Role of Rho-kinase in reexpansion pulmonary edema in rabbits

Makoto Sawafuji,1 Akitoshi Ishizaka,2 Mitsutomo Kohno,1 Hidefumi Koh,2 Sadatomo Tasaka,2 Yoshiki Ishii,3 and Koichi Kobayashi1

Departments of 1Surgery and 2Medicine, Keio University School of Medicine, Tokyo; and 3Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi, Japan

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Sawafuji, Makoto, Akitoshi Ishizaka, Mitsutomo Kohno, Hidefumi Koh, Sadatomo Tasaka, Yoshiki Ishii, and Koichi Kobayashi. Role of Rho-kinase in reexpansion pulmonary edema in rabbits, Am J Physiol Lung Cell Mol Physiol 289:L946–L953, 2005. First published July 8, 2005; doi:10.1152/ajplung.00188.2004.—Reexpansion of a collapsed lung increases the microvascular permeability and causes reexpansion 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, although their roles in reexpansion 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-to-plasma (T/P) and bronchoalveolar lavage (BAL) fluid-to-plasma (B/P) ratios with 125I-labeled albumin. After intratracheal instillation of 125I-albumin, epithelial permeability was also assessed from the plasma leak (PL) index, the ratio of 125I-albumin in plasma/total amount of instilled 125I-albumin. T/P, B/P, and PL index were significantly increased in the reexpanded lung. These increases were attenuated by pretreatment with Y-27632, a specific ROCK inhibitor. However, neutrophil influx, neutrophil elastase activity, and malondialdehyde concentrations in BAL fluid collected from the reexpanded lung were not changed by Y-27632, a specific ROCK inhibitor. However, neutrophil influx, neutrophil elastase activity, and malondialdehyde concentrations in BAL fluid collected from the reexpanded lung were not changed by Y-27632. In endothelial monolayers, Y-27632 significantly attenuated 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 pro-

Address for reprint requests and other correspondence: M. Sawafuji, Dept. of Surgery, Keio Univ., 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan (e-mail: msawafuji@nifty.com).

Methods

The experiments were performed in 45 male Japanese White rabbits, weighing between 900 and 1,800 g. All procedures were performed in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
reviewed and approved by the Laboratory Animal Care Panel of Keio University. The animals were divided into the following three groups: 1) a reexpansion group (RE), 2) a Rho-kinase inhibitor pretreatment group (RE + Y), which received 10 mg/kg Y-27632 intraperitoneally before reexpansion of the left lung, and 3) a sham-operated group (sham) that 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; Shinano, 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, St. Paul, MN) to produce complete collapse of the left lung by absorption of the alveolar gas in the pulmonary circulation. A 4-0 nylon string attached to the clip was exteriorized 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 experiment 1 is provided in Fig. 1. Endothelial permeability was assessed from the transvascular flux of $^{125}$I-labeled albumin administered intravenously. Epithelial permeability was measured as the flux of $^{125}$I-albumin from intravascular space to airspace. To correct blood contamination in the lung tissue or bronchoalveolar 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 and placed in the supine position, and a 24-gauge catheter was inserted in an ear vein. $^{125}$I-labeled BSA (2 ml of 37 kBq/ml; Life Science Products, Boston, MA) was injected intravenously 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 declamped after 36 h of clamping. On the day of experiments, 5 ml blood were collected from 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 was 7.9 ± 0.3, 8.1 ± 0.4, and 8.0 ± 0.5 ml, respectively. There was no significant difference in the recovery of BAL fluid among the groups. The fluid was centrifuged at 400 g 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.

Fig. 1. Protocol for experiment 1. The experimental groups consist of the following: sham-operated group (Sham), reexpansion group (RE), and Rho-kinase inhibitor pretreatment group (RE + Y). Sham group, only thoracotomy was performed. RE group, left main bronchus was declamped after 36 h of clamping, and the collapsed lung was reexpanded for 2 h. RE + Y group, 10 mg/kg specific Rho-kinase inhibitor (Y-27632) was administered in the peritoneal cavity 30 min before reopening the left main bronchus. $^{125}$I-albumin was injected iv 10 min before reopening the left main bronchus to assess transvascular permeability. $^{131}$I-albumin was injected iv 5 min before death to correct blood contamination.
We confirmed that most of the dye was distributed in the left lung after death of the animal. Blood samples were obtained for measurements of $^{125}$I-albumin activity at baseline and 10, 30, 60, 90, and 120 min after instillation. Because, clinically, the onset of reexpansion 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 airspace to intravascular space.

**Pulmonary Endothelial Permeability**

Pulmonary endothelial permeability was ascertained by two separate methods used to measure the bidirectional flux of albumin across the alveolar epithelial barrier. 1) In experiment 1, the flux of albumin from intravascular space to lung airspace was assessed by measuring the changes in flux of albumin from lung airspace to intravascular space. The extravascular $^{125}$I-albumin in the superior lobe was calculated by subtracting the amount of intravascular $^{125}$I-albumin from the total $^{125}$I-albumin in the lung tissue sample. The intravascular $^{125}$I-albumin in the superior lobe was corrected by calculating the ratio of $^{125}$I to $^{131}$I counts in the unit weight of blood and $^{131}$I-albumin in the unit weight of superior lobe parenchyma. We assumed that all of the $^{131}$I-albumin was confined to the circulation, since the effect of leakage of the plasma volume marker in the extravascular space is negligible when calculating $T/P$ (13). Therefore, the $^{131}$I counts must reflect the residual blood in the lung tissue sample. The following formula was used to calculate the $T/P$ ratio (10)

\[
T/P = \frac{^{131}I_{\text{tissue}} - ^{125}I_{\text{blood}}}{^{131}I_{\text{blood}}/(1 - \text{Hct})}
\]

where $^{125}$I tissue is $^{125}$I cpm from a unit weight of superior lobe lung tissue, $^{125}$I blood is $^{125}$I cpm from a unit weight of superior lobe lung tissue, $^{131}$I blood is $^{131}$I cpm from a unit weight of superior lobe lung tissue, $^{131}$I blood is $^{131}$I cpm from a unit weight of blood, and Hct is hematocrit.

**Pulmonary Epithelial Permeability**

Pulmonary epithelial permeability was ascertained by two separate methods used to measure the bidirectional flux of albumin across the alveolar epithelial barrier. 1) In experiment 1, the flux of albumin from intravascular space to lung airspace 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 airspace 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)

\[
B/P = \frac{^{125}I_{\text{BAL}} - ^{131}I_{\text{BAL}}}{^{125}I_{\text{BAL}}/(1 - \text{Hct})}
\]

where $^{125}$I BAL is $^{125}$I cpm from a unit weight of BAL fluid and $^{131}$I BAL is $^{131}$I cpm from a unit weight of BAL fluid.

2) In experiment 2, the flux of albumin from airspace to intravascular space, the plasma leak (PL) index, was calculated as the ratio of $^{125}$I-albumin in circulating plasma to that instilled in the left lung, a modification of a previously described method (30, 39). The amount of $^{125}$I-albumin in circulating plasma was calculated by assuming the plasma volume as volume (liter) = body weight (kg) $\times$ 0.071 - hematocrit (30, 39).

**Extravascular Lung Water**

Extravascular lung water was measured as the blood-free wet-to-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, and 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 death. Because 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 $W/D$ 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 $W/D$ ratio of each superior lobe lung tissue sample.

**Neutrophil Elastase Activity and MDA Concentration in the BAL Fluid**

Neutrophil elastase activity was determined with the highly specific synthetic substrate N-methoxy succinil-Ala-Ala-Pro-Val-p-nitroaniline by the method of Yoshimura et al. (42). MDA was measured with a BIOXYTECH LPO-586 kit (Oxis International, Portland, OR) by the method of Gerard-Monnier et al. (9). Briefly, N-methyl-p-pheny lindole 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 2,500 g at 4°C for 10 min, the absorbance of the clear supernatant was measured at 586 nm. The standard curve was constructed with an MDA standard, and the MDA concentration was expressed in micromoles per liter.

**Histopathological Examination**

Histopathological examinations were performed to assess pulmonary neutrophil sequestration. Twelve animals were divided among the three experimental groups described earlier, and treated similarly. The right and left lungs were fixed by inflation with formalin, gravimetrically instilled at 25 cmH$_2$O. The lung was fixed for 24 h before the preparation of 2- to 5-mm sagittal sections embedded in paraffin, from which 5-$\mu$m sections were sliced and stained with hematoxylin and eosin. The number of neutrophils was counted under 1,000 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 (34).

**In Vitro Permeability Study**

Human pulmonary artery endothelial cells in their fourth passage were obtained from KURABO Industries (Osaka, Japan) and were

### Table 1. $^{125}$I and $^{131}$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>$^{125}$I, cpm/g</td>
<td>13,696 ± 9,094</td>
<td>4,411 ± 395</td>
<td>10,897 ± 5,032</td>
</tr>
<tr>
<td>$^{131}$I, cpm/g</td>
<td>8,225 ± 952</td>
<td>7,351 ± 1,021</td>
<td>10,650 ± 1,290</td>
</tr>
<tr>
<td>R-lung, $^{125}$I, cpm/g</td>
<td>7,367 ± 4,746</td>
<td>3,091 ± 291</td>
<td>6,996 ± 2,936</td>
</tr>
<tr>
<td>$^{131}$I, cpm/g</td>
<td>2,200 ± 211</td>
<td>2,264 ± 418</td>
<td>3,095 ± 526</td>
</tr>
<tr>
<td>L-lung, $^{125}$I, cpm/g</td>
<td>9,208 ± 6,330</td>
<td>3,851 ± 261</td>
<td>7,553 ± 3,224</td>
</tr>
<tr>
<td>$^{131}$I, cpm/g</td>
<td>1,959 ± 232</td>
<td>1,945 ± 436</td>
<td>3,488 ± 378</td>
</tr>
<tr>
<td>R-BAL, $^{125}$I, cpm/ml</td>
<td>227 ± 90</td>
<td>110 ± 24</td>
<td>300 ± 176</td>
</tr>
<tr>
<td>$^{131}$I, cpm/ml</td>
<td>84 ± 43</td>
<td>22 ± 7</td>
<td>210 ± 202</td>
</tr>
<tr>
<td>L-BAL, $^{125}$I, cpm/ml</td>
<td>523 ± 241</td>
<td>618 ± 135</td>
<td>605 ± 251</td>
</tr>
<tr>
<td>$^{131}$I, cpm/ml</td>
<td>93 ± 43</td>
<td>279 ± 164</td>
<td>161 ± 55</td>
</tr>
</tbody>
</table>

Values are means ± SE. R, right; L, left; lung, superior lobe lung tissue; BAL, bronchoalveolar lavage; RE, reexpansion; RE + Y, Y-27632 treatment and reexpansion.
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 FBS (GIBCO-BRL Life Technologies, Grand Island, NY). Monolayers of endothelial cells were prepared on filters as previously described (41). In brief, 12-mm diameter Millicell-HA tissue culture plate well inserts were obtained from Millipore (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 μg/cm² 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 × 10⁵ cells/insert (upper chamber). The inserts were placed in a six-well culture plate (Falcon; Becton-Dickinson, Lincoln Park, NJ), with each well filled with 2 ml culture medium, and incubated at 37°C in a humidified 5% CO₂ atmosphere for 2 wk 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 μM, in a humidified 5% CO₂ atmosphere. A 0.50-mM H₂O₂ solution was then added to the upper chamber. The culture medium was aspirated 1 h later, and 500 μl PBS containing 0.1% BSA were 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 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-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

Morphological 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, 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 data are expressed as means ± SE. One-way ANOVA followed by Fisher’s least significant difference test was used for among-groups and between-lung comparisons. One-way ANOVA with repeated-measurements analysis was used to estimate the significance of PL and mean arterial pressures. Differences with P values <0.05 were considered statistically significant.
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 Fig. 2. In the left or reexpanded lung, pretreatment with Y-27632 inhibited the increase in T/P ratio after reexpansion. 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 the 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 in 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 the B/P ratio in the right lung.

The PL from airspace to intravascular space up to 120 min after instillation of $^{125}$I-albumin in the left lung is shown in Fig. 4. PL was significantly higher at 10 min after instillation of $^{125}$I-albumin in the RE group than in the other two groups. Furthermore, in the RE group, the W/D ratio was significantly greater than in the RE + Y group (Fig. 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 (Fig. 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 twofold greater than in the sham group, although the difference did not reach statistical significance (Fig. 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, whereas there was no significant difference in neutrophil elastase activity among the three 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, although it 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 four monolayers in each group and show representative findings in Fig. 9. Close cell-to-cell contact was present in the control preparations of human pulmonary artery endothelial cells (Fig. 9A). Treatment with H$_2$O$_2$ caused the development of randomly oriented stress fibers, cell contraction, and intercellular gaps (Fig. 9B), and treatment with Y-27632 mitigated these changes (Fig. 9C).

Table 2. Total and differential white blood cell counts at time of animal death

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Neutrophil</th>
<th>Lymphocyte</th>
<th>Monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</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</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</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 (×10⁵/mm³) are means ± SE; n = 7 rabbits in each group.

Table 3. Malondialdehyde concentration and neutrophil elastase activity in BAL fluid

<table>
<thead>
<tr>
<th></th>
<th>NE Activity, nmol p-NA/ml</th>
<th>MDA, µmol/l</th>
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<tbody>
<tr>
<td>Sham</td>
<td></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></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></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>
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</table>

Values are means ± SE. NE, neutrophil elastase; MDA, malondialdehyde; p-NA, p-nitroaniline. *P < 0.05 vs. sham group; n = 4 in each group.
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 reexpansion pulmonary edema. Treatment with Y-27632, a ROCK-specific inhibitor, attenuated ALI after reexpansion of a lung collapsed for 36 h. We used the $^{125}$I-albumin T/P ratio as an index of vascular endothelial permeability, measured the bidirectional flux of albumin across the alveolar epithelium by a modification of the method described by Smedira et al. (30) and Wiener-Kronish et al. (39), and calculated the $^{125}$I-albumin B/P ratio and the PL index as indexes of epithelial permeability. These indexes were all increased in the reexpanded lung, increases that were suppressed by pretreatment with Y-27632 (Figs. 2–4). However, Y-27632 neither prevented the accumulation of neutrophils nor inhibited the effects of neutrophil elastase activity and MDA production in the reexpanded lung (Figs. 6 and 7 and Table 2).

The T/P ratio represents the amount of $^{125}$I-albumin that traverses the pulmonary vascular endothelium per unit of time. The transendothelial flux of albumin is mainly determined by endothelial permeability, capillary surface area, and capillary pressure (4, 35). Although 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 intraperitoneal 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 pretreatment in the reexpanded lung was mainly the result of suppression of an increased pulmonary vascular permeability to albumin. These findings confirm that the Rho/ROCK pathway may

<table>
<thead>
<tr>
<th>Time After Continuous Suction, min</th>
<th>Baseline</th>
<th>30</th>
<th>60</th>
<th>90</th>
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<td>RE</td>
<td>68±3</td>
<td>67±1</td>
<td>73±2</td>
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<td>RE + Y</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 means ± SEM; n = 3 rabbits in each group. Units are Torr.

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

Fig. 9. Morphological changes in pulmonary endothelial cells. Rhodamine-phalloidin stain of monolayers. A: control; B: monolayers 60 min after treatment with 0.50 mM H$_2$O$_2$; C: pretreatment with Y-27632 (10 μM) for 30 min before H$_2$O$_2$ treatment. Scale bar = 20 μm.

Table 4. Mean arterial pressures

<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</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</td>
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<td>67±1</td>
<td>73±2</td>
<td>73±5</td>
<td>71±2</td>
</tr>
<tr>
<td>RE + Y</td>
<td>67±1</td>
<td>70±1</td>
<td>72±2</td>
<td>67±2</td>
<td>73±4</td>
</tr>
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</table>

Values are means ± SEM; n = 3 rabbits in each group. Units are Torr.

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contribute to the increase in vascular endothelial permeability that develops in reexpanded lungs.

In patients with reexpansion pulmonary edema, the concentrations of neutrophil chemoattractant, such as IL-8 and leukotriene B4, are increased in edema fluid and are accompanied by higher numbers of pulmonary neutrophils (19, 31). In addition, the administration of antioxidants before lung reexpansion mitigates the development of edema, supporting the hypothesis that reactive oxygen species produced by pulmonary neutrophils play important roles in the development of reexpansion 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 reexpanded lung (Figs. 6 and 7 and 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 reexpansion injury.

Several studies, including ours, suggest that reexpansion pulmonary edema is associated with an increase in pulmonary vascular endothelial permeability (20, 24, 40). However, few have examined the alveolar epithelial permeability in this disorder. Pulmonary edema often develops rapidly after reexpansion, 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) and Wiener-Kronish et al. (39), we were able to examine the epithelial permeability in reexpanded lungs by measuring the bidirectional flux of 125I-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 reexpanded 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 reexpansion pulmonary edema. Because the PL index began to rise immediately after reexpansion (Fig. 4), the increase in epithelial permeability may occur immediately after reexpansion. 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 reexpansion may be an indication that the mechanical stretch of the alveolar epithelial barrier plays a role in the increased epithelial permeability of the reexpanded lung (Fig. 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, pretreatment with Y-27632 did not prevent the accumulation of neutrophils and production of reactive oxygen species in the lung reexpanded after 36 h of collapse. In a preliminary study, we observed neutrophil influx in collapsed lungs before their reexpansion. 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 reexpanded lungs, starts at the time of lung collapse (20). Treatment instituted immediately before reexpansion may be insufficient to inhibit recruitment and activation of neutrophils.

The increase in microvascular permeability is associated with the formation of an 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 GTPase Rho plays an important role in the regulation of actomyosin contractile elements (27, 33). Rho and its downstream effector, ROCK, are involved in signal transduction in endothelial cells, linking extracellular stimuli to a dynamic rearrangement of cytoskeletal actin (33). 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, actomyosin contraction, and endothelial cell barrier dysfunction (1, 5, 16). In a 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. (2) also reported a suppressive effect of Y-27632 on H2O2-induced pulmonary edema in isolated rabbit lungs. In cultured pulmonary endothelial cells, MLCK 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 hyperpermeability induced by reactive oxygen species in the reexpanded 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 perijunctional 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 reexpansion pulmonary edema. Y-27632 may be effective in treating reexpansion pulmonary edema and other types of ALI associated with an increase in alveolar barrier permeability.

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