Expanded Materials & Methods belonging to Beckers et al.

ROCK2 Regulates Thrombin Receptor-mediated Vascular Permeability: Role of Baseline Junctional Forces

All animal studies were approved by the Institutional Animal Care Committee of the University of Illinois.

Materials

EBM2 and medium Medium 199 supplemented with 20 mmol/L HEPES, L-glutamine and penicillin/streptomycin were obtained from Lonza (Basel, Switzerland); newborn calf serum (NBCS) was obtained from Gibco (Grand Island, NY). Tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus as described by Maciag et al.(1) Human serum albumin (HSA) and human serum were obtained from Sanquin CLB (Amsterdam, The Netherlands). Serum was prepared from 10 to 20 healthy donors, pooled, heat-inactivated and stored at 4°C. Trypsin was purchased from Gibco/Invitrogen (Grand island, NY), heparin from Leo Pharmaceutical Products (Weesp, The Netherlands) Thrombin from Sigma (Missouri, USA). Ac-TFLLRNPNDK-NH2 was from Biosource/Invitrogen (Carlsbad, CA). FITC- and HRP-labeled secondary antibodies were from Dako (Ostrup, Denmark). Rhodamine-phalloidin was from Molecular Probes (Eugene, Oregon). Antibodies against VE-cadherin, ROCK1 and ROCK2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). pS1366ROCK antibody was kind gift of dr.H.H. Lee (Taipei, Taiwan).(2) SLx-2119 was Surface Logix (Brighton, MA). Y-27632 was obtained from Calbiochem (Amsterdam, The Netherlands).

Cell Culture

Unless indicated otherwise, experiments were performed with human umbilical vein endothelial cells (HUVECs). Key findings were verified in human pulmonary microvascular endothelial cells (HPMVECs).

HUVECs were isolated, cultured, and characterized as previously described.(3) HUVECs were cultured on fibronectin- or gelatin-coated dishes in Medium 199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 40 µg/mL crude endothelial cell growth factor, 2 mmol/L L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C under 5% CO2 /95% air atmosphere. Media were changed every other day. Cells were cultured up to passage 2. Before all experiments cells were washed once with Medium 199 and preincubated for 1 hour in Medium 199 + 1% HSA.

Human pulmonary microvascular endothelial cells (HPMVECs) were isolated from human lung tissue, as previously described.(4) Cells were cultured in EGM2-MV culture medium (EBM2 medium

supplemented with 5% fetal bovine serum, human epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, hydrocortisone, ascorbic acid, gentamicin and amphotericin according to the manufacturers protocol, and with 100 IU/mL penicillin and 100mg/mL streptomycin) and seeded on gelatine-coated 25cm^2 culture flasks. Cells were grown to confluence at 37^{0}C and 5% CO2, with a change of culture medium every other day. They were extensively characterized as endothelial cells by the presence of endothelial markers and the absence of epithelial, lymphatic and smooth muscle cell markers. Cells were cultured up to passage 7, for experiments passage 4-7 cells were used.

HUVEC transfections

For *in vitro* studies of RNA interference, transfections were performed with *ROCK1*– and *ROCK2*–validated short interfering (si) RNA duplexes (50 nM) or a scrambled nonsilencing siRNA as negative control (Santa Cruz Biotechnology, Santa Cruz, CA). HUVECs were transfected using Amaxa nucleofection according to the manufacturer's protocol (www.amaxa.com) as described previously.(5) At 48 hours after siRNA transfection endothelial barrier function was evaluated or cells were harvested to determine the level of ROCK1 and ROCK2 proteins by immunoblotting. The efficiency of the transfection was monitored by immunoblotting 48 hours after transfection. A net decrease in ROCK protein expression in each experiment of at least 90% was observed in ROCK1-silenced ECs and of 75% in ROCK2-silenced ECs.

Western blotting

Proteins were separated by gel electrophoresis, blotted onto a nitrocellulose membrane, and stained with the indicated primary antibodies. Proteins were detected by chemi-luminescence according to the manufacturer's protocol (Amersham), and images were obtained using a charge-couple device camera (Fuji Science Imaging Systems). Signals were quantified with AIDA Image Analyzer software (Isotopenmessgeräte; Staubenhardt, Germany).

Evaluation of the barrier function

For the evaluation of the barrier function, confluent monolayers of HUVEC (first and second passage) were trypsinized and seeded in high density on polycarbonate filters of the Transwell^{TM} system pre-coated with fibronectin and gelatin, and cultured. Medium was renewed every other day. Monolayers were used between 4 and 6 days after seeding. Passage of macromolecules across the endothelial monolayers during a one hour-period was investigated by assay of the transfer of HRP and was performed as described previously.(6) Preceding the HRP passage, monolayers were preincubated with the Rho kinase inhibitors (10 microM/L) Y-27632, and SLx-2119 for 30 minutes.

Electrical cell substrate impedance sensing (ECIS)

ECIS^{TM} Model 1600R (Applied BioPhysics, Troy, NY, USA) was used to measure transendothelial electrical resistance (TEER) in confluent monolayers according to Tiruppathi *et al*.(7) In short, 250 µl of cell suspension ($8x10^5$ cells per ml) was seeded to each well of an 8W1 ECIS array equilibrated with cystein solution according to the manufacturer's protocol and coated with gelatin. When monolayers reached maximum resistance endothelial integrity was measured in real-time as described. Resistance was measured at multiple frequencies to allow for calculation of resistance attributable to cell-cell adhesion (Rb) and to cell-matrix interaction (Alpha).(8, 9)

3D Digital imaging microscopy

EC were fixed with 2% formaldehyde in PBS for 10 minutes at room temperature, and stained for F-actin with rhodamine-phalloidin. Stained cells were washed in PBS and mounted in Vectashield® Mounting medium for fluorescence with DAPI from Vector Laboratories, Inc. (Burlingame, California, USA). Digital imaging microscopy was performed essentially as described before.(10) In short, HUVECs were examined with a ZEISS Axiovert 200 Marianas inverted microscope, equipped with a motorized stage (stepper-motor z-axis increments: 0.1 micron). A cooled CCD camera [Cooke Sensicam (Cooke, Tonawanda, NY), 1280x1024 pixels] recorded images with true 16-bit capability. The camera is linear over its full dynamic range (up to intensities of over 4000) while dark/background currents (estimated by the intensity outside the cells) are typically < 100. Exposures, objective, montage, and pixel binning were automatically recorded with each image stored in memory. The microscope, camera, data viewing/processing were conducted/controled by SlidebookTM software (Intelligent Imaging Innovations, Denver, CO). Images were taken with a custom ZEISS 40x air (NA 0.75) and 63X oil lens (NA 1.4).

Quantification of monolayer contractility

To measure EC monolayer contraction, we used monolayer traction microscopy.(11) Briefly, ECs were seeded cells at confluence on 4kPa stiff collagen-coated polyacrylamide gel substrates. Within those gels, we embedded fluorescent microbeads. Images of microbeads obtained during the experiment were compared with a second image of the same microbeads obtained after detachment of cells at the end of the experiment. From these two images, a monolayer displacement field was computed. From the displacement field, we computed the monolayer traction field.(12) This computation circumvents limitations of the classical boundary value problem(13) through a new algorithm that accounts for unbalanced forces within the microscope field of view. From the traction field, we calculated the root mean square (RMS) value of monolayer traction. RMS tractions were reported for the following groups: Control, SLx-2119 pretreatment, and Y-27632 pretreatment. The monolayers were subsequently treated with thrombin and RMS tractions were computed 10 minutes after treatment.

Quantification of intercellular stress

To measure intercellular stress in intact monolayers we used Monolayer Stress Microscopy as previously described.(14) Briefly, each cell in the monolayer exerts local forces on the substrate (here called traction forces) which are balanced across the entire monolayer – giving rise to the local intercellular stress. Since the lateral extent of the monolayers studied here is much greater than their height we calculate the force balance in two demensions by treating the monolayer as a continuous, 2-D ,thin elastic sheet. The local intercellular stress at any point in the sheet is represented by the local stress tensor, σ_{ij} where i,j run over the coordinates x,y. Stresses in the Z direction are assumed to be zero. As above, there is no net force on the monolayer, σ_{ij} balances the measured traction forces and the key equation giving the intercellular force becomes:

$$\frac{\partial \sigma_{ij}}{\partial x_j} = T_i$$

Where we sum over repeated indices. The only assumption in the above treatment is that the monolayer can be treated as a continuum. However, since in the present work we study cells in the interior of the monolayer a problem arises at the boundary of the field of view where we do not know the stresses. We impose a boundary condition of zero normal displacement, which could lead to inaccurate calculation of intercellular forces close to the edge. We have previously shown that the intercellular forces are dominated by the local source terms (the tractions) and any influence from cells outside of the field of view generally decays as 1/r where r is the distance from the edge.(14) In the stress maps presented, we have discarded the calculated stresses from a region roughly 100um wide around the outer edge of the image, thus minimizing this error.

Liposomal delivery of siRNA in the mouse lung.

Cationic liposomes were made using a mixture of dimethyldioctadecyl-ammonium bromide (DDAB) and cholesterol in chloroform, as described previously.(15) Control, ROCK1 or ROCK2 siRNA (75 µg) or ROCK1 + ROCK2 (37.5 µg each siRNA) were mixed with 100 µL of liposomes. As the ROCK1 and ROCK2 that were used for *in vitro* studies with human ECs were less effective in cultured mouse NIH3T3 cells, we designed new siRNAs (siRNAROCK1: CUACCACUUUCCUGCCAAUUU and siRNA ROCK2: UGAAGAAAGUCAAGAGAU (Dharmacon, Herlev, Denmark)), that resulted in a >80% downregulation of ROCK1 and ROCK2 *in vitro*, Figure 1. The mixture of liposomes and siRNA were injected intravenously (via retro-orbital injection) into C57/Bl6 mice. After 48 hrs mice were used for determining lung microvascular permeability or were used for immunoblotting and immunohistochemistry analysis.

Assessment of lung capillary leakage.

PAR1 specific peptide (TFRLLN) (1 mg/kg) or control peptide (FTLLRN) was injected retroorbitally followed by injection of Evans blue conjugated albumin (EBA) (20 mg/kg) as described.(16, 17) After 30 min, mice were sacrificed and blood was collected from the right ventricle into heparinized syringes. Plasma was separated by centrifugation at 1,300 x *g* for 10 minutes. Right lung lobe was homogenized as described.(17) Lung homogenates and plasma were incubated with 2 volumes of formamide (18 hr, 60ºC), centrifuged at 5,000 x *g* for 30 min, and the optical density of the supernatant was determined spectrophotometrically at 620 nm and at 740 nm (to correct for hemoglobin). EBA extravasation was calculated as the ratio of EBA extravasated in lung versus that in plasma.

Lung weight determination.

Left lungs from the same mice used for EBA extravasation were excised and completely dried in the oven at 60°C overnight for calculation of lung wet-dry ratio**.(18)**

Immunohistochemistry

Right lungs were flushed with saline and embedded in paraffin. For immunohistochemistry 5µm slices were cut and stained for ROCK1 and ROCK2. In short, paraffin slices were deparaffinized with xylene, and rehydrated with 100% ethanol. Endogenous peroxydase was blocked with 0.3% H_2O_2/methanol solution. After antigen retrieval (60min autoclave in 10mM sodium-citrate solution, pH 6.0), slices were overnight (4°C) incubated with primary antibody against ROCK1 (1:100, #611136, BD Transduction Laboratories, New Jersey, USA), ROCK2 (1:250, #610623, BD Transduction Laboratories), or antibody diluent alone (negative control). Detection of primary antibody was performed with Envision® (ROCK1, DAKO Netherlands, Heverlee, Belgium) or Powervision® (ROCK2, Immunologic, Duiven, The Netherlands), according to the manufacturers protocol, and subsequently counterstained with hematoxylin. Slices were evaluated with a Leica DMRB light microscope (Leica Microsystems, Wetzlar, Germany) at 20x (air, NA 0.40) and 40x (air, NA 0.65) magnification. For imaging, a Nikon D50 digital camera (Nikon Corporation, Tokyo, Japan) was used. For quantification, line scans were drawn over the vessel wall from the lumen to the cytosplasm of underlying smooth muscle cells (see Figure 1C). Staining intensity over the line scan was calculated using Image J software. The intensity of staining in the endothelial cell layer was compared with luminal staining intensity (background signal) and with cytoplasmic staining intensity of smooth muscle cells (as non-targeted tissue). The difference in staining intensity with smooth muscle cells was used to measure effectivity of knock down of indicated siRNAs. Effectivity of knockdown was measured in arterioles. Per arteriole three line scans were drawn, and the average staining intensity (endothelial cell – smooth muscle cell) was taken.

Statistical analysis.

All data are reported as mean ± SD. Comparisons between 2 experimental groups were made by student t-test and between >2 groups by one way ANOVA with bonferoni post-hoc test. Differences in mean values were considered significant at $p < 0.05$.

Supplementary figures:

Figure S1. The endothelial barrier-disruptive effects of thrombin are recapitulated by activation of the thrombin receptor PAR1 with a PAR1-activating peptide.

Representative tracings of alterations in trans-endothelial electrical resistance of HUVEC monolayers evoked by 1 U/mL thrombin (blue) and 45 microg/mL PAR1-peptide (red), as measured by the ECIS method, see Materials and Methods section.

Figure S2. Thrombin-induced ROCK2 activation in HUVECs is ablated by the ROCK2 inhibitor SLx-2119, and by siROCK2, but not siROCK1.

Representative Western blot showing changes in the phosphorylation of ROCK2 upon stimulation with 1U/mL thrombin for the indicated time points in the presence or absence of 10 microM SLx-2119 as indicated. Blot was probed with a phospho-specific ROCK2 antibody (upper panel). Blot was reprobed with ROCK2 (middle panel) antibody to verify reduced ROCK2 expression upon siRNA transfection and with ERK1/2 antibody (lower panel) as a loading control.

Figure S3 Dose- and time dependency of the effects of SLx-2119 on hyperpermeability of primary human pulmonary microvascular endothelial cell (HPMVEC) monolayers.

A Representative experiment showing the effect of 0.1, 1 and 10 microM SLx-2119 and 10 microM Y-27632 on (thrombin-enhanced) HRP passage across HPMVEC monolayers

B Time-dependent effects of Rho kinase inhibitors on the passage of HRP across control and thrombin-stimulated human pulmonary microvascular endothelial monolayers. Confluent monolayers were preincubated for 1 hour in medium 199+1% HSA, pre-treated with 10 microM Y-27632 (blue), 10 microM SLx-2119 (red) or sham-treated (blue) for 30 minutes. Subsequently, the cumulative HRP passage across the monolayers was measured in the presence of 1 U/mL thrombin, as described in Materials and Methods. The baseline permeability of monolayers that were not treated with thrombin is shown for comparison (black). Right panel is color-shaded to better visualize the individual contributions of ROCK1 and ROCK2 to the thrombin-enhanced permeability. Values are the mean + SD (experiment performed in triplicate).

Figure S4. Effect of silencing of ROCK1 and/or ROCK2 on thrombin-induced changes in the F-actin cytoskeleton.

HUVECS were treated with siRNAs for ROCK1 and ROCK2 as described in the Materials & Methods section, seeded on glass cover slips in a confluent density and grown for 48 hrs. ECs were preincubated for 1 hour in medium 199 + 1% HSA, subsequently stimulated for 30 minutes with 1 U/mL thrombin or sham-treated, and subsequently fixed with paraformaldehyde. HUVECs were stained for F-actin by rhodamine-phalloidin (red). Nuclei were stained with DAPI (blue).

Figure S5A. Protrusive activity of ROCK2-inhibited HUVECs in thrombin-induced inter-endothelial gaps.

Shown are enlarged images from the boxed areas in Figure 3.

Figure 5B: 3D images were constructed by the 3D rendering mode in Slidebook software and show thin protrusion close to the glass surface.

Figure S5C: Cartoon showing the protrusion filling inter-endothelial gaps in ROCK2-inhibited endothelial cells.

Figure S6. Silencing of ROCK2 does not affect thrombin-induced contractile stress enhancements.

Representative phase contrast images (A) and cell-substrate traction stress maps (B), at baseline and post thrombin treatment from each group. Traction force microscopy was preformed 72 hours post-transfection for 15 minutes (baseline) and subsequently stimulated with thrombin (1 U/mL) for 30 min. Inter-endothelial gap are indicated with arrows. Bar = 50 um.

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