Materials and Methods

**Mice** – All animal studies were approved by the Institutional Animal Care Committee of University of Illinois. Mice were housed under standard conditions in the Biological Resources Laboratory facilities. The generation and characteristics of the RhoGDI-1^{−/−} mice are described elsewhere. Because of the impairment in reproduction capacity of RhoGDI-1^{−/−} mice, RhoGDI-1 heterozygous mice (RhoGDI-1^{+/−}), were used for mating. RhoGDI-1 heterozygous mice (F4) were intercrossed to generate litters of WT, RhoGDI-1^{+/−}, and RhoGDI-1^{−/−} mice. The genotype of resultant knockout mice was identified on DNA extracted from tail biopsy according to the manufacturer’s protocol Wizard Genomic DNA purification kit Promega (Madison, WI) by polymerase chain reaction assay. We used RhoGDI-1^{−/−} mice on C57Blk/6J background and their WT littermates in the experiments. Male littermate mice (8-14 wks old) were used in all studies.

**Determination of pulmonary microvascular permeability** – Lung preparation: C57BL/6 mice or RhoGDI-1^{−/−} mice weighing between 25 and 35 grams were anesthetized with an i.p. injection of ketamine (100 mg/kg), xylazine (2.5 mg/kg), and acepromazine (2.5 mg/kg). A tracheal cannula was inserted for positive-pressure ventilation and 100U of heparin, injected into the internal jugular vein for anticoagulation. The diaphragm was exposed through a midline abdominal incision and excised from the rib cage. A sternotomy was performed and the thoracic cavity exposed. We inserted a polyethylene perfusion cannula (PE-90; Becton Dickinson, Sparks, MD) into the pulmonary artery via the pulmonic valve, which we accessed through a small incision in the right ventricle. The cannula was secured by means of a suture encompassing the pulmonary artery and underlying aorta. The left atrium was cut free for drainage of venous effluent. The lungs were perfused with RPMI 1640 solution at constant flow (2 ml/min), temperature (37°C), and pH (7.4), using a peristaltic pump (Model Minipuls 3; Gilson, City, State). Preparations received positive-pressure ventilation (rate, 120 breaths/min; peak inspiratory pressure, 8-10 cm H₂O; end-expiratory pressure, 2 cm H₂O) by means of a pressure-controlled ventilator (Kent Scientific, Litchfield, CT). The lungs and heart were rapidly removed from the thoracic cavity and suspended en bloc from a 6-cm lever arm.
extending from a force-displacement transducer (FT03, Grass Telefactor, and Warwick, RI). The lung-weight transducer was zeroed, so that subsequent weight changes indicated gain or loss of fluid from the lung preparation. Pulmonary arterial pressure was recorded using a Gould pressure transducer. The two transducers were connected to amplifiers (CP122 AC/DC strain gage Amplifier, Grass Instrument Co, West Warwick, RI) and the recordings were displayed on a computer monitor with the aid of an analog-to-digital converter (DAS 1800ST board; Keithly Metabyte, Solon, OH) and data acquisition software (Notebook Pro for Windows; Labtech, Andover, MA). Lung preparations underwent a 20-minute equilibration perfusion to establish isogravimetric conditions; those preparations that failed to achieve an isogravimetric state by the end of the equilibration period were discarded.

**Determination of lung microvessel filtration coefficient:** We made four determinations of the microvessel filtration coefficient \( K_{fc} \) in each mouse lung preparation at 20-min intervals. To determine \( K_{fc} \), we produced a step-increase in pulmonary arterial pressure of 8-10 cm H₂O for 5 min. Lung preparations gained weight in response to the pressure increment with a characteristic biphasic time course: an initial fast phase of vascular volume expansion followed by a much slower phase of net fluid filtration. We measured the filtration rate off-line from the slope of the slow phase of lung weight gain using a LOTUS 1-2-3© macro developed in this laboratory. We normalized the observed filtration rate by the imposed vascular pressure increase and the lung dry weight (lung tissue dried in a 60°C oven for 48-72 hrs) to obtain \( K_{fc} \) in units of ml/min/cm H₂O/dry lung g. We averaged the four \( K_{fc} \) determinations made and recorded a mean \( K_{fc} \) value for each lung preparation.

**Drug infusions** – Drug infusions were made at a rate of 0.2 ml/min through a side-port in the perfusion cannula. The perfusion rate was decreased to 1.8 ml/min during drug infusions, such that total flow through the lung vasculature remained constant at 2 ml/min. In experiments testing the effects of Y-27632 on lung liquid permeability in RhoGDI-1 \(^{-/-}\) mice, drug infusions were begun 15 min before the first \( K_{fc} \) trial and 5 min before the succeeding three \( K_{fc} \) trials (see above). In experiments testing the effects of the PAR-1 agonist peptide, infusion was begun 5 min before first \( K_{fc} \) measurement. Drug infusions were discontinued during \( K_{fc} \) trials. For biochemical assays, infusion of
PAR-1 peptide or Y-27632 was started after the 20-min equilibration period. In each case, the drug infusion was performed for 5 min prior to harvesting the lung tissue. Four different concentrations (0.1, 0.5, 1, or 2 µM) of Y-27632 were used. The PAR-1 peptide was used at a concentration of 6 µM in all experiments.

**Electron microscopy and morphometric analysis** – After removal of blood from the vascular space with a 10-min HBSS perfusion, lungs were fixed in situ (20 min at RT) by injecting through the pulmonary artery cannula a mixture of 3% formaldehyde + 1.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Excised specimens were further fixed in the same mixture (1hr at RT) then post-fixed in 2% OsO4 in acetate veronal buffer, pH 6.8 (1h on ice), stained in the dark (1hr at RT) with Kellenberg uranyl acetate, dehydrated through graded ethanol and then embedded in Epon 812. Tissue blocks were cured (72 hr at 90º C), cut with a Leica microtome, and 60 nm sections were counterstained with uranyl acetate and lead citrate and examined and photographed in a JEOL 1220 transmission electron microscope at 80 KeV.

Six or seven Epon blocks of lung specimens were used for thin sectioning, and 6-8 grids per block (every grid with 12-20 sections) were examined. We photographed and printed at a final magnification 38,000x capillary profiles (5-8 µm in diameter) with a full circumference on the examined section, and venular profiles (10-38 µm in diameter). We counted the sealed and opened inter-endothelial junctions. We regarded sealed inter-endothelial junctions as those with more than one fusion point, and as opened inter-endothelial junctions as those that allowed direct and uninterrupted communication between the vascular lumina and the perivascular space.

**Transfection and culture of human umbilical vein endothelial cells (HUVECs)** – HUVECs were obtained at first passage from Cambrex (Walkersville, MD, culture line CC-2519), and were utilized at passages 6-10. Cells were cultured in EBM-2 medium (Cambrex) supplemented with 10% (v/v) fetal bovine serum (Cellgro) and EGM-2
SingleQuots (Cambrex) and maintained at 37°C in humidified atmosphere of 5% CO2-95% air. Transient transfections of HUVECs were performed with SuperFect Transfection reagent (Qiagen) according to the manufacturer’s protocols. Transfection of siRNAs was performed using DharmaFect1 (Dharmacon) or via electroporation (Amata) and experiments were performed 36 hr after transfection.

**Rho GTPases activation assay** – Activation of Rho proteins in vivo was determined by using pull-down assays as described 3. Briefly, extracted lungs were homogenized in the lysis buffer (50mM Tris, pH 7.2, 1% Tryton X-100. 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, protease inhibitor cocktail). Lysates were then clarified by centrifugation at 14000 rpm for 3 min in a centrifuge. Supernatants were then immediately removed and added to the aliquots of Rho binding domain (RBD) or PAK binding domain PBD-GST beads. Samples were incubated on a rotator for 1hr, washed with washing buffer, and analyzed by SDS-Page followed by Western blotting. Western blotting was performed as described 3.

**Immunocytochemistry** was performed as described 3. HUVECs grown to confluency on coverslips coated with gelatin were serum-starved for 6 hr before each experiment. Cells were washed with Hank’s balanced salt solution (HBSS) and fixed with 3.3% paraformaldehyde for 30 min. Cells were permeabilized for 5 min with 0.1% Triton X-100/ PBS and washed extensively with HBSS. After blocking with 1%BSA/0.2% fish skin gelatin in HBSS for 1 h at RT, cells were incubated with primary antibody in blocking solution for 1 h at RT followed by incubation with secondary antibodies. Slides were mounted using ProLong Antifade Kit (Molecular Probes). Microscopy was performed using Zeiss LSM 510 confocal microscope equipped with 63x water-immersion objective with appropriate filter sets.

**References**

2. Gao X, Kouklis P, Xu N, Minshall RD, Sandoval R, Vogel SM, Malik AB. Reversibility of increased microvessel permeability in response to VE-cadherin