Original Article

Role of Rho-kinase in Re-expansion Pulmonary edema in Rabbits

Makoto Sawafuji, Akitoshi Ishizaka*, Mitsutomo Kohno, Hidefumi Koh*, Sadatomo Tasaka*, Yoshiki Ishii ** and Koichi Kobayashi

From: Department of Surgery, Keio University, Tokyo, Japan 160-8582
*Department of Medicine, Keio University, Tokyo, Japan 160-8582
**Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University, Tochigi, Japan 321-0293

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Address all correspondences and reprint requests to:

Makoto Sawafuji, MD.
Department of Surgery, Keio University
35 Shinanomachi, Shinjuku-ku, TOKYO 160-8582 Japan
Phone: 81-3-3353-1211 or 3-4356-6281(direct) Fax: 81-3-4356-6424
Email: msawafuji@nifty.com

Key words: acute lung injury, acute respiratory distress syndrome, re-expansion pulmonary edema, Rho, Rho-kinase
**Abstract**

Re-expansion of a collapsed lung increases the microvascular permeability and causes re-expansion pulmonary edema. Neutrophils and their products have been implicated in the development of this phenomenon. The small GTP-binding proteins Rho and its target Rho-kinase (ROCK) regulate endothelial permeability, though their roles in re-expansion pulmonary edema remain unclear. We studied the contribution of ROCK to pulmonary endothelial and epithelial permeability in a rabbit model of this disorder. Endothelial and epithelial permeability was assessed by measuring the tissue/plasma (T/P) and broncho-alveolar lavage (BAL) fluid/plasma (B/P) ratio with $^{125}$I-albumin. After intratracheal instillation of $^{125}$I-albumin, epithelial permeability was also assessed from the plasma leak (PL) index, the ratio of $^{125}$I-albumin in plasma/total amount of instilled $^{125}$I-albumin. T/P, B/P and PL index were significantly increased in the re-expanded lung. These increases were attenuated by pre-treatment with Y-27632, a specific ROCK inhibitor. However, neutrophil influx, neutrophil elastase activity, and malondialdehyde concentrations in BAL fluid collected from the re-expanded lung were not changed by Y-27632. In endothelial monolayers, Y-27632 significantly attenuated the H$_2$O$_2$-induced increase in permeability, and mitigated the morphologic changes in the actin microfilament cytoskeleton of endothelial cells. These *in vivo* and *in vitro* observations suggest that the Rho/ROCK pathway contributes to the increase in alveolar barrier permeability associated
with re-expansion pulmonary edema. (208 words)

**Key words:** acute lung injury, acute respiratory distress syndrome, re-expansion pulmonary edema, Rho, Rho-kinase
**Introduction**

Re-expansion of a collapsed lung occasionally causes acute lung injury (ALI) known as re-expansion pulmonary edema (3, 17, 18). The main characteristic of this disorder is an increase in pulmonary microvascular permeability (24, 39). Vascular endothelial permeability is related to the cellular cytoskeleton. An increase in vascular permeability is accompanied by a reorganization of the actin-based cytoskeleton and contraction of endothelial cells, resulting in the formation of intercellular gap (7, 28). A calmodulin-dependant myosin light chain kinase (MLCK) contributes to the regulation of cell contraction and endothelial cell permeability. Activation of MLCK causes the phosphorylation of the myosin light chains, resulting in the contraction of acto-myosin, in the formation of stress fibers and in subsequent hyperpermeability of endothelial cells (8). A signaling pathway of the small GTPase Rho and its target protein, Rho-kinase (Rho-associated coiled-coil forming protein kinase; ROCK), also regulates the contraction of endothelial cells (5). The activation of Rho leads to the phosphorylation of the myosin binding subunit of myosin light chain phosphatase via the action of ROCK, inactivating the myosin light chain phosphatase and blocking the dephosphorylation of myosin light chains (16). Continued phosphorylation of myosin light chains results in sustained cell contraction.

In studies in isolated rat lungs, a myosin light chain kinase inhibitor attenuated the injury associated with ischemia-reperfusion (15) and that
induced by ventilators (23). These observations suggest that the intracellular signal transduction which regulates the cellular cytoskeleton is involved in the pathophysiology of some forms of ALI. However, the role of the Rho/ROCK-mediated pathway in re-expansion pulmonary edema in vivo remains unclear, while more attention has been paid to the role of alveolar epithelial injury in the pathophysiology of ALI (38). The Rho/ROCK-mediated pathway has been implicated in the organization of perijunctional actin and in the regulation of permeability of tight-junction of the epithelial cells (6, 22). Therefore, we hypothesized that the Rho/ROCK-mediated pathway is involved in the changes in alveolar endothelial and epithelial barrier permeability in re-expansion pulmonary edema.

Y-27632, a highly selective inhibitor of ROCK in vitro and in vivo (36), was found useful to examine the role of the Rho/ROCK-mediated pathway in vivo (11, 29). To test our hypothesis, we examined the inhibitory effect of Y-27632 in our rabbit model of re-expansion lung injury (20). We measured the flux of transvascular albumin as an indicator of endothelial permeability (10). Alveolar epithelial permeability was assessed by measuring the bi-directional flux of albumin across the alveolar epithelial barrier (10, 30, 40). We also performed in vitro experiments in human pulmonary artery endothelial cell monolayers to study the effects of Y-27632 on hyper-permeability and endothelial cytoskeletal rearrangement induced by hydrogen peroxide (H₂O₂). These experiments were performed since reactive oxygen species appear
involved in the injury to pulmonary endothelial cells observed in re-expansion pulmonary edema (12). We observed morphologic changes in the microfilament cytoskeleton using a rhodamine phalloidin stain (25). The main objective of this study was to ascertain the role of the Rho/ROCK pathway in the increased alveolar endothelial and epithelial barrier permeability present in re-expanded lungs.

**Methods**

The experiments were performed in 45 male Japanese White rabbits, weighing between 900 and 1800 g. All procedures were reviewed and approved by the Laboratory Animal Care Panel of Keio University. The animals were divided into three groups: 1) a re-expansion group (RE), 2) a Rho-kinase inhibitor pre-treatment group (RE+Y), which received 10 mg/kg of Y-27632 intraperitonealy before re-expansion of the left lung, and 3) a sham-operated group (sham), which underwent thoracotomy only. The surgical techniques have been previously reported in detail (20). Briefly, general anesthesia was induced with intramuscular ketamine, 75 mg/kg, and xylazine, 5 mg/kg. A tracheostomy was performed and a 3.5-mm diameter Portex LTD tracheal cannula (Hythe, Kent, UK) was inserted. The rabbits were mechanically ventilated (SN-480-7, Shinano Co., Tokyo, Japan) with room air at 30 breaths/min and a tidal volume of 10 ml/kg.

In the RE and RE+Y groups, after completion of the left posterolateral
thoracotomy, the left main bronchus was clamped with an atraumatic vascular clip (Vascu-statt, Scanlan International, Inc., St Paul, MN) to produce complete collapse of the left lung by absorption of the alveolar gas into the pulmonary circulation. A 4-0 nylon string attached to the clip was exteriorised for later retraction. In the sham group, a left posterolateral thoracotomy was performed and the left main bronchus was dissected from the surrounding tissue. The chest wall was closed. The rabbits were allowed to recover for 36 h before the experiments began. The tracheal cannula was removed after the recovery of spontaneous breathing.

**Experimental protocol**

**Experiment 1**

Twenty-one animals were divided into three groups listed above to measure endothelial and epithelial permeability. The protocol for the experiment 1 is provided in Figure 1. Endothelial permeability was assessed from the transvascular flux of $^{125}$I-albumin administered intravenously. Epithelial permeability was measured as the flux of $^{125}$I-albumin from intravascular space to air space. To correct blood contamination in the lung tissue or broncho-alveolar lavage (BAL) fluid samples, $^{131}$I-albumin was injected intravenously. On the day of experiments, the rabbits were sedated with intramuscular ketamine, 100 mg/kg, 30 min before the experiment, placed in the supine position and a 24-gauge catheter was inserted in an ear vein. Two
ml of 37-kBq/ml $^{125}$I-labeled bovine serum albumin (Life Science Products Inc., Boston, MA) was injected i.v. to assess the pulmonary extravasated albumin in all experimental groups. In the RE and RE+Y groups, 10 min after $^{125}$I-albumin injection, the left main bronchus was re-opened by retracting the string. At this time, a 9-F drainage chest tube (Sumitomo Bakelite CO., Tokyo, Japan) was inserted into the left pleural cavity and continuous suction at -10cmH$_2$O was applied through a chest tube to re-inflate the collapsed lung. In the RE+Y group, 10 mg/kg of Y-27632 was administered into the peritoneal cavity, 30 min before re-opening the left main bronchus. An identical volume of saline was injected intraperitoneally in the sham and RE group. Room air breathing resumed spontaneously. To prevent hypotension and dehydration, 50 ml/kg of normal saline was infused i.v. throughout the period of continuous suction. Two ml of 37-kBq/ml $^{131}$I-labeled human serum albumin (Daiichi radioisotope Laboratories, Tokyo, Japan) was injected i.v., 115 min after re-opening the left main bronchus. We used $^{131}$I-albumin as a plasma volume marker to correct for residual blood content in excised lung tissue samples. Heparin, 2000 IU i.v., was injected simultaneously. The animals were sacrificed 120 min after re-opening the left main bronchus by injection of 100 mg of pentobarbital i.v. The chest was immediately opened, 5 ml of blood were collected by cardiac puncture, each pulmonary hilus was clamped at the end of expiration, and the lungs were removed and divided into superior and inferior lobes. Blood and superior lobe lung samples were placed in a pre-weighed glass tube for
measurements of weight and radioactivity.

Total blood cells were counted with a Sysmex K-1000 counter adapted for rabbit cells (Sysmex Co., Kobe, Japan). Differential counts of 200 cells were performed on glass slide smears stained by a modified Wright’s stain (Diff-Quick; American Scientific Product, McGraw Park, IL).

BAL was performed with 10 ml normal saline through a catheter secured within the bronchus of the excised inferior lobe. The volume of fluid recovered from the left lung of the sham, RE and RE+Y groups were 7.9±0.3 ml, 8.1±0.4 ml and 8.0±0.5 ml, respectively. The corresponding volumes recovered from the right lung were 7.9±0.4 ml, 8.3±0.3 ml and 8.3±0.5 ml, respectively. There was no significant difference in the recovery of BAL fluid among the groups. The fluid was centrifuged at 400g and 4°C for 10 min. The supernatant was used for gamma counting of $^{125}$I and $^{131}$I to measure the pulmonary epithelial permeability, and for the analysis of neutrophil elastase activity and malondialdehyde (MDA) concentration. The cell pellet was re-suspended in 1 ml of saline, and the cells were counted by a modified hemocytometer method (Unopet Microcollection System; Becton Dickson, Rutherford, NJ). Cell differential in BAL was counted in smears obtained by cytocentrifugation. Differential counts were made on 200 cells from smears stained with a modified Wright’s stain.

In 9 additional animals, divided into the 3 experimental groups and treated similarly, the mean arterial pressure was measured with a polygraph
Experiment 2

Twelve animals were divided among the 3 experimental groups described earlier to measure the epithelial permeability. Epithelial permeability was measured as the flux from air space to intravascular space of $^{125}$I-albumin instilled into the left lung. On the day of experiments, 5 ml of blood was collected from a catheter inserted in the right carotid artery, heparinized and centrifuged to obtain plasma. The blood was replaced with an equal volume of Ringer lactate. Five min after the onset of continuous suction, the rabbits were placed in the left lateral decubitus position to facilitate the collection of liquid in the left lung. Two ml/kg of autologous plasma containing 3 mg of Evan’s blue dye and 212 kBq of $^{125}$I-labeled bovine serum albumin were instilled into the left inferior lobe over 5 min with an 18-gauge tube advanced through the tracheostomy, as previously described (30, 40) We confirmed that most of the dye was distributed in the left lung after sacrifice of the animal. Blood samples were obtained for measurements of $^{125}$I-albumin activity at baseline and 10, 30, 60, 90, 120 min after instillation. Since, clinically, the onset of re-expansion pulmonary edema takes place immediately or within a few hours (17), we chose these time points of observation to examine the early changes in flux of albumin from lung air space to intravascular space.

Pulmonary endothelial permeability

Pulmonary endothelial permeability was calculated as the ratio of $^{125}$I-albumin
per unit weight of lung tissue extravascular space over that in plasma (T/P ratio) in the left and right superior lobes. The activities of $^{125}\text{I}$ and $^{131}\text{I}$ in superior lobe parenchyma and blood were counted with a gamma counter (ARC-300; Aloca, Tokyo, Japan), with appropriate corrections for crossover between radionuclides. The extravascular $^{125}\text{I}$-albumin in the superior lobe was calculated by subtracting the amount of intravascular $^{125}\text{I}$-albumin from the total $^{125}\text{I}$-albumin in the lung tissue sample. The intravascular $^{125}\text{I}$-albumin in the superior lobe was corrected by calculating the ratio $^{125}\text{I}$ to $^{131}\text{I}$ counts in the unit weight of blood and $^{131}\text{I}$-albumin in the unit weight of superior lobe parenchyma. We assumed that all of the $^{131}\text{I}$-albumin was confined to the circulation, since the effect of leakage of the plasma volume marker into the extravascular space is negligible when calculating T/P (13). Therefore the $^{131}\text{I}$ counts must reflect the residual blood in the lung tissue sample. The following formula was used to calculate the T/P ratio (10):

$$\frac{[^{125}\text{I} \text{ tissue} - ^{125}\text{I} \text{ blood} \times ^{131}\text{I} \text{ tissue} / ^{131}\text{I} \text{ blood}]}{^{125}\text{I} \text{ blood} / (1 - \text{Hct})},$$

where $^{125}\text{I} \text{ tissue} = ^{125}\text{I} \text{ cpm from a unit weight of superior lobe lung tissue}$; $^{125}\text{I} \text{ blood} = ^{125}\text{I} \text{ cpm from a unit weight of blood}$; $^{131}\text{I} \text{ tissue} = ^{131}\text{I} \text{ cpm from a unit weight of superior lobe lung tissue}$; $^{131}\text{I} \text{ blood} = ^{131}\text{I} \text{ cpm from a unit weight of blood}$; and Hct = hematocrit.

**Pulmonary epithelial permeability**

Pulmonary epithelial permeability was ascertained by two separate methods used to measure the bi-directional flux of albumin across the alveolar epithelial
barrier: 1) In experiment 1, the flux of albumin from intravascular space to lung air space was assessed by measuring the BAL fluid to plasma $^{125}$I-albumin concentration ratio (B/P ratio). We hypothesized that, if epithelial permeability was increased, the amount of $^{125}$I-albumin entering the air space from the circulation would increase, thus increasing the amount of $^{125}$I-albumin collected in BAL fluid. Blood contamination in BAL fluid was also corrected by $^{131}$I activity of BAL fluid (10). The following formula was used to calculate the B/P ratio (10):

$$\frac{[^{125}\text{I BAL} - ^{125}\text{I blood} \times ^{131}\text{I BAL} / ^{131}\text{I blood}]}{[^{125}\text{I blood}/(1-\text{Hct})]}$$

where $^{125}$I BAL = $^{125}$I cpm from a unit weight of BAL fluid; $^{125}$I blood = $^{125}$I cpm from a unit weight of blood; $^{131}$I BAL = $^{131}$I cpm from a unit weight of BAL fluid; $^{131}$I blood = $^{131}$I cpm from a unit weight of blood; and Hct = hematocrit.

2) In experiment 2, the flux of albumin from air space to intravascular space, the plasma leak (PL) index, was calculated as the ratio of $^{125}$I-albumin in circulating plasma to that instilled into the left lung, a modification of a previously described method (30, 40). The amount of $^{125}$I-albumin in circulating plasma was calculated by assuming the plasma volume as [volume (L) = body weight (kg) x 0.07(1 - hematocrit)] (30, 40).

**Extravascular lung water**

Extravascular lung water was measured as the blood-free wet/dry lung weight ratio (W/D). The superior lobe parenchyma and blood samples were weighed immediately after excision, dried for 72 h in a vacuum oven (DP22; Yamato,
Tokyo, Japan) at 90 °C and 200 mmHg, then weighed again. We estimated the weight of residual blood contained in the tissue samples by counting $^{131}$I in the tissue and blood samples obtained at sacrifice. Since we assumed that all of the $^{131}$I-albumin was confined to the circulation, we were able to correct for the wet weight of the blood-free tissue sample. In addition, using the wet/dry weight ratio of the blood sample, we were also able to correct for the dry weight of the residual blood in the lung tissue sample. We calculated the dry weight of blood-free lung tissue by subtracting the weight of the residual blood from that of lung tissue sample. This allowed us to calculate the blood-free wet/dry weight ratio of each superior lobe lung tissue sample.

**Neutrophil elastase activity and malondialdehyde concentration in the broncho-alveolar lavage fluid**

Neutrophil elastase activity was determined with the highly specific synthetic substrate N-methoxysuccinli-Ala-Ala-Pro-Val p-nitroanilline (p-NA), by the method of Yoshimura et al (42). MDA was measured with a BIOXYTECH LPO-586 kit (Oxis International Inc., Portland, OR) by the method of Gerard-Monnier (9). Briefly, N-methyl-2-phenylindole was added to the BAL fluid, followed by the addition of 12 N HCl, and incubated at 45 °C for 60 min. After centrifugation at 2,500g at 4 °C for 10 min, the absorbance of the clear supernatant was measured at 586nm. The standard curve was constructed with an MDA standard, and the MDA concentration was expressed in µmol/L.
Histopathologic examination

Histopathologic examinations were performed to assess pulmonary neutrophil sequestration. Twelve animals were divided among the 3 experimental groups described earlier, and treated similarly. The right and left lungs were fixed by inflation with formalin, gravimetrically instilled at 25 cmH₂O. The lung was fixed for ≥48 h, before the preparation of 2-5 mm sagittal sections embedded in paraffin, from which 5-μm sections were sliced and stained with hematoxyline and eosin. The number of neutrophils was counted under oil, at x1000 magnification, among 60 randomly selected fields per rabbit. The neutrophil count was divided by the number of alveoli in the corresponding field to compensate for variable lung inflation (33).

In vitro permeability study

Human pulmonary artery endothelial cells in their 4th passage were obtained from KURABO Industries Ltd. (Osaka, Japan), and were cultured in a humidified 5% CO₂ atmosphere with RPMI-1640 medium, supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated, endotoxin free, fetal bovine serum (Gibco/BRL Life Technologies, Grand Island, NY). Monolayers of endothelial cells were prepared on filters as previously described (41). In brief, 12-mm in diameter Millicell™-HA tissue culture plate well inserts were obtained from Millipore Corp. (Bedford, MA). The inserts consist of a surfactant-free 0.45-μm pore size microporous cellulose membrane filter sealed to a cylindrical polystyrene holder. They were
incubated for 3 h with 7 $\mu$g/cm$^2$ of human fibronectin (Sigma Chemical, St. Louis, MO) at 37 °C to facilitate the cell attachment. The fibronectin solution was aspirated and the human pulmonary artery endothelial cells suspended in the culture medium were seeded on the membrane filter at a density of 4 x 10$^5$ cells/insert (upper chamber). The inserts were placed into a 6-well culture plate (Falcon; Becton Dickinson, Lincoln Park, NJ), with each well filled with 2 ml of the culture medium, and incubated at 37 °C in a humidified 5% CO$_2$ atmosphere for 2 weeks before the measurements of permeability.

To assess the permeability, we measured the albumin transferred across the monolayers of human pulmonary artery endothelial cells cultured on a porous filter. These monolayers were incubated at 37 °C for 30 min in the culture medium containing the Y-27632 solution in concentrations of 1 and 10 $\mu$M, in a humidified 5% CO$_2$ atmosphere. A 0.50-mM H$_2$O$_2$ solution was then added to the upper chamber. The culture medium was aspirated 1 h later, and 500 $\mu$l of phosphate-buffered saline (PBS) containing 0.1% bovine albumin was added to the upper chamber. The insert was placed in 1 well of a 24-well culture plate (Falcon; Becton Dickinson), where each well was filled with 0.7 ml of PBS alone. After incubation for 20 min, the insert was removed from the well, and the albumin concentration of the lower chamber was measured with a Bio-Red Protein Assay kit (BIO-RED, Hercules, CA).

**Morphologic observations using rhodamine phalloidin stain**

The changes in the actin microfilament cytoskeleton of the monolayers of
human pulmonary artery endothelial cells grown on filters were examined with a rhodamine phalloidin stain (Molecular Probes, Inc., Eugene, OR) as described previously (25), and photographed with a fluorescence microscope equipped with epi-illumination (Nikon Optiphot; Nikon, Garden City, NY).

**Statistical analysis**

All date are expressed as means ± standard error (SE). One-way analysis of variance followed by Fisher’s least significant difference test was used for among-groups and between-lung comparisons. One-way analysis of variance with repeated measurements analysis was used to estimate the significance of plasma leak and mean arterial pressures. Differences with $P$ values <0.05 were considered statistically significant.

**Results**

**In vivo study**

The measurements of $^{125}$I and $^{131}$I activities in blood, superior lobe lung tissue and BAL fluid are shown in Table 1. The T/P ratios are shown in Figure 2. In the left or re-expanded lung, pre-treatment with Y-27632 inhibited the increase in T/P ratio after re-expansion. Endothelial permeability in the RE group was significantly higher than in the sham and RE+Y groups. In contrast, in the right lung, there was no significant difference in T/P ratio among the three groups.

Figure 3 shows the B/P ratio measured in each lung. In the left lung of the RE group, the albumin flux into the airspace was significantly higher than
in the left lung of the sham and the RE+Y groups. There was no difference among groups in B/P ratio in the right lung.

The PL from air space to intravascular space up to 120 min after instillation of $^{125}$I-albumin into the left lung is shown in Figure 4. PL was significantly higher at 10 min after instillation of $^{125}$I-albumin in the RE group than in the other 2 groups. Furthermore, in the RE group, the W/D ratio was significantly greater than in the RE+Y group, though was similar to the W/D in the sham group (Figure 5).

The mean total peripheral neutrophil counts at the end of experiments are shown in Table 2. There were no significant differences among the groups. On light microscopy, the number of neutrophils per alveolus in the left lung of both the RE and RE+Y groups was significantly greater than in the sham group ($P<0.01$, Figure 6). There was no difference, in the right lung, in neutrophil counts among the groups. The neutrophil count in the left lung BAL fluid in the RE and RE+Y groups was more than two-fold greater than in the sham group, though the difference did not reach statistical significance (Figure 7).

The BAL fluid neutrophil elastase activity in the left lung of the RE and RE+Y groups was significantly higher than in the left lung of the sham group, while there was no significant difference in neutrophil elastase activity among the 3 groups in the right lung (Table 3). In the left lung, the MDA concentration of BAL fluid was significantly higher in the RE than in the sham group, though was similar in the RE and the RE+Y groups (Table 3).
Mean arterial pressure did not differ significantly among the experimental groups (Table 4).

**Permeability in pulmonary endothelial cell monolayers**

Figure 8 shows the endothelial permeability index estimated from the albumin transfer across human pulmonary endothelial cell monolayers. H$_2$O$_2$ increased the permeability of the monolayer above control levels, and treatment with Y-27632 in concentrations of 1 and 10 µM significantly attenuated the increase induced by H$_2$O$_2$.

**Morphological changes**

We used 4 monolayers in each group and show representative findings in Figure 9. Close cell-to-cell contact was present in the control preparations of human pulmonary artery endothelial cells (Figure 9A). Treatment with hydrogen peroxide caused the development of randomly oriented stress fibers, cell contraction and intercellular gaps (Figure 9B), and treatment with Y-27632 mitigated these changes (Figure 9C).

**Discussion**

We examined the role of the Rho/ROCK-mediated pathway in the changes in vascular endothelial and alveolar epithelial permeability, in a new rabbit model of re-expansion pulmonary edema. Treatment with Y-27632, a ROCK-specific inhibitor, attenuated ALI after re-expansion of a lung collapsed for 36 h. We used the $^{125}$I-labeled albumin T/P ratio as an index of vascular endothelial
permeability, measured the bi-directional flux of albumin across the alveolar epithelium by a modification of the method described by Smedira et al (30, 40), and calculated the $^{125}\text{I}$-labeled albumin BAL B/P ratio and the PL index as indices of epithelial permeability. These indices were all increased in the re-expanded lung, increases which were suppressed by pre-treatment with Y-27632 (Figures 2, 3 & 4). However, Y-27632 neither prevented the accumulation of neutrophils nor inhibited the effects of neutrophil elastase activity and MDA production in the re-expanded lung (Figure 6, 7, Table 2).

The T/P ratio represents the amount of $^{125}\text{I}$-labeled albumin that traverses the pulmonary vascular endothelium per unit of time. The trans-endothelial flux of albumin is mainly determined by endothelial permeability, capillary surface area and capillary pressure (4, 34). While the administration of Y-27632 did not lower the systemic blood pressure in this study, it may have lowered the pulmonary artery and capillary pressures and increased the capillary surface area, since it relaxes smooth muscles by its ROCK inhibitory action (2). However, if the intra-peritoneal administration of Y-27632 had changed the T/P ratio by relaxing the smooth muscle in this model, the ratio in the right lung should have differed among the groups. Therefore, the suppression of the increased T/P by Y-27632 pre-treatment in the re-expanded lung was mainly due to the suppression of an increased pulmonary vascular permeability to albumin. These findings confirm that the Rho/ROCK pathway may contribute to the increase in vascular endothelial permeability which develops in re-expanded lungs.

In patients with re-expansion pulmonary edema, the concentrations of neutrophil chemoattractant, such as IL-8 and LTB$_4$, are increased in edema
fluid, and are accompanied by higher numbers of pulmonary neutrophils (19, 31). In addition, the administration of antioxidants before lung re-expansion mitigates the development of edema, supporting the hypothesis that reactive oxygen species produced by pulmonary neutrophils may play important roles in the development of re-expansion pulmonary edema (12). In our study, neutrophils accumulated together with a rise in BAL fluid neutrophil elastase activity and MDA concentration, which reflects lipid peroxidation caused by reactive oxygen species in the re-expanded lung (Figure 6, 7, Table 2). These observations suggest that fluid mediators released from activated neutrophils, such as elastase and/or reactive oxygen species, contribute to the development of lung re-expansion injury.

Several studies, including ours, suggest that re-expansion pulmonary edema is associated with an increase in pulmonary vascular endothelial permeability (20, 24, 39). However, few have examined the alveolar epithelial permeability in this disorder. Pulmonary edema often develops rapidly after re-expansion, and the protein concentration in edema fluid is increased (17, 19, 31), suggesting a concomitant increase in epithelial permeability. Injury to the alveolar epithelial barrier markedly influences the severity of ALI (38). By modifying the method of Smedira et al (30, 40), we were able to examine the epithelial permeability in re-expanded lungs by measuring the bi-directional flux of $^{125}$I-labeled albumin through the alveolar septal barrier. We found that both the B/P and PL index were increased, which is consistent with an increase in epithelial permeability in the re-expanded lung. The simultaneous increase in vascular endothelial and alveolar epithelial permeability is likely to be the source of severe and acute disease manifestations at the very onset of
re-expansion pulmonary edema. Since the PL index began to rise immediately after re-expansion (Figure 4), the increase in epithelial permeability may occur immediately after re-expansion. It has been reported that alveolar epithelium is more resistant to injury than the pulmonary vascular endothelium (26). The immediate increase in PL index after re-expansion may be an indication that the mechanical stretch of the alveolar epithelial barrier plays a role in the increased epithelial permeability of the re-expanded lung (Figure 4).

Y-27632 interacts with ROCK in human neutrophils to suppress the production of superoxide and the chemotactic-peptide-induced cell motile function in vitro (14, 21). In the rat liver, Y-27632 attenuated ischemia and reperfusion injury by limiting the infiltration by neutrophils and rise in tissue MDA concentration (32). However, in the present study, pre-treatment with Y-27632 did not prevent the accumulation of neutrophils and production of reactive oxygen species in the lung re-expanded after 36 h of collapse. In a preliminary study, we observed neutrophil influx into collapsed lungs before their re-expansion. We also have reported that the production of IL-8 by macrophages and alveolar epithelial cells, which plays an important role in the migration of neutrophils in re-expanded lungs, starts at the time of lung collapse (20). Treatment instituted immediately before re-expansion may be insufficient to inhibit recruitment and activation of neutrophils.

The increase in microvascular permeability is associated with the formation of endothelial paracellular gap. An actin-myosin contraction of the endothelial cells is important to the regulation of paracellular gap formation (8, 28). It has been reported that the small GTAase Rho plays an important role in
the regulation of actomyosin contractile elements (27, 35). Rho and its downstream effector, ROCK are involved in signal transduction in endothelial cells, linking extracellular stimuli to a dynamic rearrangement of cytoskeletal actin (35). The activation of Rho, via the action of ROCK, causes the phosphorylation of the myosin-binding subunit of myosin light chain phosphatase, inactivating the latter, and increasing the phosphorylation of myosin light chains, acto-myosin contraction and endothelial cell barrier dysfunction (1, 5, 16). In cultured cell model of thrombin-induced endothelial cell barrier dysfunction, the ROCK inhibitor, Y-27632 decreases endothelial hyperpermeability, and suppresses the reorganization of F-actin and the formation of intercellular gaps (37). Chiba et al. also reported a suppressive effect of Y-27632 on H₂O₂-induced pulmonary edema in isolated rabbit lungs (2). In cultured pulmonary endothelial cells, myosin light chain kinase contributes to H₂O₂-induced actin rearrangement (43). These observations suggest that the Rho/ROCK pathway is involved in the increased vascular endothelial permeability induced by reactive oxygen species. In this study, Y-27632 attenuated significantly both the increase in endothelial permeability and the reorganization of F-actin. These observations suggest that Y-27632 downregulates the Rho/ROCK signal transduction pathway in endothelial cells and suppresses the hyper-permeability induced by reactive oxygen species in the re-expanded lung by inhibiting the reorganization of F-actin, subsequently blocking the contraction of vascular endothelial cells. In addition, Rho protein
regulates the epithelial tight junction and peri-junctional actin organization (6, 22). Y-27632 may influence epithelial cells by protecting the cellular barrier, although this was not examined in the present study.

In summary, this is the first report of the involvement of the Rho/ROCK-mediated pathway in the increase in pulmonary vascular endothelial and alveolar epithelial permeability in re-expansion pulmonary edema. Y-27632 may be effective in treating re-expansion pulmonary edema and other types of ALI associated with an increase in alveolar barrier permeability.

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39. Wilkinson PD, Keegan J, Davies SW, Bailey J, and Rudd RM. Changes in pulmonary microvascular permeability accompanying re-expansion


**Figure legends**

**Figure 1.** Protocol for the experiment 1. The experimental groups consist of: sham-operated group (Sham), re-expansion group (RE) and Rho-kinase inhibitor pre-treatment group (RE+Y). Sham group: only thoracotomy was performed. RE group: left main bronchus was declamped after 36h of clamping and the collapsed lung was re-expanded for 2h. RE+Y group: 10 mg/kg of specific Rho-kinase inhibitor (Y-27632) was administered into the peritoneal cavity, 30 min before re-opening the left main bronchus. $^{125}$I-albumin was injected intravenously 10 min before re-opening the left main bronchus to assess transvascular permeability. $^{131}$I-albumin was injected intravenously 5 min before sacrifice to correct blood contamination.

**Figure 2.** T/P ratio, as an index of pulmonary endothelial permeability. The left lung is the test lung, and the right lung is the control. Values are mean ± SE; n=7 in each group. *$P <0.01$ vs. sham group. #*$P <0.01$ vs. Y-27632-treated (RE+Y) group.

**Figure 3.** B/P ratio, as an index of pulmonary epithelial permeability. The left lung is the test lung, and the right lung is the control. Values are mean ± SE; n=7 in each group. *$P <0.05$ vs. sham group. #*$P <0.05$ vs. Y-27632-treated (RE+Y) group.

**Figure 4.** Changes in PL index up to 2 h. Values are mean ± SE; n=4 in each group. *$P <0.01$ vs. sham group. #*$P <0.05$ vs. Y-27632-treated (RE+Y) group.
\( ^{+} P <0.01 \) vs. Y-27632-treated (RE+Y) groups.

**Figure 5.** Lung wet/dry ratio, an index of pulmonary edema, in the sham, re-expansion (RE) and Y-27632-treated (RE+Y) groups. The left lung is the test lung, and the right lung is the control. Values are mean + SEM; n=7, each group. * \( P <0.05 \) vs. the sham group. \( ^{#}P<0.05 \) vs. Y-27632-treated (RE+Y) group.

**Figure 6.** Tissue neutrophil counts per alveolus in lung. Values are mean ± SE; n=4 in each group. * \( P <0.01 \) vs. sham group.

**Figure 7.** Neutrophil counts (x 10\(^5\)/ml) in BAL fluid. Values are mean ± SE; n=7 in each group.

**Figure 8.** Effect of Y-27632 on the \( \text{H}_2\text{O}_2 \)–induced (0.50mM) increase in permeability in pulmonary endothelial cell monolayers. Values are means ± SE; n=5 in each group. * \( P <0.005 \) vs. \( \text{H}_2\text{O}_2 \) + Y-27632, 0 µM. \( ^{#} P <0.0001 \) vs. \( \text{H}_2\text{O}_2 \) + Y-27632, 0 µM.

**Figure 9.** Morphologic changes in pulmonary endothelial cells. Rhodamine phalloidin stain of monolayers. A) Control, B) monolayers 60 min after treatment with 0.50mM \( \text{H}_2\text{O}_2 \), and C) pre-treatment with Y-27632, 10 µM, for 30 min before \( \text{H}_2\text{O}_2 \) treatment. Scale bar = 20µm.
Table 1

\(^{125}\text{I and }^{131}\text{I activities in blood, lung and BAL fluid}\)

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=7)</th>
<th>RE (n=7)</th>
<th>RE+Y (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{125}\text{I (cpm/g)})</td>
<td>13696 ± 9094</td>
<td>4411 ± 395</td>
<td>10897 ± 5032</td>
</tr>
<tr>
<td>(^{131}\text{I (cpm/g)})</td>
<td>8225 ± 952</td>
<td>7351 ± 1021</td>
<td>10650 ± 1290</td>
</tr>
<tr>
<td><strong>R-Lung</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{125}\text{I (cpm/g)})</td>
<td>7367 ± 4746</td>
<td>3091 ± 291</td>
<td>6996 ± 2936</td>
</tr>
<tr>
<td>(^{131}\text{I (cpm/g)})</td>
<td>2200 ± 211</td>
<td>2264 ± 418</td>
<td>3095 ± 526</td>
</tr>
<tr>
<td><strong>L-Lung</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{125}\text{I (cpm/g)})</td>
<td>9208 ± 6330</td>
<td>3851 ± 261</td>
<td>7553 ± 3224</td>
</tr>
<tr>
<td>(^{131}\text{I (cpm/g)})</td>
<td>1959 ± 232</td>
<td>1945 ± 436</td>
<td>3488 ± 378</td>
</tr>
<tr>
<td><strong>R-BAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{125}\text{I (cpm/ml)})</td>
<td>227 ± 90</td>
<td>110 ± 24</td>
<td>300 ± 176</td>
</tr>
<tr>
<td>(^{131}\text{I (cpm/ml)})</td>
<td>84 ± 43</td>
<td>22 ± 7</td>
<td>210 ± 202</td>
</tr>
<tr>
<td><strong>L-BAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{125}\text{I (cpm/ml)})</td>
<td>523 ± 241</td>
<td>618 ± 135</td>
<td>605 ± 251</td>
</tr>
<tr>
<td>(^{131}\text{I (cpm/ml)})</td>
<td>93 ± 43</td>
<td>279 ± 164</td>
<td>161 ± 55</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. R=Right, L=Left, Lung = superior lobe lung tissue, BAL=broncho-alveolar labage fluid.
Table 2
Total and differential white blood cell counts at time of animal sacrifice

<table>
<thead>
<tr>
<th></th>
<th>Total (10^2/mm^3)</th>
<th>Neutrophil (10^2/mm^3)</th>
<th>Lymphocyte (10^2/mm^3)</th>
<th>Monocyte (10^2/mm^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham  (n=7)</td>
<td>31.7 ± 10.5</td>
<td>17.2 ± 6.0</td>
<td>13.2 ± 4.1</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>RE (n=7)</td>
<td>52.0 ± 10.0</td>
<td>22.3 ± 7.1</td>
<td>26.5 ± 5.8</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>RE+Y (n=7)</td>
<td>41.4 ± 14.8</td>
<td>24.3 ± 5.4</td>
<td>16.5 ± 6.7</td>
<td>2.2 ± 1.3</td>
</tr>
</tbody>
</table>

Values (10^2/mm^3) are mean ± SEM.
Table 3
Malondialdehyde concentration and neutrophil elastase activity in BAL fluid

<table>
<thead>
<tr>
<th></th>
<th>NE activity (nmol pNA/ml)</th>
<th>MDA (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.28 ± 0.03</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>L</td>
<td>0.40 ± 0.27</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>RE</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.11 ± 0.07</td>
<td>0.56 ± 0.11*</td>
</tr>
<tr>
<td>L</td>
<td>1.95 ± 0.49*</td>
<td>2.40 ± 0.88*</td>
</tr>
<tr>
<td>RE+Y</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.02 ± 0.02</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>L</td>
<td>2.63 ± 0.41*</td>
<td>1.49 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. R=Right lung, L=Left lung, NE=Neutrophil elastase, MDA= malondialdehyde, *P<0.05 vs. control group.
**Table 4**

Mean arterial pressures (Torr)

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n=3)</td>
<td>65 ± 2</td>
<td>67 ± 3</td>
<td>68 ± 2</td>
<td>68 ± 1</td>
<td>73 ± 6</td>
</tr>
<tr>
<td>RE (n=3)</td>
<td>68 ± 3</td>
<td>67 ± 1</td>
<td>73 ± 2</td>
<td>73 ± 5</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>RE+Y (n=3)</td>
<td>67 ± 1</td>
<td>70 ± 1</td>
<td>72 ± 2</td>
<td>67 ± 2</td>
<td>73 ± 4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
Sham operation

Collapse left lung

Y-27632 i.p.

Saline i.p. 125I-albumin i.v.

131I-albumin i.v.

Re-expansion of the left lung

Sacrifice

Figure 1.
Figure 2

125I-albumin tissue-plasma ratio (T/P)
125I-albumin BALF-plasma ratio (B/P)

- **Sham**
- **RE**
- **RE+Y**

**Figure 3**
Figure 4
Figure 6

Sham
RE
RE+Y

Neutrophils / Alveolus

Right

Left

* *
Neutrophils in BAL fluid (×10^5/ml)

- **Sham**
- **RE**
- **RE+Y**

**Figure 7**
Figure 8

Albumin concentration (µg/ml)

H$_2$O$_2$ (0.50mM)

Y-27632 (µM)

- 0 1 10

+ + +

* * *

# # #
Figure 9 (B)