



Protective Effect of Inhaled Rho-Kinase Inhibitor on Lung Ischemia-Reperfusion Injury

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Background. Rho-kinase, an intracellular serine/threonine kinase, is a key regulator of cytoskeletal dynamics. Recent studies have demonstrated that Rho-kinase is involved in the ischemia-reperfusion injury (IRI) pathogenesis of many organs; however, its involvement with lung IRI remains unclear. This study assessed the association of Rho-kinase with lung IRI and evaluated the protective effect of inhaled Rho-kinase inhibitors in lung IRI.

Methods. The study included isolated rat lung perfusion models, divided into three groups: sham, Rho-kinase inhibitor, and warm ischemia (n = 6 each). The lungs were exposed to 60 minutes of warm ischemia by perfusion cessation. At the onset of ischemia, nebulized fasudil, a novel Rho-kinase inhibitor, and saline were inhaled in the Rho-kinase inhibitor and warm ischemia groups, respectively. Perfusion was restarted after the ischemic period, and physiologic data were collected for 90 minutes. Lungs in the sham group were continuously perfused without ischemia or drug administrations.

Protein expression in tissue specimens related to the Rho-kinase pathway was evaluated by Western blotting.

Results. Warm ischemia and subsequent reperfusion enhanced Rho-kinase activity, and this was suppressed by fasudil inhalation. Fasudil inhalation significantly attenuated IRI pathophysiology, including pulmonary vascular contraction, dynamic compliance, lung edema, and oxygenation. Molecular analysis showed that Rho-kinase suppressed myosin phosphatase and endothelial nitric oxide synthase activities, suggesting these are downstream targets of Rho-kinase during lung IRI pathogenesis.

Conclusions. The present study suggests that Rho-kinase activation is involved in lung IRI pathogenesis and that inhaled Rho-kinase inhibitors may attenuate this pathogenesis.

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Despite significant advances in organ preservation, ischemia-reperfusion injury (IRI) remains one of the major causes of primary graft dysfunction after lung transplantation [1–3]. IRI pathogenesis is multifaceted, and the exact molecular mechanisms remain unclear [4, 5]. Previous reports have shown that xanthine oxidase [6], nicotinamide adenine dinucleotide phosphate oxidase [7], nitric oxide synthases [8], nuclear factor- κ B [9], Toll-like receptor 4 [10], and proinflammatory cytokines [11] are involved in the development of lung IRI. Therefore, a thorough understanding of the physiologic, cellular, and molecular changes in lung IRI is crucial for a good prognosis in lung transplant recipients.

Rho-kinase is an intracellular serine/threonine kinase that was identified as one of the effectors of the small

guanosine triphosphate-binding protein Rho [12, 13]. It is ubiquitously present in mammalian cells and forms a molecular switch in the cells for regulating cytoskeletal dynamics. Rho-kinase plays a significant role in the development of various vascular diseases, such as coronary hyperconstriction [14], vasospasm after subarachnoid hematoma [15], systemic hypertension [16], and pulmonary hypertension [17]. Ischemia and subsequent reperfusion also induce pathophysiologic changes to the vasculature of the lungs, including pulmonary vascular contraction and increased vascular permeability [18]. Studies have reported that Rho-kinase is related to IRI pathogenesis of several organs, including the heart [19], liver [20], and kidney [21]; however, the role of Rho-kinase in lung IRI pathogenesis is still not determined.

We hypothesized that Rho-kinase plays a key role in the development of lung IRI and that inhibition of Rho-kinase would have a protective effect against IRI. To test this hypothesis, we examined the effect of Rho-kinase and its inhibition on lungs by using an isolated rat lung perfusion model.

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Material and Methods

Animals

The study used specific pathogen-free male Lewis rats (Japan SLC, Hamamatsu, Japan), weighing 290 to 330 g. All animals received care in compliance with the “Principles of Laboratory Animal Care” (National Society for Medical Research) and the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication No. 86–23, revised 1996, Bethesda, MD). The Kyoto University Faculty of Medicine Ethical Committee approved the study protocol.

Set-up of Isolated Lung Perfusion System

An isolated lung perfusion system (Hugo Sachs Elektronik Harvard Apparatus, March Hugstetten, Germany) was used, as previously reported [22–24]. The perfusate consisted of heparinized blood obtained from 2 donor rats and saline with 4% bovine serum albumin, with pH adjusted to between 7.2 and 7.3 by sodium bicarbonate. Rats anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) were intubated after tracheotomy and ventilated during the operation. After heparinization (500 IU per rat), a first cannula was inserted into the pulmonary artery and a second cannula was inserted into the left atrium through a median sternotomy. The 2 cannulas were connected to the perfusion circuit driven by 2 roller pumps, and lung perfusion was started with a low flow rate (1 mL/min).

The lung and heart block was procured and placed in an artificial thorax after starting perfusion (Fig 1A). It was ventilated with peak inspiratory and expiratory chamber pressures of –8 and –4 cm H₂O and a respiratory rate of 60 cycles/min. In the set-up period, the perfusion flow was gradually increased every 2 min in a stepwise fashion (1, 3, 5, 7, and 10 mL/min), and the lung was stabilized for 15 min. The perfusate drained from the left atrium was deoxygenated in a deoxygenator filled with a gas mixture

of 92% nitrogen and 8% carbon dioxide. Pulmonary venous pressure was maintained at 2 cm H₂O against the hilum. The artificial thorax, perfusate circuit, and the airway were maintained at 37°C.

Protocol After Set Up

Rats were randomly assigned into one of the following three groups: (1) a nondamaged sham group, (2) group exposed to warm ischemia and treated with the Rho-kinase inhibitor fasudil [25], termed as the Rho-kinase inhibitor (RKI) group, and (3) group exposed to warm ischemia but not treated with fasudil, termed as the warm ischemia (WI) group ($n = 6$ in each group). Perfusion in the RKI and WI groups was interrupted in the 37°C artificial thorax after the stabilization period, and the lungs were exposed to warm ischemia for 60 minutes (Fig 1B). At the onset of ischemia, fasudil solution was administered to the RKI group and normal saline was administered to the WI group. After 60 minutes of ischemia, the lungs were reperfused for 90 minutes with a five-step increase in flow rates as follows: 1 mL/min (0 to 5 minutes), 3 mL/min (5 to 8 minutes), 5 mL/min (8 to 10 minutes), 7 mL/min (10 to 12 minutes), and 10 mL/min (12 to 90 minutes). The lung in the sham group was continuously perfused for 90 minutes after the stabilization period. After 90 minutes of reperfusion, the lungs were collected for further analyses.

Drug Administration

Fasudil was administered by inhalation according to the successful results achieved in a previous report [17]. An Aerogen Pro ultrasonic nebulizer (Aerogen, Galway, Ireland) was placed in the inspiratory loop of the ventilator. The diameter of the aerosolized particles was 1 to 5 μ m in this system. Fasudil hydrochloride was purchased from LC Laboratories (Woburn, MA). A 100 mmol/L fasudil solution (obtained by dissolving fasudil in double-distilled water) or normal saline were aerosolized for the first 5 minutes of warm ischemia.

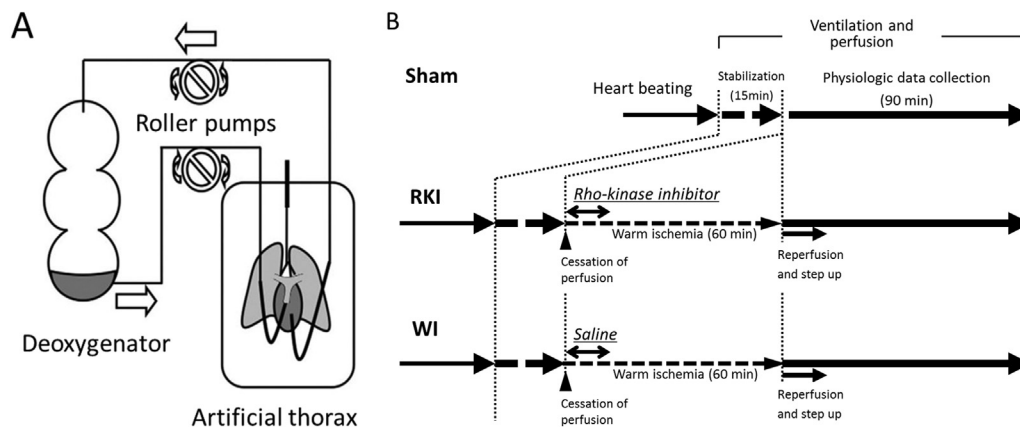


Fig 1. A diagram of (A) the isolated rat lung perfusion model and (B) the experimental protocol. Cannulas were inserted into the pulmonary artery and left atrium and were connected to the perfusion circuit driven by 2 roller pumps. The lung was placed in an artificial thorax and ventilated. (RKI = Rho-kinase inhibitor group; WI = warm ischemia group.)

Measurements

PHYSIOLOGIC DATA. Pulmonary arterial pressure and dynamic compliance were automatically recorded every 10 minutes after the onset of reperfusion. The pulmonary vascular resistance (PVR; $\text{cm H}_2\text{O} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$) was calculated according to the following equation: $\text{PVR} = [\text{pulmonary arterial pressure} - \text{pulmonary venous pressure}] / \text{perfusate flow}$. Partial pressure of arterial oxygen (PaO_2), which was obtained from blood gas analysis of the left atrium effluent, was recorded every 15 minutes. The baseline time is defined as the end of the stabilization period.

WET/DRY LUNG WEIGHT RATIO AND HISTOLOGIC EVALUATION. The right upper and middle lobes were dried using a 100°C stove for 24 hours. These were weighed before and after thermal dehydration to calculate the wet/dry lung weight ratio, which reflected the severity of lung edema. The right lower lobes were intratracheally instilled with 10% formalin for subsequent microscopic examination. These were then embedded in paraffin and stained with hematoxylin and eosin.

RHO-KINASE ACTIVITY. The left lungs were stored at -80°C for molecular analysis using Western blotting. Myosin phosphatase is a substrate of Rho-kinase, which is inactivated by phosphorylation at Thr696 (Fig 2). Phosphorylated myosin phosphatase leads to enhancement of myosin light chain (MLC) phosphorylation, which results in stress fiber formation [26]. We adopted the phosphorylation ratio of myosin phosphatase as a marker of Rho-kinase activity [27, 28].

Tissue specimens were homogenized in a lysis buffer consisting of 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2 ethylenediaminetetraacetic acid, 1% nonyl phenoxypolyethoxylethanol-40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerophosphate, 1 mmol/L Na_3VO_4 , 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ pepstatin, and 1 mmol/L phenylmethane sulfonyl fluoride.

Protein concentration was standardized according to quantification by the Bradford method (Eppendorf BioPhotometer; Eppendorf, Hamburg, Germany). Each sample was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were subsequently transferred to polyvinylidene difluoride membranes (Immobilon-P membrane; Merck Millipore, Darmstadt, Germany).

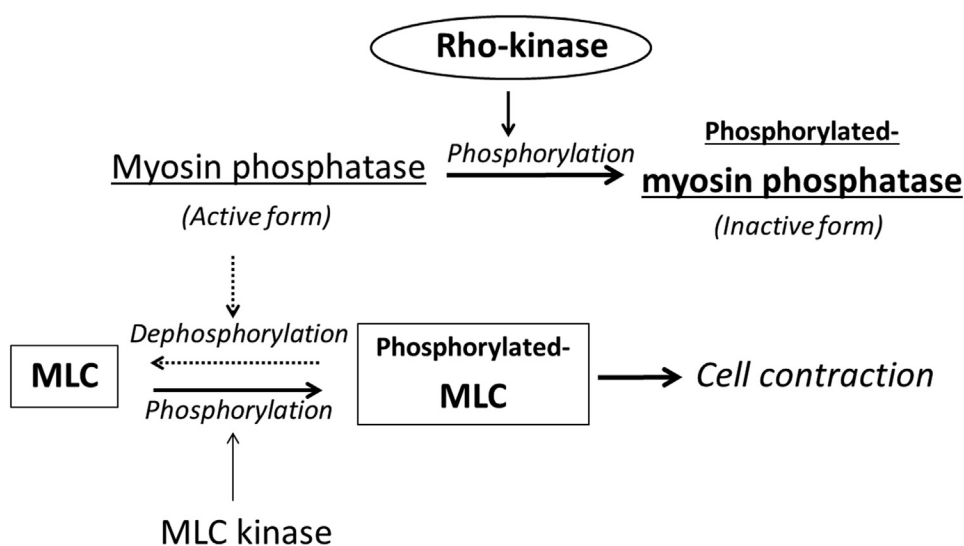
The first antibodies used in this study were rabbit phosphorylated and total myosin phosphatase target subunit-1 antibodies purchased from Cell Signaling Technology (Danvers, MA). Membranes were then incubated with secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), and immunoreactivity was detected using a LAS-3000 system (Fujifilm, Tokyo, Japan). Acquired protein bands were quantified by using ImageJ (National Institutes of Health, Bethesda, MD) [29]. β -Actin was used for confirmation of the validity of Western blotting, for which anti- β -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

ENDOTHELIAL NITRIC OXIDE SYNTHASE ACTIVITY. Protein expression of the phosphorylated form of endothelial nitric oxide synthase (eNOS), which is activated after phosphorylation of Ser1177, and total eNOS expression were also compared by Western blotting, as described above. The primary antibodies were purchased from Abcam (London, United Kingdom).

Statistical Analyses

All values are presented as the mean $\pm$ standard error of the mean. The differences of physiologic parameters were analyzed by repeated-measures analysis of variance. Wet/dry lung weight ratio and values of protein expressions obtained by Western blotting were analyzed by one-way analysis of variance with the Tukey post hoc test to differentiate among the three groups. A p value of less than 0.05 was considered statistically significant. All

Fig 2. Diagram shows the molecular mechanism of Rho-kinase in cell contraction. Rho-kinase inactivates myosin phosphatase by phosphorylation and increases the phosphorylation ratio of myosin light chain (MLC), which results in cell contraction.



statistical analyses were performed with EZR [30], which is a graphical user interface for R software (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Physiologic Data

PULMONARY VASCULAR RESISTANCE. PVR in the WI group showed an abrupt increase after reperfusion, followed by a more gradual increase, which was significantly higher than that in the sham group ($p < 0.001$, Fig 3a). The lungs from the RKI group suppressed the increase of PVR ($p < 0.001$) and maintained a low PVR in similarity with the sham group.

DYNAMIC COMPLIANCE. Dynamic compliance in the WI group decreased and was significantly lower than in the sham group ($p < 0.001$, Fig 3b). The decrease in dynamic compliance was to a lesser extent in the RKI group ($p < 0.001$).

PARTIAL PRESSURE OF ARTERIAL OXYGEN. The PaO_2 of perfusate in the WI group was significantly lower than in the sham group ($p < 0.001$, Fig 3c); however, this decrease of PaO_2 was less in the RKI group ($p < 0.001$).

Wet/dry lung weight ratio

The wet/dry lung weight ratio, reflective of lung edema, was higher in the WI group than in the sham group ($p < 0.001$, Fig 3d). In the RKI group, the wet/dry lung weight ratio was also increased but not to the same extent as that of the WI group ($p < 0.001$).

Macroscopic and Microscopic Findings

Macroscopic findings showed the lungs of the WI group were reddish and edematous. This was not present in the sham or RKI groups (Fig 4A). Microscopically, mild edema in the perivascular space was observed in the sham and RKI groups. In contrast, severe perivascular edema with sporadic hemorrhage was observed in the WI group (Fig 4B).

Phosphorylated Myosin Phosphatase Expression and Rho-Kinase Activity

Figure 5 shows representative protein bands for phosphorylated-myosin phosphatase, total myosin phosphatase, and β -actin as a protein loading control and the mean values of phosphorylated-myosin phosphatase

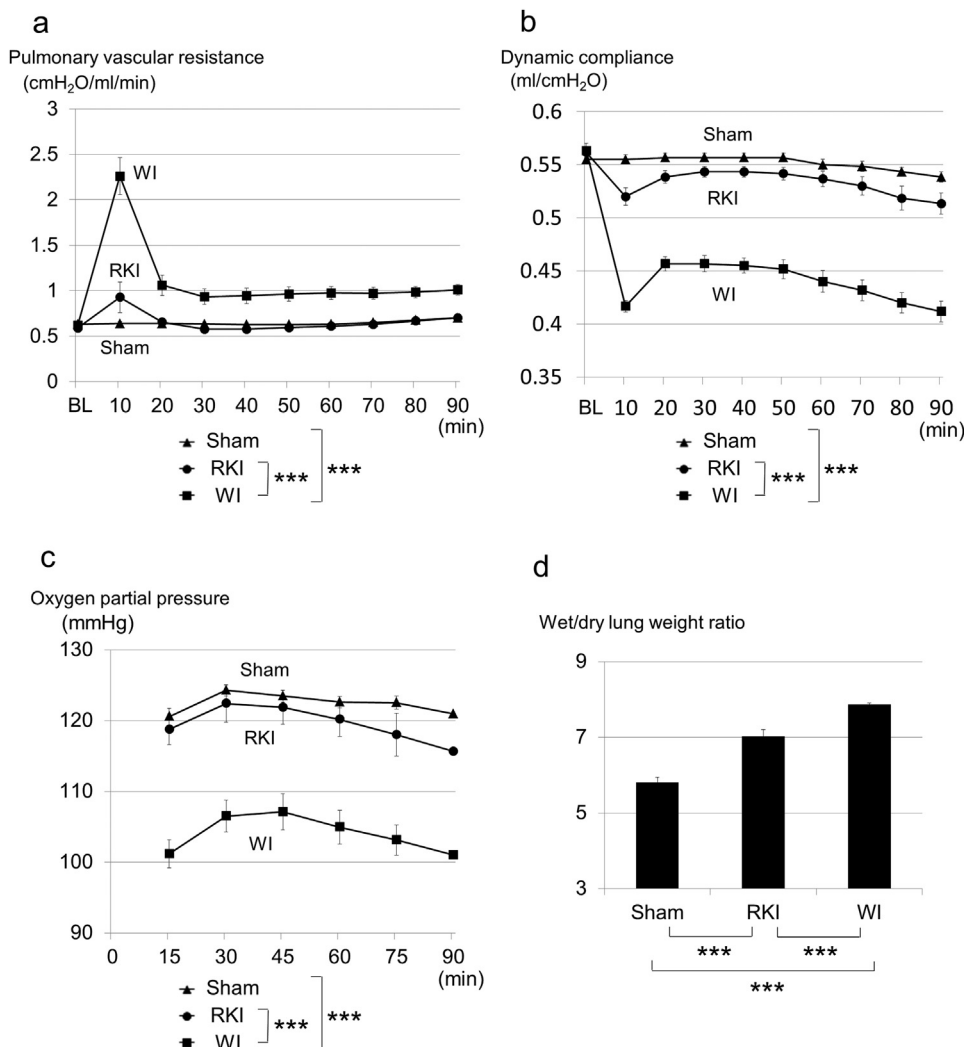


Fig 3. Physiologic data after reperfusion is shown for (a) pulmonary vascular resistance, (b) dynamic compliance, (c) partial pressure of arterial oxygen, and (d) wet/dry lung weight ratio in the sham, Rho-kinase inhibitor (RKI), and warm ischemia (WI) groups. The error bars show the standard error of the mean. *** $p < 0.001$ for differences between the two groups indicated. (BL = baseline.)

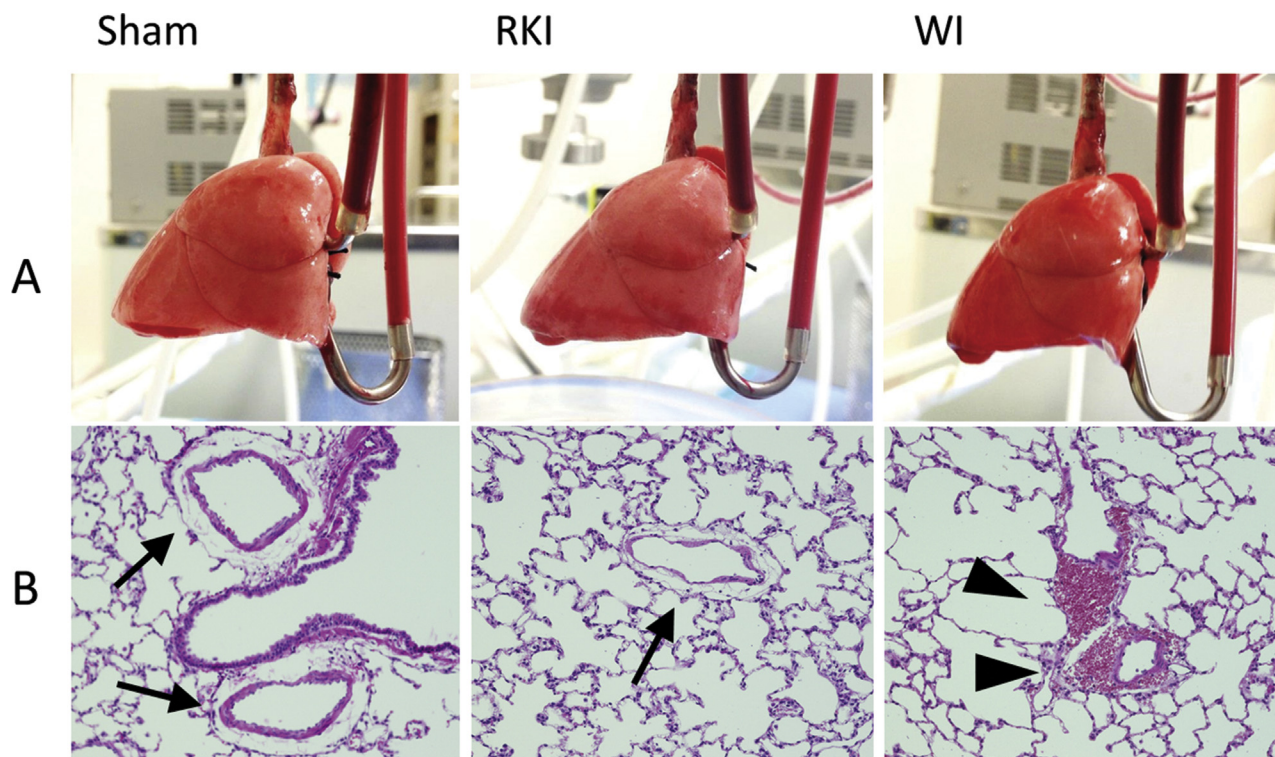


Fig 4. (A) Macroscopic and (B) histologic (hematoxylin and eosin staining, original magnification $\times 200$) findings in rat lungs after reperfusion. Macroscopic findings showed the lungs of the warm ischemia (WI) group were reddish and edematous. This was not present in the sham or Rho-kinase inhibitor (RKI) groups. Microscopically, mild edema in the perivascular space was observed in the sham and RKI groups (arrow). In contrast, severe perivascular edema with sporadic hemorrhage was observed in the WI group (arrowhead).

expression, total myosin phosphatase expression, and the phosphorylation ratio of myosin phosphatase reflecting Rho-kinase activity. Rho-kinase activity and phosphorylated-myosin phosphatase expression were each greater in the WI group than in the sham group. However, these readouts were less pronounced in the RKI group compared with the WI group.

eNOS activity as measured by phosphorylated eNOS expression

Figure 6 shows the mean values of phosphorylated eNOS expression, total eNOS expression, and the phosphorylation ratio of eNOS. Phosphorylated eNOS expression (active form) in the WI group was significantly lower than in the sham and RKI groups. Total eNOS expression in the RKI and WI groups tended to be lower than in the sham group, and the phosphorylation rate of eNOS in the WI group tended to be lower than in other groups, although the differences were not significant in either case.

Comment

In the present study, we showed that Rho-kinase, which is an intracellular serine/threonine kinase regulating cytoskeletal dynamics, plays an important role in the development of lung IRI. Moreover, we observed that inhibition of Rho-kinase alleviated several pathophysiologic features of lung IRI, including alleviation of pulmonary vascular

contraction, dynamic compliance, lung edema, and oxygenation. This study provides new evidence of the biomolecular mechanisms underlying lung IRI.

In this study, we assessed the association of Rho-kinase in lung IRI using Western blotting of specimens taken from an isolated lung perfusion model. We showed that Rho-kinase activity was enhanced after reperfusion following warm ischemia and that this could be suppressed by the Rho-kinase inhibitor fasudil. Furthermore, Rho-kinase inhibition alleviated several negative physiologic changes that occur in IRI. Our results suggest that the activation of Rho-kinase is associated with IRI pathogenesis in the lung with the ensuing pathogenesis a result of myosin phosphatase and eNOS inactivation.

A well-described function of Rho-kinase is vasoconstriction [31]. The unique molecular function of Rho-kinase described below plays a significant role in the development of various vascular diseases [14–17]. Vasoconstriction of the pulmonary artery is also a hallmark physiologic change occurring during lung IRI. In our study, the collected real-time physiologic parameters showed abrupt increases in PVR soon after reperfusion. Cell contraction in vascular smooth muscles is caused by actin-myosin interaction, which is primarily regulated by phosphorylation of MLC. The phosphorylation level of MLC is determined by the balance between the activities of MLC kinase and myosin phosphatase. In lung IRI, activated Rho-kinase decreases myosin phosphatase

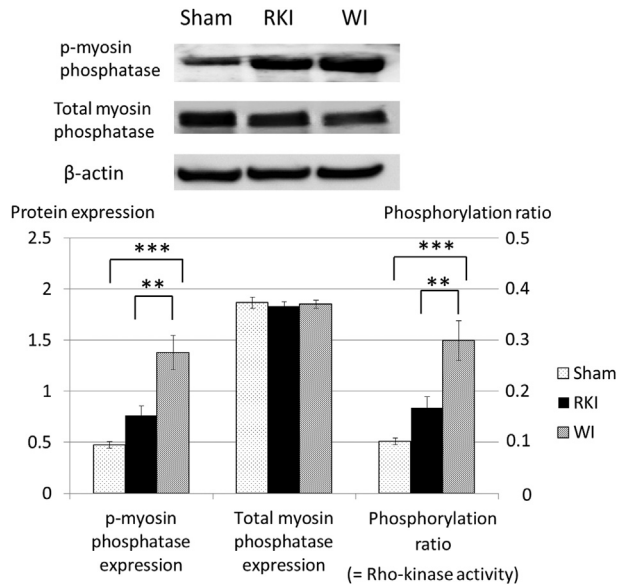


Fig 5. Representative protein bands for phosphorylated (p)-myosin phosphatase, total myosin phosphatase, and β -actin as the protein loading control and the mean values of p-myosin phosphatase expression, total myosin phosphatase expression, and the phosphorylation ratio of myosin phosphatase, which reflects Rho-kinase activity. Rho-kinase activity and p-myosin phosphatase expression were greater in the warm ischemia (WI) group than in the sham and Rho-kinase inhibitor (RKI) groups. The error bars show the standard error of the mean. ** $p < 0.01$, and *** $p < 0.001$ for differences between two groups.

activity through phosphorylation of myosin phosphatase target subunit-1. This leads to increased phosphorylation of MLC and a resulting contraction of vascular smooth muscle (Fig 2). This theory corresponds to our results and is considered to be one of the molecular mechanisms behind vasoconstriction in lung IRI. Through inhibiting this mechanism, Rho-kinase inhibitors act as vasodilators in injured lungs.

The microscopic perivascular edema, decreased pulmonary compliance, and increased wet/dry lung weight ratio that we observed are typical findings describing lung edema. The extraalveolar vessels, which showed perivascular edema in our study, are reported to have greater permeability responses than microcapillaries have in response to a variety of inflammatory stimuli [32]. In general, increased vascular permeability is another physiologic change in lung IRI, which is the major cause of lung edema [18]. Recent studies have shown that Rho-kinase in endothelial cells is involved in endothelial barrier function [33]. Rho-kinase also enhances actin-myosin interactions in endothelial cells, thus inducing cell contraction. Endothelial cell contraction creates intercellular gaps and results in endothelial barrier disruption. Rho-kinase inhibitors are considered to maintain endothelial barrier function by inhibition of this mechanism.

In addition to the inactivation of myosin phosphatase, decreased eNOS activity is likely to be another molecular mechanism under the control of Rho-kinase during lung

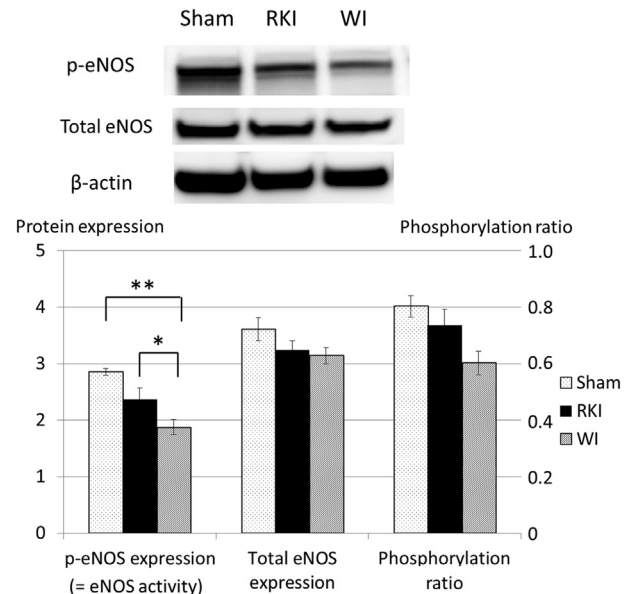


Fig 6. Representative protein bands for phosphorylated (p) endothelial nitric oxide synthase (eNOS), total eNOS, and β -actin and the mean values of p-eNOS expression, total eNOS expression, and the phosphorylation ratio of eNOS. Expression of p-eNOS in the warm ischemia (WI) group was significantly lower than in the sham and Rho-kinase inhibitor (RKI) groups. The error bars show the standard error of the mean. * $p < 0.05$, ** $p < 0.01$ between groups.

IRI. Nitric oxide created by eNOS has multiple physiologic functions, such as regulation of vascular tone, inhibition of leukocyte adhesion, and the scavenging of superoxide anions [34]. Nitric oxide inhalation therapy is in widespread clinical use to improve the lung allograft condition. Rho-kinase is reported to decrease eNOS activity because it shortens the half-life of eNOS messenger RNA and downregulates the gene expression. As a result, eNOS protein expression is decreased [35]. Furthermore, Rho-kinase dephosphorylates eNOS at Ser1177 through the Rho-kinase-Akt pathway [36]. This adds to inhibition of eNOS activity, because phosphorylated Ser1177 positively regulates Rho-kinase activity [37]. The current study confirmed a decrease in phosphorylated eNOS expression in injured lungs, which likely resulted from the increased tendencies of eNOS expression and phosphorylation rate of eNOS.

We made use of the novel Rho-kinase inhibitor fasudil [25], which has already been safely used for the clinical treatment of vasospasm after subarachnoid hemorrhage [38]. In our study, fasudil was administered by inhalation for a specific targeting of the respiratory system. We confirmed a concentration-dependent efficacy of nebulized fasudil on injured lungs in a preliminary experiment (data not shown). Nagaoka and colleagues [17] previously showed the efficacy of inhaled Rho-kinase inhibitor in a rat pulmonary hypertension model. Here, inhalation of nebulized 100 mmol/L fasudil, which was adopted in our study, decreased pulmonary arterial pressure without reducing systemic arterial pressure

[17]. Fasudil has two important characteristics required for inhalation: a low molecular weight and high solubility in water and lipid. We consider the fasudil particles reach alveoli, infiltrate lung cells, and gradually produce the beneficial effect. Inhalation is considered the best method for Rho-kinase inhibitor administration for several reasons: First, airway administration is effective even to injured lungs where intravascular administration is not possible or ineffective because of severe vasoconstriction and thromboses. Second, airway administration of fasudil minimizes its most common adverse effect, decreased systemic arterial pressure. Third, this can be the fastest clinical administration method for cases of lung transplantation with donation after cardiac death, in which there has been a cessation of blood circulation.

This study had several limitations. First, our study made use of an ex vivo lung perfusion system. Further in vivo studies, including a model of lung transplantation, will be required to verify the role of Rho-kinase and its inhibition in IRI. Experiments with genetically engineered mice are also required.

Second, the molecular evaluations of specimens were performed only after 90 minutes of reperfusion following 60 minutes of ischemia. More detailed examinations of the temporal changes in Rho-kinase activity and the effects from the ischemic time length are therefore necessary.

Third, IRI pathogenesis is considered to be multifaceted; therefore, large-animal experiments will be required before the clinical use of such approaches.

In conclusion, our study suggests that activation of Rho-kinase is involved in lung IRI pathogenesis and that Rho-kinase inhibitors may potentially be a useful strategy to reverse this pathogenesis in the clinic.

References

- de Perrot M, Bonser RS, Dark J, et al. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part III: donor-related risk factors and markers. *J Heart Lung Transplant* 2005;24:1460–7.
- Barr ML, Kawut SM, Whelan TP, et al. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part IV: recipient-related risk factors and markers. *J Heart Lung Transplant* 2005;24:1468–82.
- Sato M, Okada Y, Oto T, et al. Registry of the Japanese Society of Lung and Heart-Lung Transplantation: official Japanese lung transplantation report, 2014. *Gen Thorac Cardiovasc Surg* 2014;62:594–601.
- Weyker PD, Webb CA, Kiamanesh D, Flynn BC. Lung ischemia reperfusion injury: a bench-to-bedside review. *Semin Cardiothorac Vasc Anesth* 2013;17:28–43.
- Chen F, Date H. Update on ischemia-reperfusion injury in lung transplantation. *Curr Opin Organ Transplant* 2015;20:515–20.
- Adkins WK, Taylor AE. Role of xanthine oxidase and neutrophils in ischemia-reperfusion injury in rabbit lung. *J Appl Physiol* (1985) 1990;69:2012–8.
- Al-Mehdi AB, Zhao G, Dodia C, et al. Endothelial NADPH oxidase as the source of oxidants in lungs exposed to ischemia or high K⁺. *Circ Res* 1998;83:730–7.
- Suda T, Mora BN, D'Ovidio F, et al. In vivo adenovirus-mediated endothelial nitric oxide synthase gene transfer ameliorates lung allograft ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 2000;119:297–304.
- Ishiyama T, Dharmarajan S, Hayama M, Moriya H, Grapperhaus K, Patterson GA. Inhibition of nuclear factor kappaB by IkappaB superrepressor gene transfer ameliorates ischemia-reperfusion injury after experimental lung transplantation. *J Thorac Cardiovasc Surg* 2005;130:194–201.
- Shimamoto A, Pohlman TH, Shomura S, Tarukawa T, Takao M, Shimpo H. Toll-like receptor 4 mediates lung ischemia-reperfusion injury. *Ann Thorac Surg* 2006;82:2017–23.
- de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med* 2003;167:490–511.
- Ishizaki T, Maekawa M, Fujisawa K, et al. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *Embo J* 1996;15:1885–93.
- Maekawa M, Ishizaki T, Boku S, et al. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 1999;285:895–8.
- Shimokawa H, Seto M, Katsumata N, et al. Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. *Cardiovasc Res* 1999;43:1029–39.
- Sato M, Tani E, Fujikawa H, Kaibuchi K. Involvement of Rho-kinase-mediated phosphorylation of myosin light chain in enhancement of cerebral vasospasm. *Circ Res* 2000;87:195–200.
- Uehata M, Ishizaki T, Satoh H, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;389:990–4.
- Nagaoka T, Fagan KA, Gebb SA, et al. Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. *Am J Respir Crit Care Med* 2005;171:494–9.
- den Hengst WA, Gielis JF, Lin JY, Van Schil PE, De Windt LJ, Moens AL. Lung ischemia-reperfusion injury: a molecular and clinical view on a complex pathophysiological process. *Am J Physiol Heart Circ Physiol* 2010;299:H1283–99.
- Yada T, Shimokawa H, Hiramatsu O, et al. Beneficial effect of hydroxyfasudil, a specific Rho-kinase inhibitor, on ischemia/reperfusion injury in canine coronary microcirculation in vivo. *J Am Coll Cardiol* 2005;45:599–607.
- Takeda K, Jin MB, Fujita M, et al. A novel inhibitor of Rho-associated protein kinase, Y-27632, ameliorates hepatic ischemia and reperfusion injury in rats. *Surgery* 2003;133:197–206.
- Versteilen AM, Blaauw N, Di Maggio F, et al. Rho-Kinase inhibition reduces early microvascular leukocyte accumulation in the rat kidney following ischemia-reperfusion injury: roles of nitric oxide and blood flow. *Nephron Exp Nephrol* 2011;118:e79–86.
- Chen F, Nakamura T, Fujinaga T, et al. Protective effect of a nebulized beta2-adrenoreceptor agonist in warm ischemic-reperfused rat lungs. *Ann Thorac Surg* 2006;82:465–71.
- Ohsumi A, Chen F, Nakajima D, et al. Therapeutic effect of surfactant inhalation during warm ischemia in an isolated rat lung perfusion model. *Transpl Int* 2012;25:1096–105.
- Motoyama H, Chen F, Hijiya K, et al. Plasmid administration during ex vivo lung perfusion ameliorates lung ischemia-reperfusion injury. *J Heart Lung Transplant* 2014;33:1093–9.
- Mueller BK, Mack H, Teusch N. Rho kinase, a promising drug target for neurological disorders. *Nat Rev Drug Discov* 2005;4:387–98.
- Muranyi A, Derkach D, Erdodi F, Kiss A, Ito M, Hartshorne DJ. Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells. *FEBS Lett* 2005;579:6611–5.
- Duong-Quy S, Dao P, Hua-Huy T, Guilluy C, Pacaud P, Dinh-Xuan AT. Increased Rho-kinase expression and activity and pulmonary endothelial dysfunction in smokers with normal lung function. *Eur Respir J* 2011;37:349–55.

28. Cazzola M, Calzetta L, Rogliani P, et al. High glucose enhances responsiveness of human airways smooth muscle via the Rho/ROCK pathway. *Am J Respir Cell Mol Biol* 2012;47:509–16.
29. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9:671–5.
30. Kanda Y. Investigation of the freely available easy-to-use software 'EZ' for medical statistics. *Bone Marrow Transplant* 2013;48:452–8.
31. Somlyo AP, Somlyo AV. Ca²⁺ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;83:1325–58.
32. Lowe K, Alvarez DF, King JA, Stevens T. Perivascular fluid cuffs decrease lung compliance by increasing tissue resistance. *Crit Care Med* 2010;38:1458–66.
33. Hirase T, Kawashima S, Wong EY, et al. Regulation of tight junction permeability and occludin phosphorylation by RhoA-p160ROCK-dependent and -independent mechanisms. *J Biol Chem* 2001;276:10423–31.
34. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993;329:2002–12.
35. Laufs U, Endres M, Stagliano N, et al. Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J Clin Invest* 2000;106:15–24.
36. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601–5.
37. Ming XF, Viswambharan H, Barandier C, et al. Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol Cell Biol* 2002;22:8467–77.
38. Satoh S, Ikegaki I, Kawasaki K, Asano T, Shibuya M. Pleiotropic effects of the rho-kinase inhibitor fasudil after subarachnoid hemorrhage: a review of preclinical and clinical studies. *Curr Vasc Pharmacol* 2014;12:758–65.

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