**Original
Article**

Attenuation of Lung Ischemia-Reperfusion Injury by Rho-Associated Kinase Inhibition in a Rat Model of Lung Transplantation

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Background: A signaling pathway of the small GTPase Rho and Rho-associated coiled-coil-forming protein kinase (ROCK), regulates the contraction of endothelial cells. We studied the effects of Y-27632, a specific ROCK inhibitor, to clarify the role of Rho/ROCK in the pathogenesis of ischemia-reperfusion lung injury in a rat model of single-lung transplantation (LTx).

Methods: We flushed 5 donor rat lungs with Euro-Collins solution, and 5 donor lungs with Euro-Collins + Y-27632, 0.03 mg/ml, and preserved the lungs for 6 h at 4°C before reperfusion for 4 h. The 5 rat recipients of Y-27632-treated lungs also received a 10-mg/kg bolus of Y-27632 i.p. 30 min before reperfusion.

Results: Pretreatment of the donor lungs and recipient rats with Y-27632 prominently suppressed the post-LTx edema, while the permeability index was only slightly decreased. The (1) numbers of neutrophils and macrophages, and (2) tumor necrosis factor (TNF)- α concentration, were significantly lower in the bronchoalveolar lavage fluid of treated than untreated lungs.

Conclusions: Y-27632 (1) inhibited the migration of inflammatory cells into the alveolar space, (2) decreased the production of TNF- α , and (3) attenuated the edema after LTx. Endothelial Rho and ROCK may play an important role in the pathogenesis of post-LTx injury.

Keywords: ischemia reperfusion injury, lung transplantation, rat model, tumor necrosis factor- α , Rho/Rho-associated coiled-coil-forming protein kinase

Introduction

Lung transplantation is a treatment option for patients with end-stage lung disease.^{1–3)} Despite considerable

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Received: April 29, 2013; Accepted: June 26, 2013

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recent progress made in the operative management of lung transplant (LTx) candidates, lung injury due to ischemia-reperfusion (I-R) remains an important cause of postoperative morbidity and mortality.⁴⁾ Furthermore, graft dysfunction occurring early after LTx promotes the rejection of the graft.^{5,6)} Experimental and clinical observations suggest that the main dysfunctional characteristic of I-R injury is an increase in pulmonary microvascular permeability, and that the transendothelial migration of inflammatory cells may be a critical step in its development after LTx, as well as a source of inflammatory mediators.^{7–10)} However, the mechanisms of post-transplant lung injury remain poorly known, and an effective treatment of I-R injury has yet to be found.

The contraction of endothelial cells is regulated by a signaling pathway of the small GTPase Rho and its target protein, Rho-kinase, or Rho-associated coiled-coil-forming protein kinase (ROCK).^{11–13} We have recently observed that, in endotoxin-induced lung injury or re-expansion pulmonary edema, Rho and Rho kinase appear to regulate the transendothelial cell migration and control endothelial permeability by modulating the intracellular cytoskeleton via the phosphorylation of endothelial myosin light chain.^{14,15} Although the role played by the Rho/ROCK-mediated pathway in I-R injury after LTX remains unclear, we hypothesized that it is implicated in the changes in endothelial permeability. We tested our hypothesis in lung preservation experiments with Y-27632, a highly selective inhibitor of ROCK,¹⁶ which allows to examine the role played by the Rho/ROCK-mediated pathway in vivo.^{17,18} We specifically ascertained, in a rat model of LTX, the role played by the Rho/ROCK-mediated pathway in the transvascular migration of inflammatory cells into the alveoli, and the production of tumor necrosis factor (TNF)- α in the bronchoalveolar lavage (BAL) fluid, which plays a critical role in post-transplantation lung injury.¹⁰

Materials and Methods

Animal model

Specific, pathogen-free, inbred Lewis rats, 10-weeks of age, weighing 300 g to 350 g (Charles River Breeding Laboratories, Tokyo, Japan) were used as donor and recipient animals. All procedures described in this report were approved by our institutional review board for animal studies. For our orthotopic left LTX in rats, we have modified previously described surgical techniques.^{19,20} Under general anesthesia, the donor animals were intubated, artificially ventilated, and administered heparin, 1000 U/kg, i.v. Via median sternotomy, a 14-gauge catheter was inserted into the main pulmonary artery through the right ventricle. After section of the inferior vena cava and amputation of the left and right atrial appendages, the pulmonary artery was flushed with 100 ml/kg of one of the preservation solutions described later, at a temperature of 4°C and pressure of 18 cm H₂O. The trachea was ligated and cut at end-inspiration of the ventilator cycle, and the donor heart and lungs were removed en bloc. The left lung was wrapped in gauze soaked with 50 ml of preservation solution, and stored at 4°C. An orthotopic left LTX was then performed in the recipient rats, using a cuff technique for the vessel and bronchial

anastomosis. Blood flow and ventilation to the transplanted lung were reestablished after 6 h of cold ischemia. After chest closure and awakening from the anesthesia, the recipient animals were housed freely in room air, and sacrificed after 4 h of reperfusion. The grafted lungs were harvested and used for the evaluation of lung injury.

Experimental groups

Lewis rats were divided between 2 experimental groups. In 5 rats assigned to LTX alone, the pulmonary artery was flushed with 100 ml/kg of Euro-Collins solution (LTX group), while in 5 rats assigned to the treatment group (LTX + Y-27632), 0.03 mg/ml of Y-27632 (Mitsubishi Pharma Co., Osaka, Japan), was added to the Euro-Collins solution to preserve the lungs. This latter group also received a 10-mg/kg bolus of Y-27632 i.p., 30 min before reperfusion of the lungs. Human serum albumin (Buminate, Baxter Healthcare Corporation, Glendale, California, USA), was injected i.v., 1 h before sacrifice of the animals, for measurement of the lung vascular permeability index. The animals were sacrificed by i.p. injection of 50 mg of pentobarbital, 4 h after reperfusion of the lungs. Blood samples were collected by cardiocentesis, and the transplanted left lung was harvested and lavaged with 2.5 ml of saline. The BAL fluid was centrifuged at 3000 rpm and 4°C for 15 min, and the supernatant was stored at –80°C until further analyses.

Study measurements

The wet-to-dry weight ratio of the transplanted lung was calculated as the lung edema index. The vascular permeability index of the grafts was calculated as the human serum albumin concentration in BAL fluid/plasma concentration ratio. Commercially available enzyme linked immunosorbent assay kits (Rat TNF- α Assay Kit, Takara Bio Inc., Shiga, Japan) were used to measure the concentrations of TNF- α in BAL fluid.

Bronchoalveolar lavage fluid cytology and histopathologic studies

To evaluate the effects of Y-27632 on the migration of inflammatory cells into the alveolar space, we compared the number of cells in the BAL fluid recovered from the treated versus the control rats. Smears of BAL fluid obtained by cytocentrifugation were stained with a modified Wright's stain for total and 200-cell differential counts, using a modified hemacytometer method

(Unopette™ Microcollection System; Becton Dickinson, Rutherford, New Jersey, USA).

The histopathologic effects of Rho kinase inhibition were examined by comparing lung specimens harvested from rats treated with Y-27632, with specimens obtained from control rats. The lung specimens were inflated with 10% buffered formalin at a pressure of 10 cm H₂O, fixed, embedded in paraffin, cut in 4-μm sections, and stained with hematoxylin and eosin.

Statistical analyses

The data are expressed as means ± SE. Between-groups differences in measurements at specific time points were examined by Student's *t*-test. A *P* value <0.05 was considered statistically significant. The analyses were performed with the StatView-J, version 5.0 (Abacus Concepts Inc., Berkeley, California, USA).

Results

Lung water and permeability index

The amount of water retained in the transplanted lungs was measured after 6 h of ischemia and 4 h of reperfusion. Addition of Y-27632 to the lung preservation solution and its i.v. administration to the grafts recipients significantly decreased the lung edema index (*P* <0.05) compared with the LTX group (**Fig. 1**). The permeability index in the group pre-treated with Y-27632 was also lower than in the control group, though the difference was not statistically significant (**Fig. 2**).

Bronchoalveolar fluid examination

The results of the BAL fluid cytology are shown in **Fig. 3**. After 6 h of ischemia and 4 h of reperfusion, the overall number of cells was significantly lower in the group of rats treated with Y-27632 than in the LTX group (*P* = 0.01). While neutrophils were the predominant cell type in both study groups, the neutrophil and macrophage counts were both lower in the LTX + Y-27632-treated than in the control group (*P* <0.05). Similarly, pretreatment with Y-27632 significantly lowered the concentration of TNF-α in BAL fluid (**Fig. 4**) compared with exposure to the Euro-Collins solution only (*P* <0.05).

Histopathologic observations

On histologic examination, prominent alveolar hemorrhages, infiltration of inflammatory cells, and interstitial

Fig. 1 Mean (± SE) wet-to-dry weight ratio of transplanted lung measured after 6 h of lung ischemia and 4 h of reperfusion, in 5 control (LTX) and 5 treated (LTX + Y-27632) rats. **P* <0.05.

Fig. 2 Mean (± SE) permeability index measured after 6 h of lung ischemia and 4 h of reperfusion, in 5 control (LTX) and 5 treated (LTX + Y-27632) rats.

Fig. 3 Mean (± SE) total and differential cell counts in the bronchoalveolar lavage fluid (BALF) recovered from the transplanted lungs after 6 h of ischemia and 4 h of reperfusion in 5 control (LTX) and 5 treated (LTX + Y-27632) rats. **P* <0.05.

Fig. 4 Mean ($\pm$ SE) TNF- α concentration in bronchoalveolar lavage (BAL) fluid measured after 6 h of lung ischemia and 4 h of reperfusion, in 5 control (LTX) and 5 treated (LTX + Y-27632) rats. * P < 0.05.

Fig. 5 Representative histology of transplanted lungs after 6 h of lung ischemia and 4 h of reperfusion in (A) a control (LTX), and (B) a Y-27632-treated (LTX + Y-27632) rat.

thickening were found in the transplanted lungs of the LTX group (**Fig. 5A**). In contrast, only faint alveolar hemorrhages and less marked inflammatory infiltration, and interstitial thickening were observed in the rats treated with Y-27632 (**Fig. 5B**).

Discussion

In this study of I-R lung injury after LTX in rats, pretreatment with the ROCK-specific inhibitor, Y-27632, attenuated (a) the accumulation of macrophages and neutrophils, and (b) the formation edema, in the transplanted lung. Furthermore, the production of TNF- α in the BAL fluid recovered from the grafts was suppressed by pretreatment with Y-27632. These observations strongly suggest that the Rho/ROCK-mediated pathway is implicated in the pathogenesis of I-R lung injury after LTX.

We have previously found, in a rabbit model, that the Rho/ROCK-mediated pathway is involved in the increase in pulmonary vascular permeability associated with reexpansion pulmonary edema.¹⁵⁾ In that model, Y-27632 significantly limited the increase in endothelial permeability and inhibited the reorganization of F-actin and formation of intercellular gaps. This suggests that Y-27632 downregulates the Rho/ROCK signal transduction pathway in endothelial cells, and suppresses the hyperpermeability of the reexpanded lung by inhibiting the reorganization of F-actin, and by subsequently blocking the contraction of the vascular endothelial cells. In this study, the addition of Y-27632 to the lung preservation solution and its intravenous administration before LTX significantly attenuated the severity of pulmonary edema, though had a lesser effect of vascular permeability. Since, unlike in the case of re-expansion pulmonary edema, the transplantation model requires a complete interruption of the pulmonary blood flow, other mechanisms than the Rho/ROCK-mediated pathway might be operative in post-transplant lung injury, which would explain the absence of a significant decrease in pulmonary vascular permeability conferred by Y-27632.

We have previously found, in a rat model of LTX, that the production of TNF- α begins in alveolar macrophages, stimulated by reperfusion after ischemia, and that the lung injury cascade triggered by soluble TNF- α begins after the cleavage of membranous TNF- α by a TNF- α -converting enzyme.¹⁰⁾ We have also observed the mitigation of endothelial and alveolar septal injury by inhibition of the release of soluble TNF- α by a TNF- α -converting enzyme inhibitor. In this study, the concentrations of soluble TNF- α in the BAL fluid recovered from the transplanted lungs were significantly lower in the Y-27632-treated than in the LTX group, along with a decrease in the accumulation of inflammatory cells in the lung tissue. This suggests that inhibition of the Rho/ROCK-mediated pathway suppressed the increase in local concentrations of TNF- α by

preventing the infiltration of the grafts by inflammatory cells, including macrophages. Therefore, in the LTX model, Y-27632 may limit the development of edema by suppressing the production of cytokines, such as TNF- α .

Recent studies showed that ROCK is involved in the regulation of apoptosis.^{21,22)} Okumura, et al., found that the culture of endothelial cells in medium containing Y-27632 inhibited apoptosis and increased the number of proliferating cells.²³⁾ In addition, ROCK inhibition was anti-apoptotic in some models of spinal cord injury,²⁴⁾ dissociated human embryonic stem cells,²⁵⁾ and grafted neural precursors.²⁶⁾ These observations may support the efficacy of adding Y-27632 to the lung preservation solution. On the other hand, the systemic administration of Y-27632 alone decreased I-R injury in hepatic²⁷⁾ and cardiac transplantation models.²⁸⁾ Therefore we believe that (a) adding Y-27632 to the lung preservation solution and (b) administering it i.v. to the graft recipient might synergistically attenuate lung I-R injury in a lung transplantation model. Further research is needed to examine the effects of pre-treating with Y-27632 the donor lungs, the recipient rats, or both, on lung I-R injury.

The inhibition of I-R lung injury by pretreatment of our lung-transplanted rats with Y-27632 was incomplete, suggesting a limited protective effect. While Y-27632 is also known to inhibit the contraction of smooth muscles and normalize the blood pressure of hypertensive rats,¹⁶⁾ we observed no manifestation of systemic hypotension, whether after its intravenous administration, or after the transplantation of lungs pretreated with Y-27632. More detailed studies are needed before attempts are made to use a ROCK inhibitor clinically in LTX.

Conclusion

In a rat model of LTX, Y-27632 attenuated I-R lung injury by suppressing the development of edema and infiltration of the grafts by inflammatory cells. These effects seemed attributable to a mitigation of endothelial cell injury. Pretreatment with Y-27632 also lowered the concentrations of TNF- α in BAL fluid recovered from the grafts. These observations suggest that the inhibition of the Rho/ROCK-mediated pathway confers therapeutic effects in LTX-related I-R lung injury.

Disclosure Statement

Mitsutomo Kohno and other co-authors have no conflict of interest.

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