more beneficial effects in the treatment of PAH.

Bosentan, a dual inhibitor of ETs and ETs endothelin receptors, is another effective drug for the treatment of PAH in the clinical setting.46-49 However, not only endothelin but also many other vasoactive substances (eg, serotonin, thrombin and platelet-derived growth factor) are likely to be involved in the pathogenesis of PAH, and importantly, all of them could activate the Rho-kinase pathway.9,26,37,38 Because Rho-kinase inhibitors could inhibit signal transductions initiated by all these vasoactive substances, it is possible that they exert more broadly beneficial effects than each single receptor antagonist.9,26,37,38

Study Limitations

Several limitations should be mentioned. First, although we were able to demonstrate Rho-kinase activation in both circulating neutrophils and lung tissues from PAH patients, the interactions between the 2 remain to be examined. It is conceivable that Rho-kinase in circulating neutrophils is activated when they go through the lung where Rho-kinase is activated. Indeed, we have previously demonstrated that Rho-kinase can be upregulated by inflammatory stimuli (eg, angiotensin II and interleukin-1beta).50 It was recently reported that Rho-kinase activity in circulating neutrophils is increased in patients with metabolic syndrome.17 However, in the present study, plasma levels of CRP were only slightly increased in patients with PAH. Thus, enhanced systemic Rho-kinase activity may not be a simple result of the systemic inflammatory responses in those patients. The underlying mechanism of Rho-kinase activation in the circulating neutrophils of PH patients remains to be examined in future studies. Second, because we obtained the control lung tissues from patients with lung cancer, we were unable to match the age and gender of the PAH patients and controls in protocol 2 for obvious ethical reasons. We have previously demonstrated that loss of estrogen (eg, menopause) in protocol 2 for obvious ethical reasons. We have previously demonstrated that loss of estrogen (eg, menopause) could be involved in the pathogenesis of PAH, and importantly, all of them could activate the Rho-kinase pathway.9,26,37,38 Because Rho-kinase inhibitors could inhibit signal transductions initiated by all these vasoactive substances, it is possible that they exert more broadly beneficial effects than each single receptor antagonist.9,26,37,38

Disclosures

None.

References


