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Activation of Rho Kinase Isoforms in Lung Endothelial Cells during Inflammation¹

Phyllus Y. Mong and Qin Wang²

Rho kinase (ROCK)³ is among the best-characterized downstream effector of Rho family small GTPases. Two isoforms of ROCK, ROCK1 and ROCK2, encoded by two different genes have been identified. Human ROCK1 and ROCK2 share 64% overall sequence identity with 89% identity in the catalytic domain on the N terminus. The C terminus of ROCK negatively regulates ROCK activity, possibly by binding to the catalytic domain (1). ROCK contains a Rho binding domain, and binding of GTP-bound active RhoA induces conformational changes in ROCK and stimulates ROCK activity. However, ROCK activity can be stimulated independently of RhoA. Binding of lipid messengers such as arachidonic acid or sphingosine phosphorylcholine, or proteolytic cleavage of the C terminus results in ROCK activation (1, 2). Moreover, ROCK activation can be modulated through phosphorylation at various serine/threonine/tyrosine residues, further demonstrating the complex nature in regulating ROCK activity (3, 4). Despite the high sequence homology between ROCK1 and ROCK2, ROCK1 or ROCK2 activation can occur through isoform-specific mechanisms. For example, proteolytic cleavage by caspase 3 leads to ROCK1 activation during apoptosis, whereas proteolytic cleavage by granzyme B leads to ROCK2 activation (5, 6). This specificity is due to the lack of consensus sequence for caspase 3 cleavage in ROCK2 or for granzyme B cleavage in ROCK1. Activation of ROCK in turn phosphorylates various downstream targets to regulate cell motility and gene transcription. One of the best-characterized substrates of ROCK is myosin phosphatase target (MYPT-1) subunit, and phosphorylation of MYPT-1 inhibits myosin phosphatase, leading to subsequent myosin L chain (MLC) phosphorylation and stress fiber formation (2). The high sequence homology in the kinase domain has led to the assumptions that ROCK1 and ROCK2 share similar substrates.

Mice singly deficient in ROCK1 and ROCK2 show developmental defects. Both ROCK1- and ROCK2-deficient mice show failure of eye closure and closure of the ventral body wall (7–9). Most ROCK1 mice die soon after birth (9). In addition, ROCK2-deficient mice show placental dysfunction caused by thrombus formation in the labyrinth layer of the placenta, leading to intrauterine growth retardation and fetal death (7). However, those animals that survive develop normally and are fertile. The developmental defects associated with ROCK1 and ROCK2 deficiency in mice have limited the use of these animals to evaluate the functions of these two molecules in physiology and diseases in an isoform-specific manner.

Abnormal activation of ROCK has been demonstrated in a variety of cardiovascular disorders such as atherosclerosis and pulmonary hypertension, and inhibition of ROCK activity using pharmacologic inhibitors has shown protective effects in animal models (1, 10, 11). Fasudil, the only ROCK inhibitor used clinically, was approved in Japan in 1995 for the treatment of vasospasm after subarachnoid hemorrhage. Fasudil has since been tested in various clinical trials and shown great safety profile (12). Despite these studies suggesting ROCK as a target for therapeutic interventions, a better understanding of the role of ROCK isoforms in physiology...
and disease processes is required to design more selective ROCK inhibitors. Using pharmacologic inhibitors that target ROCK, previous studies have demonstrated an important role for ROCK in regulating endothelial cell (EC) actin cytoskeleton organization and permeability increases during an inflammatory response in vivo and in vitro, especially during the early and acute phase of the inflammatory responses (13, 14). All these inhibitors similarly inhibit ROCK1 and ROCK2. This study examined isoform-specific activation of ROCK in cultured lung ECs induced by TNF-α in vitro and in freshly isolated lung ECs from mice during LPS pneumonia ex vivo. Using small interfering RNA (siRNA) that specifically targets ROCK1, or ROCK2 siRNA were stimulated with 20 ng/ml TNF-α and ROCK activity, 20 μM Y27632, a ROCK inhibitor, using immunoprecipitated ROCK1 or ROCK2 and purified recombinant MYPT-1 (714-1004, 80 kDa) as a substrate. The amount of phosphorylated MYPT-1, or immunoprecipitated ROCK1 or ROCK2 was examined. C. Isoform-specific ROCK activation in ECs following TNF-α stimulation. ECs were treated with vehicle or 20 ng/ml TNF-α for the indicated times, and each sample was divided into half for ROCK1 or ROCK2 activity assay. The amount of phosphorylated MYPT-1 from each kinase activity assay or immunoprecipitated ROCK1 or ROCK2 was examined. The total ROCK1 and ROCK2 activity in untreated ECs was defined as one, and the relative amount of ROCK1 and ROCK2 activity was presented (n = 3 samples for each time point).

**Materials and Methods**

**Cells, pharmacologic inhibitors, and siRNA**

Human pulmonary microvascular ECs (Cambrex) were grown on dishes precoated with 4 μg/ml fibronectin. Confluent ECs were treated with either vehicle (PBS containing 0.1% BSA) or 20 ng/ml recombinant human TNF-α for 5 min to 24 h by adding vehicle or a stock solution directly to EC cultures (15).

The siRNA targeting ROCK1, ROCK2, or a control siRNA was obtained as a pool of four siRNA duplexes from Dharmacon. ECs were treated for 4 h with 10 nM ROCK1 or ROCK2 siRNA or their corresponding control siRNA premixed with 6 μg/ml lipofectin (15). The cells were then incubated with normal culture medium for 72 h before experiments.

**Evaluation of the activity of ROCK1 or ROCK2**

Activation of ROCK1 or ROCK2 was evaluated by immunoprecipitating ROCK1 or ROCK2 followed by an in vitro kinase activity assay. Cells were lysed using a Triton X-100 lysis buffer containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 0.5 M NaCl and 1% Triton X-100. The cleared cell lysates were incubated for 1 h at 4°C with 2 μg of isotype control IgG, goat anti-ROCK1, or goat anti-ROCK2 Ab (Santa Cruz Biotechnology). The mixture was then incubated with protein A/G agarose beads at 4°C overnight. The beads were pelleted, washed, and incubated for 30 min at 30°C with kinase reaction buffer containing 200 μM ATP and 0.5 μg of purified MYPT-1 (714-1004), a ROCK-specific substrate (Upstate Biotechnology). The reaction was terminated by boiling the samples in SDS samples. The amount of phosphorylated MYPT-1 or immunoprecipitated ROCK1 or ROCK2 was examined using immunoblot. To confirm that MYPT-1 phosphorylation was due to ROCK activity, 20 μM Y27632, a specific ROCK inhibitor, or control vehicle was included in the kinase reaction mix in some experiments.

**Measurement of EC permeability to fluxes of dextran**

The fluxes of dextran across ECs were evaluated using Transwell chambers (15). ECs were seeded onto Transwell inserts (6.5 mm diameter and 0.4 μm pore size) coated with 4 μg/ml fibronectin. The cells treated with either control siRNA, ROCK1 or ROCK2 siRNA were stimulated with 20 ng/ml TNF-α or buffer. At the same time, 250 μg of FITC-conjugated dextran (10 kDa) was added to the top well, and 100-μl aliquots of samples were taken from the bottom chamber every hour for 6 h. A 24-h time point was also measured. The amount of dextran in the samples was measured using a fluorescence plate reader and calculated after constructing a standard curve. Each time after taking a sample, 100 μl of medium was added back to the bottom chamber. For each time point, the amount of dextran present in the bottom wells as well as the amount in all the samples removed for measurement was calculated, added up and plotted. The amount of dextran in the bottom wells in each sample was expressed relative to the amount of dextran in the control siRNA-treated samples without TNF-α stimulation at 24 h.

**Isolation of ECs from mouse lungs and evaluation of ROCK activity in freshly isolated lung ECs**

Lung ECs were isolated from total lung digest and ROCK activity in freshly isolated lung ECs was evaluated. All the animal experiments were...
performed using wild-type C57BL/6 mice (Charles River Breeding Laboratories) and approved by the Institutional Animal Care and Use Committee. The animals were sacrificed using inhaled isofluorane followed by cervical dislocation. The lungs were perfused through right ventricle using 10 ml of sterile PBS and isolated. Disperse II (1.2 U/ml PBS; Roche Diagnostics) was instilled into the lungs, and trachea was ligated. The lungs were incubated for 30 min at 37°C before the lung tissues were separated from large bronchi, minced and incubated with 1 mg/ml collagenase/dispase for 5 min at 37°C (Roche Diagnostics). The digested lung tissues were separated into single cell suspensions using 18-gauge and 20-gauge needles. After RBC lysis, lung ECs were isolated immediately for ROCK activity measurement.

Lung ECs were isolated from single lung cell suspension using an anti-CD105 Ab. CD105 is a surface protein and an EC marker (16, 17). The single cell suspension was incubated with 2 μg/ml biotin-rat anti-mouse CD105 Ab (eBioscience) for 10 min on ice, washed, and incubated with 20 μl anti-biotin coated magnetic beads (Miltenyi Biotec) for 15 min on ice. The CD105+ cells bound to the beads were separated from the CD105− cells using magnetic separation with MS columns (Miltenyi Biotec). Both CD105+ and the CD105− cells were collected. To evaluate total ROCK activity, the sorted cells were lysed immediately using SDS lysis buffer containing 1% SDS, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml pepstatin in 10 mM Tris (pH 8.0).

The protein concentration of each sample was determined, and ROCK activity was examined by measuring the phosphorylation state of a major ROCK substrate, MYPT-1, at Thr850 by immunoblotting (Upstate Biotechnology). To evaluate the activity of ROCK isoforms, ROCK1 or ROCK2 was immunoprecipitated from CD105+ cells followed by in vitro kinase assay as described for the human ECs.

Flow cytometry analysis was performed to characterize CD105 expression on lung cells and to evaluate the purity and viability of sorted cells. CD105 expression, acetylated low-density lipoprotein (Ac-LDL) uptake, and CD45 expression were examined in whole lung cell population, sorted cells, and flow through cells. ECs as well as lung macrophages are positive for Ac-LDL uptake, but only macrophages are positive for leukocyte marker CD45. CD105 expression was examined by incubation with 1 μg of PE-conjugated CD105 Ab; Ac-LDL uptake was examined by incubation with 5 μg of Alexa Fluor 488-conjugated Ac-LDL (Molecular Probes); and CD45 expression was examined by incubation with 1 μg of PE-conjugated anti-CD45 Ab. Isotype IgG was used as controls. All cells excluding cell debris were gated for analysis. The proportion of CD105+ cells that take up Ac-LDL and are CD45− was determined by flow cytometry using a FACScalibur (BD Biosciences). Cell viability was determined by Annexin V-FITC and propidium iodide staining using an Annexin V-FITC apoptosis detection kit (R&D Systems) and flow cytometry analysis.

To validate the assays measuring ROCK activity, a pharmacologic inhibitor was used in vivo to inhibit ROCK activity. Fasudil was approved in 1995 in Japan for the treatment of cerebral vasospasm, and the pharmacokinetics of fasudil in rodents are well understood (18–20). When given orally or i.p. in rodents, fasudil is metabolized to hydroxyfasudil (18). The inhibitory effect of hydroxyfasudil shows more than 50% inhibition only for ROCK among the 16 kinases examined (21). The effect of fasudil on ROCK inhibition in rodents is dose-dependent (21–24). Based on these studies, the effect of fasudil on ROCK activation in lung cells during LPS pneumonia in vivo was examined in a dose-dependent manner. Three sets of experiments were performed in mice varying the dose of fasudil: a low dose of fasudil (1 mg/ml in drinking water for 6 days), an intermediate dose (1 mg/ml in drinking water for 6 days), and a high dose (1 mg/ml in drinking water for 6 days, then 2 mg/mouse daily i.p. injection starting 2 days before LPS injection), and a high dose (1 mg/ml in drinking water for 6 days, then 2 mg/mouse daily i.p. injection starting 2 days before LPS injection).

Induction of LPS pneumonia in mice and evaluation of LPS-induced inflammatory responses

Mice were anesthetized by isofluorane inhalation. The animals received an intratracheal instillation of either 50 μg Escherichia coli LPS (serotype O55: B5; Sigma-Aldrich) or sterile PBS before they were sacrificed as described. The acute inflammatory responses were evaluated by measuring 1) circulating inflammatory mediators including IL-6 and TNF-α using ELISA; 2) ICAM-1 expression on CD105+ ECs and CD105− cells in the lung; 3) neutrophil content in the bone marrow; and 4) neutrophil recruitment in the lung.

FIGURE 2. The role of ROCK1 and ROCK2 in mediating TNF-α-induced MLC phosphorylation. A, Specific inhibition of ROCK1 or ROCK2 protein expression by treatment with ROCK1 or ROCK2 siRNA. ECs were treated with a nonspecific control siRNA, ROCK1 siRNA, ROCK2 siRNA, or combined ROCK1 and ROCK2 siRNA, and the protein expression of ROCK1 or ROCK2 was examined. The expression of PAK (65 kDa), AKT (60 kDa), FAK (125 kDa), phosphorylated PKC (pan) (80–90 kDa), and actin (42 kDa) was also examined. B, TNF-α-induced MLC phosphorylation in ECs was prevented by pretreatment with 20 μM Y27632, a ROCK inhibitor. Phosphorylated MLC (Thr18/26, 18 kDa), phosphorylated PKC (pan, 80–90 kDa), and total vasodilator-stimulated phosphoprotein (VASP, unphosphorylated and phosphorylated, 46 kDa) was examined. C, The role of ROCK1 and ROCK2 in mediating TNF-α-induced MLC phosphorylation. ECs treated with control siRNA or ROCK1 or ROCK2 siRNA, either singly or in combination, were stimulated with TNF-α for 3 or 24 h, and MLC phosphorylation was examined. Data were expressed relative to the control siRNA treated samples without TNF-α stimulation (n ≥ 4 independent experiments), *p < 0.05 when compared with samples without TNF-α stimulation in each group. #p < 0.05 when compared with samples treated with control siRNA. ECs treated with control siRNA (■), ECs treated with ROCK1 siRNA (light gray), ECs treated with ROCK2 siRNA (dark gray), and ECs treated with ROCK1 plus ROCK2 siRNA (□) are shown.
To measure circulating IL-6 and TNF-α levels, blood was collected from the inferior vena cava, and plasma was separated from blood cells by centrifugation. Aliquots of plasma were snap-frozen and stored at −80°C until analyzed by ELISA. The amount of IL-6 and TNF-α was measured using an ELISA kit according to manufacturer’s instructions (eBioscience).

ICAM-1 expression on CD105+ lung ECs and CD105+ lung cells following LPS instillation was evaluated using flow cytometry. Single lung cell suspension was obtained as described, and ICAM-1 surface expression on both CD105+ ECs and CD105+ cells was evaluated by flow cytometry using a FACSCalibur (BD Biosciences).

To measure neutrophil content in the bone marrow, femurs were dissected and flushed using ice-cold PBS. Single cell suspension was analyzed for neutrophil content by staining for GR1, a cell surface marker for murine neutrophils, and CD11b using flow cytometry (Abs were obtained from eBioscience). The proportion of cells that were positively stained for GR1 and CD11b was determined using a FACSCalibur (BD Biosciences).

To evaluate neutrophil accumulation in the lungs, single lung cell suspension was obtained, and the proportion of neutrophils (GR1+) was determined using flow cytometry.

**Statistical analysis**

Data were analyzed using the Student t test or one-way ANOVA followed by posthoc comparisons (LSD test). A value for p < 0.05 is considered significant. Data are expressed as a mean value ± SEM.

**Results**

**Activation of both ROCK1 and ROCK2 isoforms in human pulmonary microvascular ECs during TNF-α stimulation**

Previous studies have demonstrated activation of ROCK in response to TNF-α that lasts for hours in primary human pulmonary microvascular ECs (15). To begin to evaluate isoform-specific ROCK activation, ROCK1 or ROCK2 was immunoprecipitated from primary human pulmonary microvascular ECs, and ROCK isoform activity was evaluated by an in vitro kinase activity assay using purified MYPT-1 (714-1004) as a substrate. Ab against ROCK1 or ROCK2 specifically and efficiently immunoprecipitated their presumed target, but not the other isoform (Fig. 1A). Immunoprecipitated ROCK1 and ROCK2 were both able to phosphorylate MYPT-1 in the kinase assay (Fig. 1B). Importantly, when Y27632, a Rho kinase inhibitor, was added during the kinase activity assay, MYPT-1 phosphorylation by either ROCK1 or ROCK2 was completely inhibited (Fig. 1B).

Using this technique, the activity of ROCK1 or ROCK2 in human pulmonary microvascular ECs following TNF-α stimulation was examined. Each sample was evenly divided into half for ROCK1 or ROCK2 activity measurement. In untreated ECs, total ROCK2 activity was greater than ROCK1 activity (Fig. 1C). Following TNF-α stimulation, the activity of both ROCK1 and ROCK2 increased, indicating that both isoforms are activated (Fig. 1C).

**The role of ROCK1 and ROCK2 in mediating MLC phosphorylation and permeability increases induced by TNF-α**

To examine the functions of ROCK1 and ROCK2 in mediating EC responses to TNF-α, siRNA was used to inhibit the protein expression of ROCK1 or ROCK2. In ECs treated with ROCK1 or ROCK2 siRNA, the protein expression of ROCK1 or ROCK2 was specifically inhibited when compared with ECs treated with a non-specific control siRNA (Fig. 2A). The relative protein expression of ROCK1 or ROCK2 in ECs treated with control siRNA, ROCK1 siRNA, ROCK2 siRNA, or combined ROCK1 plus ROCK2 siRNA measured 1, 0.28 ± 0.07, 1.07 ± 0.14, 0.24 ± 0.05 (ROCK1 expression), and 1, 0.94 ± 0.12, 0.27 ± 0.07, 0.24 ± 0.07 (ROCK2 expression), respectively. The expression of several other kinases including PAK, AKT, and FAK was not affected (Fig. 2A). In addition, immunoblot using a pan phosphorylated PKC Ab that detects phosphorylated PKCα, PKCβI, PKCβII, PKCδ, PKCe, PKCη, and PKCθ isoforms showed that ROCK siRNA treatment did not inhibit PKC phosphorylation.

One of the best described downstream responses of ROCK activation is MLC phosphorylation and F-actin polymerization. TNF-α stimulation causes prolonged changes in the F-actin cytoskeleton and stress fiber formation that last for hours (15, 25). By 3 h of TNF-α stimulation, F-actin polymerization and thickening of F-actin bundles occur along the cell periphery, whereas cell elongation and central stress fiber formation occur by 24 h (15, 25). Changes in the F-actin cytoskeleton were accompanied by MLC phosphorylation that could be detected by 1 h and lasted for at least 24 h (Fig. 2B). Pretreatment with Y27632, a pharmacologic inhibitor that inhibits both ROCK1 and ROCK2 activity, completely prevented MLC phosphorylation (Fig. 2B). Phosphorylation of PKC (pan) was not inhibited by Y27632 (Fig. 2B). These data indicate that TNF-α-induced MLC phosphorylation requires activation of ROCK. The role of ROCK isoforms in mediating MLC phosphorylation was therefore examined. Treatment with ROCK1 siRNA alone was not sufficient to inhibit TNF-α-induced MLC phosphorylation (Fig. 2C). Treatment with ROCK2 siRNA alone did not prevent TNF-α-induced early MLC phosphorylation at 3 h, but prevented TNF-α-induced late response at 24 h (Fig. 2C). Inhibition of both ROCK1 and ROCK2 protein expression completely prevented MLC phosphorylation induced by TNF-α (Fig. 2C). These data indicate differential requirements for ROCK1 and ROCK2 in mediating MLC phosphorylation following early (3 h) vs late (24 h) TNF-α stimulation.
ROCK isoforms was required and sufficient to induce MLC phosphorylation in response to early TNF-α stimulation, whereas ROCK2 was required and sufficient for TNF-α-induced late MLC phosphorylation.

Accompanied by MLC phosphorylation and F-actin cytoskeleton changes, TNF-α induces prolonged permeability increases in these ECs (15, 25). Inhibition of ROCK using Y27632 prevented early permeability increases in these ECs induced by TNF-α stimulation for up to 3 h (15). The role of ROCK1 and ROCK2 in mediating permeability increases induced by TNF-α was therefore examined. TNF-α treatment caused an increase in the fluxes of dextran across ECs treated with the control siRNA that was
apparent by 1–3 h and lasted for at least 24 h (Fig. 3). Inhibition of ROCK1 protein expression had little effect on the baseline permeability, but prevented TNF-α/H9251-induced early permeability increases (up to 6 h) (Fig. 3). By 24 h of TNF-α stimulation, a small inhibition was observed in cells treated with ROCK1 siRNA (Fig. 3, p < 0.05 only when compared with control siRNA-treated group using paired t test, but not using ANOVA). These data indicate that TNF-α/H9251-induced early permeability increases were entirely ROCK1-dependent, whereas at later times, TNF-α-induced permeability increase were largely ROCK1-independent. In ECs treated with ROCK2 siRNA, the baseline permeability tended to be higher than that of ECs treated with control or ROCK1 siRNA, although not statistically significant. Following TNF-α-stimulation, dextran fluxes across ROCK2 siRNA-treated ECs were similar to control siRNA-treated ECs (Fig. 3).

**Activation of ROCK isoforms in freshly isolated lung ECs following intratracheal LPS instillation in mice**

These studies using primary human ECs clearly demonstrate a role for ROCK isoforms in mediating EC responses to TNF-α. An enhanced ROCK activity in lung ECs may regulate F-actin remodeling, MLC phosphorylation and vascular permeability during an inflammatory response in vivo. To begin to address this question, lung ECs were isolated from mice and relative changes in ROCK activity in these freshly isolated lung ECs were evaluated. Single lung cell suspension was obtained, and ECs were then isolated using an anti-CD105 Ab (16). Endoglin (CD105) is a 180 kDa homodimeric transmembrane glycoprotein that is mainly expressed by ECs (16, 17). Double staining for CD105 and CD45 of the whole lung single cell suspension shows that 42.6 ± 0.9% of total lung cells were CD105/CD45−, 38.0 ± 0.5% of total lung cells were CD105−CD45+, and 2.8 ± 0.2% of total lung cells were CD105−CD45− (n = 3 lung samples) (Fig. 4). Double staining for Ac-LDL uptake and CD105 shows that all CD105+ cells

**Table I. Neutrophil infiltration and ICAM-1 expression on lung cells during LPS pneumonia**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mean Expression ± SEM</th>
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<tbody>
<tr>
<td>No LPS</td>
<td></td>
</tr>
<tr>
<td>LPS for 1 h</td>
<td></td>
</tr>
<tr>
<td>LPS for 4 h</td>
<td></td>
</tr>
<tr>
<td>LPS for 16 h</td>
<td></td>
</tr>
<tr>
<td>GR1*</td>
<td>1.38 ± 0.22%</td>
</tr>
<tr>
<td>1.00 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>1.21 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>1.94 ± 0.08*</td>
<td></td>
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<tr>
<td>ICAM-1 on CD105+</td>
<td>0.97 ± 0.05</td>
</tr>
<tr>
<td>0.97 ± 0.05</td>
<td></td>
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<tr>
<td>1.14 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>1.95 ± 0.20*</td>
<td></td>
</tr>
<tr>
<td>ICAM-1 on CD105−</td>
<td>0.83 ± 0.05</td>
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<tr>
<td>0.83 ± 0.05</td>
<td></td>
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<tr>
<td>1.14 ± 0.07</td>
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<tr>
<td>1.95 ± 0.20*</td>
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*p < 0.05 compared with the No LPS group (n = 5–7 mice in each group).

**FIGURE 5.** Activation of ROCK in freshly isolated lung ECs following intratracheal LPS instillation in mice. Mice were treated with LPS at the indicated times, and lung ECs were isolated. ROCK activation in freshly isolated lung ECs was examined by measuring the amount of phosphorylated MYPT1 (endogenous, 130 kDa), a major Rho kinase substrate. The expression of ROCK1 (160 kDa) or total ezrin, radixin, moesin (ERM, 80 kDa) was also examined. Data were expressed relative to animals not treated with LPS (n = 4 mice for each sample). *p < 0.05 when compared with samples not treated with LPS.

**FIGURE 6.** Activation of ROCK in lung ECs following LPS instillation was inhibited by treating the animals with a ROCK inhibitor, fasudil, in a dose-dependent manner. The animals were treated with either vehicle or fasudil before challenged with LPS or control, and lung ECs were isolated. ROCK activation in freshly isolated lung ECs was examined.
were also positive for Ac-LDL uptake (Fig. 4). Another population of cells were also positive for Ac-LDL uptake, and the majority of these cells stained positive for CD45, indicating a leukocyte population such as macrophages (Fig. 4). After sorting the cells using the CD105 Ab, 89%/110012% of the sorted cells were CD105+/H11001, CD45/H11002, and stained bright for Ac-LDL uptake (n/110053) (Fig. 4). These data indicate that the anti-CD105 Ab-sorted cells are characteristic of ECs (positive for Ac-LDL uptake) and are CD45 negative. Percentage of CD105+/H11001 cells that remained in the flow through and unsorted cell population measured 4+/H110061% (n/110053 samples) (Fig. 4). Cell viability was evaluated by Annexin V-FITC and propidium iodide staining. Percentage of cells that were negative for Annexin V and propidium iodide staining measured 93+/H110061%, 90+/H110062%, and 95+/H110061% in whole lung digest, CD105 sorted cells and flow through cells (n/110053 samples) (Fig. 4).

The responses of lung ECs during LPS pneumonia were then determined. *E. coli* LPS was instilled into the distal lungs in mice to initiate an acute inflammatory response in the lung. Single cell suspension was obtained. Flow cytometry was used to measure neutrophil infiltration and ICAM-1 expression. Intratracheal instillation of *E. coli* LPS (50 μg/animal) induced neutrophil infiltration into the lungs and expression of ICAM-1 on lung cells, consistent with many published studies (Table I). Percentage of GR1+ cells in the single cell suspension increased by 1 h of LPS instillation, and this increase persisted for at least 16 h (Table I). The early neutrophil infiltration by 1 h likely reflects the sequestration of neutrophils in the capillaries, whereas at later times, some of the neutrophils have migrated out of the vasculature into the alveolar space. Measurement of ICAM-1 expression on lung ECs and non-ECs was performed by double staining for ICAM-1 and CD105. Surface expression of ICAM-1 increased by 2-fold on CD105+ ECs as well as on CD105− cells after 16 h (Table I). Lung ECs were then isolated from mice following LPS instillation and ROCK activity was evaluated by measuring the phosphorylation state of a major ROCK substrate, MYPT-1 (15). LPS instillation into the lungs induced an increase in ROCK activity in CD105+/H11001 ECs (Fig. 5). The increase in ROCK activity was detected after 1 h LPS instillation and persisted for at least 16 h (Fig. 5). By contrast, ROCK activity did not increase in CD105− cells, and the ROCK activity in these cells measured 1.0±0.1, 0.6±0.2, 1.1±0.3, and 1.3±0.3 before or 1, 4, and 16 h following LPS instillation (n=4–9 mice in each group). These studies demonstrate that activation of ROCK occurs in mouse lung ECs during LPS pneumonia in vivo.

These experiments using isolated lung ECs ex vivo required protease digestion and cell sorting, and may therefore alter ROCK activity. To rule out the possibility that the measured ROCK activation in lung ECs was due to technical artifacts during cell isolation, fasudil, a ROCK inhibitor used in rodents and humans, was used to treat mice. The effect of fasudil on ROCK inhibition was examined (Fig. 6). Because the effect of fasudil on ROCK inhibition was dose-dependent in vivo, three sets of experiments were performed in mice varying the dose of fasudil (21–24). At a low dose of...
fasudil, ROCK activation in lung ECs at 4 or 16 h of LPS pneumonia was not inhibited. At an intermediate dose, ROCK activation in lung ECs was inhibited at 4 h, but only partially at 16 h when a more robust ROCK activation was observed. At a high dose, ROCK activation in lung ECs was inhibited at both times (Fig. 6). Examination of ROCK activity in CD105− lung cells showed that high dose fasudil also inhibited ROCK activity in CD105− cells (data not shown). These preliminary studies clearly demonstrate that fasudil treatment inhibits ROCK activation in lung cells including ECs in vivo in a manner that depends on the dose of fasudil and the strength of ROCK activation.

The effect of fasudil treatment on the inflammatory responses to intratracheal instillation of LPS was also evaluated in these animals that received low or high dose of fasudil. In mice that received treatment with vehicle, both TNF-α and IL-6 increased in plasma that was apparent by 1 h and increased further by 4 h of LPS challenge (Fig. 7). By 16 h, both cytokines returned to near-baseline levels (Fig. 7). Fasudil at the high dose, but not the low dose, prevented TNF-α and IL-6 production at early hours (by 1 and 4 h). ICAM-1 expression on CD105− lung cells was similarly inhibited by high dose fasudil treatment (Fig. 7). LPS instillation also induced the release of neutrophils from the bone marrow (Fig. 7). Neutrophil content increased by 4 h of LPS instillation, and started to return to baseline by 16 h (Fig. 7). Treatment with high dose, but not low dose of fasudil, inhibited the increase in bone marrow neutrophils by 4 h of LPS stimulation (Fig. 7). The early recruitment of neutrophils in the lung by 1 h and 4 h of LPS instillation was inhibited by high dose of fasudil, but not by low dose of fasudil (Fig. 7). Interestingly, neutrophil recruitment by 16 h of LPS instillation was not inhibited by fasudil (Fig. 7).

Which ROCK isoform was activated in lung ECs following LPS stimulation was also determined. Using the method we described for human pulmonary microvascular ECs, each sample was evenly divided to measure ROCK1 or ROCK2 activity. In lung ECs isolated from untreated mice, ROCK2 activity was greater than ROCK1 activity (Fig. 8). Following LPS instillation for 16 h, the activity of both ROCK1 and ROCK2 increased (Fig. 8). These data indicate that in lung ECs, activation of both ROCK1 and ROCK2 occurs following an inflammatory response and likely regulates downstream responses such as cytoskeletal reorganization and permeability increases in these cells.

Discussion

This study examined isoform-specific activation of ROCK in lung ECs during inflammatory responses using cultured human ECs as well as lung ECs freshly isolated from mice following LPS challenge. In unstimulated human pulmonary microvascular ECs as well as mouse lung ECs, ROCK2 activity is greater than ROCK1 activity. In response to stimulation with TNF-α in vitro, the activity of both ROCK1 and ROCK2 increases in human ECs. The activity of ROCK1 and ROCK2 also increases in mouse lung ECs following LPS challenge in mice. TNF-α-induced MLC phosphorylation requires activation of ROCK. Inhibition of ROCK1 alone is not sufficient to inhibit TNF-α-induced MLC phosphorylation, whereas inhibition of ROCK2 prevents TNF-α-induced late MLC phosphorylation at 24 h. Although ROCK1 is dispensable for TNF-α-induced MLC phosphorylation, ROCK1 was required for early permeability increases induced by TNF-α stimulation for up to 6 h.

The high sequence homology between ROCK1 and ROCK2 suggests that these two isoforms may be similarly regulated and share similar substrates. Several observations in this study on lung ECs support this notion. First, both ROCK1 and ROCK2 are activated in human ECs by TNF-α or in lung ECs freshly isolated from mice treated with LPS. Secondly, TNF-α-induced early MLC phosphorylation was not affected by singly inhibiting ROCK1 or ROCK2 protein expression. Although MLC phosphorylation clearly requires ROCK, ROCK1 and ROCK2 are functionally redundant in mediating TNF-α-induced early MLC phosphorylation. Why singly inhibiting ROCK1 or ROCK2 does not result in a partial inhibition of TNF-α-induced early MLC phosphorylation is interesting. MLC phosphorylation is dictated by the activity of MLC kinase and MLC phosphatase/MYPT-1 complex. ROCK regulates MLC phosphorylation mainly through inactivation of MLC phosphatase by phosphorylating MYPT-1 (26). Indeed, inhibition of ROCK activity or both ROCK1 and ROCK2 protein expression completely prevented MLC phosphorylation. Inhibition of MLC kinase, however, does not prevent TNF-α-induced F-actin cytoskeleton remodeling (25). The in vitro kinase activity assay performed in this study suggests that both ROCK1 and ROCK2 can efficiently phosphorylate MYPT-1, consistent with previous reports (27, 28). Therefore, a small increase in ROCK activity by TNF-α may be sufficient to induce MYPT-1 phosphorylation and subsequent MLC phosphorylation in ECs. These observations in lung ECs are different from those reported using fibroblasts (27, 28). In fibroblasts, ROCK1 activity is greater than ROCK2 activity, and ROCK1 is required for stress fiber formation and MLC phosphorylation (27, 28). These studies highlight the need to examine the functions of ROCK isoforms in a cell type-specific manner.

Despite the functional redundancy between ROCK1 and ROCK2 in mediating TNF-α-induced early MLC phosphorylation,
TNF-α-induced early permeability increases required ROCK1 activity. These data also indicate that MLC phosphorylation alone, as seen in ROCK1 siRNA-treated ECs, is not sufficient to cause permeability increases induced by TNF-α. ROCK1 siRNA did not affect the baseline permeability of human pulmonary microvascular ECs, but prevented permeability increases induced by TNF-α for up to 6 h. Our previous studies have shown that pretreatment with Y27632 prevents TNF-α-induced early permeability increases (by up to 3 h), clearly indicating that activation of ROCK1, ROCK2, or both is required for early responses (15). At later times, TNF-α-induced permeability increases do not require ROCK1, and other mechanisms may play a role. In human umbilical vein ECs, TNF-α induces protein expression of phospho- diesterase 2, an enzyme responsible for breaking down cAMP and cGMP, that can be detected by 6 h and persists for at least 18 h, and this induced phosphodiesterase 2 is required for TNF-α-induced permeability increase at later times (29). Activation of Rho/ROCK is generally thought to increase EC permeability, whereas activation of Rac1 is thought to stabilize EC barrier functions (30–34). Most studies have used pharmacologic inhibitors targeting ROCK such as Y27632 to examine the role of ROCK in mediating TNF-α-induced permeability increases. Although some studies demonstrate that pretreatment with Y27632 prevents TNF-α-induced permeability increases, a lack of an effect of Y27632 on TNF-α-induced permeability increases has been observed in others (15, 25, 35, 36). All these studies, however, demonstrated the progressive and prolonged nature of TNF-α-induced permeability increases, and the role of ROCK has to be evaluated through prolonged incubation of the ROCK inhibitors with ECs. A recent study demonstrated that prolonged incubation of ECs with Y27632 for 24 h is sufficient to cause an increase in EC permeability in the absence of any stimulus (37). Therefore, the experiments that require prolonged incubation with ROCK inhibitors may be difficult to interpret. Moreover, EC origins may also influence how activation of Rho GTPases regulates EC barrier functions, again highlighting the need to evaluate the functions of Rho and ROCK in a cell type-specific manner (38).

Future studies will be focused on identifying the signaling mechanisms that dictate the functional specificity of ROCK1 in mediating TNF-α-induced early permeability increases. ROCK1 and ROCK2 share most of their common substrates, as also shown in this study on MLC phosphorylation. The only identified ROCK isomorph-specific substrate is RhoE (39, 40). RhoE binds ROCK1, but not ROCK2, and inhibits ROCK1 activation. ROCK1 phosphorylation of RhoE increases RhoE stability (40). Phosphoinositide-dependent kinase 1 also binds ROCK1, and this interaction antagonizes RhoE binding and allows ROCK1 to localize at the plasma membrane (41). In addition, ROCK2 shows stronger binding to phosphatidylinositol 3,4,5 triphosphate than ROCK1 and is sensitive to changes in its levels (28). Therefore, the activity of ROCK1 vs ROCK2 may be restricted at specific sites in a cell, giving rise to functional specificity.

Activation of ROCK regulates actin/myosin contractility, expression of inflammatory mediators, and leukocyte adhesion and transmigration across ECs. Using a LPS pneumonia model, this study demonstrates activation of ROCK using freshly isolated lung ECs that correlated with neutrophil infiltration into the lungs. This protocol allows detection of relative changes in ROCK activity in lung ECs following LPS challenge. Activation of ROCK is detected only in sorted CD105+ cells and not in CD105− cells. Therefore, approaches using total lung tissues may not reveal activation of ROCK during an inflammatory process. The increase in ROCK activity in CD105+ cells during LPS was completely inhibited by giving the animals a specific pharmacologic inhibitor in a dose-dependent manner, indicating that the relative changes in ROCK activity in lung ECs during LPS are not due to technical artifacts. Importantly, both ROCK1 and ROCK2 activity increased in lung ECs during LPS pneumonia. Many studies have demonstrated increases in acute phase cytokines such as TNF-α and IL-1β in the lungs during the early phase of LPS pneumonia (42, 43). Moreover, neutrophil adhesion through the ligation of EC adhesion molecules such as ICAM-1 as well as neutrophil transmigration across ECs induces activation of Rho/ROCK in ECs (44–46). Therefore, the activation of ROCK in lung ECs during LPS pneumonia may occur as a result of stimulation by inflammatory mediators generated systemically or locally in the lung and by neutrophil adhesion and transmigration across vascular ECs. The ability to evaluate ROCK activation in lung ECs in an isoform-specific manner demonstrates the feasibility to evaluate the mechanisms that regulate ROCK activation in these cells in vivo during disease processes.

Early inflammatory responses including cytokine release, ICAM-1 expression, neutrophil bone marrow release and neutrophil recruitment into the lungs are inhibited by fasudil, clearly indicating a proinflammatory role of ROCK early on. However, the effect of fasudil on neutrophil recruitment into the lungs is lost by 16 h. This may be due to the complex role of ROCK-dependent inflammatory cytokines such as IL-6 in mediating early vs late phase of neutrophil-dependent responses during inflammation. IL-6 is required for proper resolution of neutrophil-dependent acute inflammatory responses (47, 48). Therefore, a complete inhibition of IL-6 expression by fasudil during LPS pneumonia may impair the clearance of neutrophils at later times. In addition, the differential requirement for ROCK activity in early vs late neutrophil accumulation may be due to the different roles of ROCK played in different cell types during inflammation. Fasudil inhibited ROCK activity in many cell types. In the lung, the ROCK activity in both CD105+ and CD105− cells was inhibited by the high dose fasudil. The fact that fasudil inhibited early TNF-α production suggests a role for ROCK in mediating TNF-α production by alveolar macrophages or alveolar epithelial cells in response to intratracheal instillation of LPS. Moreover, activation of ROCK promotes neutrophil de-adhesion, for example (49). The different effect of high dose fasudil on the early (by 4 h) vs late (by 16 h) inflammatory responses induced by LPS instillation underlined the complexity to globally inhibit a signaling pathway in vivo without targeting a specific cell type. Future studies to specifically inhibit ROCK isoforms in an EC-specific manner will be required to fully understand the physiological functions of ROCK in ECs during inflammation.

The lung ECs were sorted using an anti-CD105 Ab, and are therefore likely comprised of lung ECs from large vessels and microvessels. Functional heterogeneity between the large vessel ECs and microvessel ECs is well documented in the lung, especially on the mechanisms that regulate permeability increases in ECs that line different blood vessels in the lung (50, 51). Therefore, the differences in ROCK activation among different ECs in the lung during LPS challenge may be masked using the current approach and will be important to identify in future studies.

In summary, this study demonstrates that both ROCK1 and ROCK2 are activated in lung ECs in response to TNF-α stimulation in vitro and during LPS pneumonia in mice. Functional redundancy between ROCK1 and ROCK2 exists in processes such as TNF-α-induced early MLC phosphorylation. However, ROCK1 and ROCK2 can be functionally separated in processes leading to TNF-α-induced early permeability increases or late MLC phosphorylation.
Disclosures

The authors have no financial conflict of interest.

References


