Involvement of Rho Kinase in Endothelial Barrier Maintenance

G.P. van Nieuw Amerongen, C.M.L. Beckers, I.D. Achekar, S. Zeeman, R.J.P. Musters, V.W.M. van Hinsbergh

Objective—Rho kinase mediates vascular leakage caused by many vasoactive agents including thrombin. Enhanced Rho kinase activity induces endothelial barrier dysfunction by a contractile mechanism via inactivation of Myosin Phosphatase (MP). Here, we investigated the contribution of basal Rho kinase activity to the regulation of endothelial barrier integrity.

Methods and Results—Using a phospho-specific antibody against the myosin phosphatase targeting subunit (Thr696 MYPT1) as a marker for Rho kinase activity, basal endothelial Rho kinase activity was observed at cell-cell contact sites, in vitro and in situ. Thrombin enhanced MYPT phosphorylation at F-actin stress fibers. Inhibition of basal Rho kinase activity for 24 hours or depletion of Rho kinase (ROCK-I and -II) by siRNA disrupted endothelial barrier integrity, opposite to the previously observed protection from the thrombin-enhanced endothelial permeability. This barrier dysfunction could not be explained by changes in RhoA, Rac1, eNOS, or apoptosis. Remarkably, basal Rho kinase activity was essential for proper expression of the adhesion molecule VE-cadherin.

Conclusions—Rho kinase has opposing activities in regulation of endothelial barrier function: (1) an intrinsic barrier-protective activity at the cell margins, and (2) an induced barrier-disruptive activity at contractile F-actin stress fibers. These findings may have implications for long-term antivascular leak therapy. (Arterioscler Thromb Vasc Biol. 2007;27:2332-2339.)

Key Words: endothelial cells ■ MYPT1 ■ myosin phosphatase ■ cytoskeleton

Increased endothelial permeability is a vascular reaction to inflammatory and angiogenic stimuli, resulting in vascular leakage. Vascular leakage contributes to the pathogenesis of numerous, often life-threatening, disorders. Excessive plasma extravasation may aggravate acute life-threatening obstruction of respiratory airways during pulmonary disorders.1 Vascular leakage may also cause circulatory collapse in sepsis and contribute to intravitreous scar formation in diabetic retinopathy leading to blindness.2 The permeability of tumor vessels is well documented in tumor models and in human cancer, having implications for metastasis.3 Remarkably, few specific therapies are available today to counteract vascular leakage.

Cytoskeletal elements play a pivotal role in regulation of endothelial barrier function, principally by determining cell shape, facilitating cell adhesion to subendothelial matrix, and participating in formation of junctional complexes. A major cause of vascular leakage under inflammatory conditions is the loss of endothelial cell (EC) junctional integrity, which is accompanied by the formation of small gaps between ECs. Studies on thrombin-induced endothelial hyperpermeability in vitro have identified at least 4 independent signaling pathways that contribute to barrier dysfunction: (1) Ca2+-dependent activation of myosin light chain kinase4; (2) a RhoA/Rho kinase-signaling pathway5,6; (3) a protein tyrosine kinase/phosphatase pathway that enhances disruption of intercellular junctions7,8; and (4) a new pathway that involves protein kinase C zeta.9

During the last decade the central importance of small G proteins in regulating the endothelial barrier function has been established. First, activation of the Rho-like small GTPase RhoA was demonstrated to increase actomyosin contractility, which facilitates the breakdown of intercellular junctions causing barrier dysfunction.5,6 A wealth of information is now available, indicating that its downstream target Rho kinase is involved in endothelial hyperpermeability induced by a variety of vasoactive agents such as VEGF, bacterial toxins, and oxidized LDL. Next, the related Rho-like GTPases Rac1 and Cdc42 were shown to counteract the effects of RhoA, enforcing the barrier or stimulating barrier recovery respectively.10,11 In contrast to its barrier enforcing effects, Rac1 was also shown to mediate loss of barrier integrity by vasoactive agents such as VEGF and thrombin, via activation of its downstream target Pak1.12 More recently, the barrier-stabilizing properties of cAMP-activated small GTPase Rap1 were discovered.13,14 These data suggest that a
fine balance in the activities of the distinct small GTPases is essential to proper regulation of endothelial barrier integrity.

A striking feature of Rho activation by vasoactive agents is the formation of cytoplasmic F-actin stress fibers (SFs). SFs are long cytoskeletal cables of bundles of F-actin and myosin II/non-muscle myosin filaments, that can contract and exert tension. Myosin-II is believed to be involved in generation of contractile forces. Its activity is mainly controlled by its light chain (MLC-2) phosphorylation, which is regulated by 2 classes of enzymes, MLC kinases and myosin phosphatases. MLCK and Rho kinase are the 2 major MLC kinases, but others exist as well.

A type 1 myosin-associated phosphatase activity has been implicated in the regulation of EC gap formation in vitro, and pharmacological inhibitor studies suggested its importance in endothelial contractility. MP is a holo-enzyme consisting of a catalytic subunit of PP1c-δ of 38 kDa, a large subunit termed the myosin phosphatase targeting subunit or MYPT1, also known as myosin binding subunit or MBS, of which 2 isoforms M130/133 exist and a 20-kDa small subunit of unknown function. MYPT1 is the major regulatory subunit, as it binds both PP1c and phosphorylated myosin-II, thus targeting the substrate MLC-2 to the catalytic core of MP. Several kinases are able to phosphorylate-MYPT1 and inactivate the MP, including Pak1 and Rho kinase. The expected consequence is enhanced MLC-2 phosphorylation and contractility. MYPT1 phosphorylation has been demonstrated to occur on stimulation with thrombin in ECs.

Previous studies mainly focused on the role of enhanced Rho kinase activity in hyperpermeability induced by vasoactive agents. In the present study, we investigated the contribution of basal Rho kinase activity to the regulation of endothelial barrier function. First, the subcellular distribution of Rho kinase activity under basal conditions was compared with the distribution under thrombin-stimulated conditions. Subsequently, the involvement of Rho kinase activity in regulation of basal barrier integrity was investigated. Finally, it was investigated whether inhibition of Rho kinase modulated the adherens junctional protein VE-cadherin.

Materials and Methods

Sources of reagents are listed in the expanded Materials and Methods section in the online data supplement section (http://atvb.ahajournals.org). Human umbilical vein endothelial cells (HUVECs) were cultured as previously described. HUVECs with transfected with short interfering (si) RNA duplexes using Amaxa technology. Densitometric analyses of Western blots were performed by AIDA Image Analyzer software. Barrier function was evaluated by the transfer of HRP across HUVEC monolayers grown on polycarbonate filters of the Transwell system. Alternatively, transendothelial electrical resistance (TEER) was measured. For 3D-Digital fluorescence imaging microscopy, HUVECs were examined with a ZEISS Axiovert 200 Marians inverted microscope. The data acquisition protocol included confocal optical planes to obtain 3D definition, followed by a constrained iterative deconvolution operation of the images. Data are reported as mean±SD. Data were compared by a Student t test. Probability values of less than 0.05 were considered to be significant.

Results

Subcellular Localization of Rho Kinase Activities

To measure and visualize Rho kinase activity we used a phosphorylation site-specific antibody against the regulatory subunit of myosin phosphatase (MYPT1). Phosphorylation at T696 of MYPT1 (phospho-MYPT1) by Rho kinase has been previously reported to inactivate the MP and to serve as a surrogate marker for Rho kinase activity.

Expression of MYPT1 in HUVECs was detected by Western blotting, demonstrating a double band of ≈130kDa (supplemental Figure I), in agreement with the previous reported 130/133 kDa MYPT1 isoforms in smooth muscle.

Quantification demonstrated a 3-fold increase in the total amount of phospho-MYPT1 after stimulation with thrombin for 30 minutes (supplemental Figure II). The thrombin-induced phosphorylation of MYPT1 was largely prevented by preincubation with the Rho kinase inhibitor Y-27632 for 30 minutes, indicating that thrombin inhibited global MP activity through Rho kinase. In addition, these data support that phospho-MYPT1 serves as a proper surrogate marker for Rho kinase inhibition.

To determine the subcellular localization of phospho-MYPT1 in HUVECs in detail, we used wide-field 3D-deconvolution fluorescence microscopy. High power magnification demonstrated a punctate cytoplasmic distribution pattern enriched in perinuclear areas under control conditions, that did not colocalize with the fine cytoplasmic F-actin meshwork ECs (Figure 1A and enlargement of its white box in Figure 1C; the accompanying line intensity scan is presented at the bottom of panel C). In thrombin-stimulated cells phospho-MYPT1 decorated F-actin stress fibers (Figure 1B). This suggests that inhibition of MP in thrombin-stimulated cells contributes to contractile properties of stress fibers.

As staining for panMYPT1 in ECs revealed an intense presence of MYPT1 at marginal areas (data not shown), we carefully inspected whether MYPT1 was phosphorylated in these areas. In control cells, phospho-MYPT1 was visible as a fine peripheral lining (enlargements of the yellow boxes in Figure 1E). As can be derived from the line intensity scan (see Figure 1E) cortical phospho-MYPT1 perfectly colocalized with F-actin. To our surprise and in contrast to the global cellular increase in phospho-MYPT1 by thrombin (supplemental Figure II), phospho-MYPT1 staining was lower in junctional areas in thrombin-stimulated cells (see enlargement of the yellow box in Figure 1F). Quantification confirmed that thrombin reduced phosphorylation of MYPT1 at cell-cell contacts significantly (supplemental Figure III). Of note, thrombin did not influence the total amount of MYPT1 in these areas.

To verify these findings in intact vessels, rat renal arterioles were isolated, cannulated, and perfused with thrombin. Staining of ECs in situ confirmed the colocalization of phospho-MYPT1 with cortical F-actin in control vessels (Figure 2, arrow heads). In addition, enhanced MYPT1 phosphorylation associated with central F-actin filaments was observed after exposure of intact vessels to thrombin (Figure 2, arrows).
To test whether basal Rho kinase activity was indeed responsible for phosphorylation of MYPT1 in junctional areas, HUVECs were pretreated for 24 hours with Y-27632 and junctional phospho-MYPT1 was quantitated (supplemental Figure IV). Treatment with Y-27632 markedly reduced junctional phospho-MYPT1. This was further confirmed by staining for phospho-MLC-2. In accordance with inactivation of MP in those areas, cortical phospho-MLC-2 was enriched in resting ECs (supplemental Figure V), and reduced on treatment with Y-27632. Thrombin stimulation enhanced phospho-MLC-2 mainly at stress fibers, but reduced cortical phospho–MLC-2.

In conclusion, visualization of Rho kinase activity at the subcellular level reveals regional differences in Rho kinase activity. In postconfluent ECs basal Rho kinase activity colocalized with the cortical rim of F-actin, but did not colocalize with the fine cytoplasmic F-actin meshwork. In addition, it reveals opposite regulation by thrombin; thrombin induced a robust Rho kinase activation mainly present on...
F-actin stress fibers, whereas cortical Rho kinase activity was decreased in thrombin-stimulated ECs.

**Opposite Contribution of Distinct Rho Kinase Activities to Regulation of Endothelial Barrier Function**

As the data presented in Figure 1 point to the presence of intrinsic Rho kinase activity at the periphery of confluent endothelial cells, we wanted to evaluate whether the observed basal Rho kinase activity contributes to endothelial barrier integrity. Therefore, the effect of inhibition of Rho kinase on basal barrier function was studied. HUVECs were seeded on top of porous filters, grown 2 days postconfluent, and subsequently preincubated with Y-27632 for the indicated time periods. Barrier integrity was evaluated by HRP passage across the monolayers during a 1-hour period. As shown in Figure 3B, 30 minutes preincubation with Y-27632 had no effect on basal HRP passage, whereas 24 hours preincubation resulted in a 2-fold increase in HRP passage. Preincubation for 96 hours with Y-27632 even further increased HRP passage.

Alternatively, barrier integrity was evaluated by measurement of TEER. 24-hour preincubation with Y-27632 induced a drop in TEER of 27±2% (n=4), confirming a decreased barrier function (supplemental Figure VI). 24-hour pretreatment with the structurally unrelated Rho kinase-inhibitors fasudil (10 micromol/L) and H-1152 (1 micromol/L) induced a similar decrease in TEER (26±3%, n=4 and 17±5%, n=6).

We used siRNAs to target the Rho kinase isoforms ROCK-I and ROCK-II. The efficiency of the transfection was monitored by immunoblotting 48 hours after transfection. A net decrease in protein expression of >75% was observed in HUVECs transfected with the specific siRNA (Figure 4, inset). Targeting both Rho kinase isoforms by siRNA significantly reduced TEER (Figure 4), whereas targeting one...
isoform had no effect (ROCK-I) or even elevated TEER (ROCK-II).

In line with previous data, 30 minutes preincubation with Y-27632 reduced the thrombin-induced HRP passage in part (57±5%, see Figure 3B). The remaining increase in HRP passage reflects the Rho kinase-independent hyperpermeability response of thrombin (indicated with Δ in Figure 3B). The Rho kinase–independent increase in endothelial permeability on thrombin stimulation was not affected by preincubation with Y-27632 for 24 and 96 hours. This indicates that Rho kinase–independent aspects of barrier regulation of endothelial monolayers were not affected by treatment with Y-27632 for prolonged periods.

Alterations in RhoA, Rac1, eNOS, and Apoptosis Do Not Explain the Barrier-Disturbing Effects of Rho Kinase Inhibition

To study the mechanism of reduced basal barrier function of Rho kinase inhibitor-treated endothelial monolayers, we first measured activity levels of the Rho proteins RhoA and Rac1. RhoA activity did not change by pretreatment with Y-27632, as was evidenced by G-LISA (0.079±0.065 versus 0.109±0.061, control versus Y-27632–pretreated cells in arbitrary units, n=3, P=0.591). Rac activity was measured by pulldown-assay, and did not change either (0.98±0.10 versus 1.26±0.25, control versus Y-27632-pretreated cells, n=6, P=0.332).

Second, we wondered whether altered eNOS expression could explain the observed barrier dysfunction. Inhibition of Rho kinase previously was reported to interfere with eNOS protein expression,20 and altered eNOS activity results in alterations of barrier function.10 However, eNOS expression as evidenced by Western blotting did not change significantly by inhibition of Rho kinase with Y-27632 (94±25% of control; mean±SD out of 5 independent cultures, P=0.31) and therefore does probably not explain the observed changes in barrier integrity.

Finally, we wondered whether enhanced apoptosis could explain endothelial barrier dysfunction. However, no signs of
enhanced apoptosis were observed by pretreatment with Y-27632 as was evidenced by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (1.9 ± 0.3% versus 1.3 ± 0.7%, control versus 24 hour. Y-27632-pretreated cells, n = 6, P = 0.675).

**Basal Rho Kinase Activity Is Essential for Maintenance of EC Junctions**

To visualize integrity of adherens junctions, endothelial monolayers were stained for the major endothelial adhesion molecule VE-cadherin. VE-cadherin staining showed an intact lining at the cell periphery of confluent ECs (supplemental Figure VIIA, left panel). At places where the peripheral membrane of neighboring cells overlapped, VE-cadherin formed a honeycomb-like structure. After inhibition of Rho kinase for 24 hours, the peripheral VE-cadherin lining appeared thinner, and was no longer continuous at sites where small gaps were formed between ECs (supplemental Figure VIIA, right panel). These findings were not observed after inhibition of Rho kinase for 30 minutes (supplemental Figure VIIA, middle panel). Quantitative analysis confirmed that less VE-cadherin was accumulated in junctional areas after Rho kinase inhibition for 24 hours (Figure 5), or after targeting both Rho kinase isoforms by siRNA (supplemental Figure VIIA, right panel).

To investigate whether inhibition of Rho kinase affected the total cellular amount of VE-cadherin, VE-cadherin protein expression was measured by Western blotting. Treatment with Y-27632 significantly reduced VE-cadherin protein levels by 38 ± 12% (n = 3, P < 0.05; Figure 5). Targeting both Rho kinase isoforms by siRNA similarly reduced VE-cadherin expression, whereas targeting the single isoforms had no effect (supplemental Figure VIIIB).

To investigate whether cortical MYPT1 forms a complex with the junctional proteins VE-cadherin and β-catenin, these proteins were immunoprecipitated and precipitated complexes were analyzed by Western blotting. VE-cadherin and β-catenin form a stable complex with each other, but interaction with MYPT1 was undetectable (supplemental Figure VIIIB). Also, probing the blot for the ERM proteins ezrin/radixin/moesin, did not reveal a detectable interaction of VE-cadherin with this family of Rho kinase target molecules, known to anchor the cortical F-actin cytoskeleton to the plasma membrane (data not shown).

Taken together, these data indicate that, in addition to its established barrier-disruptive activity, Rho kinase has an unexpected barrier-protective activity under basal conditions, probably via inactivation of MP at the margins of ECs, necessary for proper recruitment of VE-cadherin to junctional areas.

**Discussion**

Major findings of the present study are that for the first time we demonstrate that Rho kinase has a dual role in regulation of endothelial barrier function with opposite effects: Rho kinase has (1) an intrinsic activity at cell margins that is essential for proper barrier integrity, and (2) an induced activity at stress fibers that mediates cell contraction resulting in barrier disruption. Based on these data and data from literature, we propose that basal Rho kinase activity contributes to barrier integrity by regulating VE-cadherin, whereas enhanced Rho kinase activity induced by vasoactive agents contributes to barrier dysfunction by inducing contractility of cytosolic located F-actin filaments through MP inhibition.

To inhibit Rho kinase, we used Y-27632 and hydroxyfasudil. When tested on a large panel of protein kinases, these inhibitors only inhibited PRK2 with similar potency as Rho kinase, excluding other kinases being responsible for the observations of our study. Most importantly, these structurally unrelated inhibitors and downregulation of ROCK1/2 expression by siRNA approach similarly reduced basal endothelial barrier integrity in our experiments.

Our data reveal an unexpected cortical activity of Rho kinase in postconfluent ECs. This localized Rho kinase activity reduces MP activity at the margins of the cells, resulting in a peripheral rim of phosphorylated MLC-2. Enhanced peripheral phosphorylation of MLC-2 is a pattern also seen when endothelial monolayers are treated with barrier-protective agents such as sphingosine 1-phosphate. This suggests that those barrier-protective agents enforce a basal active process. These elevated levels of phosphorylated MLC-2 spatially localized within cortical F-actin ring might provide an environment with increased tension in junctional areas, which previously has been suggested to contribute to development of junction integrity via enhanced affinity between adherens junctions and the cortical cytoskeleton. The initial stimulus responsible for basal Rho kinase activity is likely cell–cell interaction, as VE-cadherin engagement recently was shown to activate RhoA in ECs, resulting in tension.[25]

Targeting Rho kinase isoforms by siRNAs revealed that each isoform was dispensable for forming a proper barrier. This suggests that ROCK-I and ROCK-II can functionally replace each other mutually. Targeting both isoforms severely
disrupted barrier integrity, in line with the effects of the pharmacological inhibitors, all of them inhibiting both iso-
forms. Remarkably, single targeting of ROCK-II even im-
proved barrier function, suggesting that this is the isoform
that is mainly responsible for the barrier disruptive effects.
Indeed, it was shown in epithelial cells that ROCK-II, but not
ROCK-1 mediates disassembly of the junctions. In ECs
ROCK-II, but not ROCK-1 has been implicated in microparticle
generation. These specific functions require further
investigation.

In epithelial cells Rho kinase was shown to be necessary
for the local concentration of E-cadherin in cell–cell con-
tacts. In apparent contrast, Braga et al reported that in the
endothelial context junctional maturation is not dependent on
RhoA activity. They observed that after inhibition of RhoA
for 2 hours ECs are still able to form new cell–cell contacts.
Here, we extend these findings by determining the effects of
inhibition of Rho kinase for longer periods on junctional
integrity and evaluation of endothelial barrier function. Of
note, we chose our conditions such that allowed formation of
adherens junctions before we started the Rho kinase inhibitor
studies. Detailed analysis reveals a reduced peripherally-
localized VE-cadherin expression and an impaired endothe-
lium barrier when Rho kinase is inhibited.

Several scenarios exist for how Rho kinase activity might
contribute to a proper barrier function. Rho kinase results in
phosphorylation of ERM proteins via inactivation of MP,
and activated ERM proteins anchor the cortical F-actin cytoskel-
eton properly to the plasma membrane. A proper plasma
membrane anchorage is essential to develop actomyosin
tension, which is required for correct recruitment of adherens
junctonal components. In addition, recent data indicate that
ERM proteins can activate Rac1, and might therefore
contribute to Rac1-mediated barrier protection.

A more likely scenario, however, is that Rho kinase plays a
role in proper recycling of VE-cadherin to EC junctions.
The VE-cadherin interaction with the F-actin cytoskeleton
has a very dynamic nature. Reduced VE-cadherin recycling
recently was shown to play an important role in VEGF-
enhanced endothelial permeability. Furthermore, Rho ki-
nase has been implicated in endosomal trafficking. There-
fore, we propose that impaired VE-cadherin endosomal
recycling results in enhanced VE-cadherin degradation in
Y-27632–treated cells.

The dual regulation of Rho kinase by thrombin has several
implications. First, these findings provide a warning for the single
use of quantitative Western blotting to measure Rho kinase
activity by surrogate markers like phospho-MYPT1, as concu-
current alternative subcellular activities are masked. Second, it
indicates that timing and subcellular targeting are important
when developing pharmacological agents to inhibit vascular
leak. Therefore, our findings warrant attention to the time
window for treatment with Rho kinase interfering drugs of
patients. Although the negative effects of longer incubations
with Rho kinase inhibitors on basal barrier integrity did not
outweigh the positive effects in reducing the thrombin re-
sponse, these data indicate that—in contrast to what has been
thought—inhibition of Rho kinase might negatively influence
endothelial barrier function in the long run.

In conclusion, those data reveal a dual role for Rho
kinase–mediated MP inactivation in the regulation of barrier
integrity.

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Disclosures
None.

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Figure I
Figure II
Figure III
Figure IV: Control vs. Y-27632

A) Images showing control (left) and Y-27632 (right) conditions.
B) Bar graph indicating the junctional phospho-MYPT1 (% of control) with a significant decrease observed in Y-27632 condition.
Phospho-MLC

Figure V
Figure VII

A

Control 30' Y-27632 24 hr Y-27632

B

<table>
<thead>
<tr>
<th>IP: Beta-catenin</th>
<th>IP: NS</th>
<th>IP: VE-Cadherin</th>
<th>WCL</th>
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<tr>
<td>IB: MYPT 130 kDa</td>
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<tr>
<td>IB: VE-Cadherin 130 kDa</td>
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<tr>
<td>IB: β-catenin 90 kDa</td>
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Figure VIII

A

MOCK  siROCK-I  siROCK-II  siROCK-I+II

B

IB: ROCK-I  IB: ROCK-II  IB: VE Cad

Figure VIII
Online supplement

Materials

Medium 199 supplemented with 20 mmol/L HEPES, L-glutamine and penicillin/streptomycin were obtained from Biowhittaker (Verviers, Belgium); 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 was obtained from a local blood bank and was prepared from 10 to 20 healthy donors, pooled, and stored at 4°C. Human serum albumin (HSA) was from Sanquin CLB (Amsterdam, The Netherlands). Trypsin was purchased from Gibco, heparin, histamine, and thrombin from Leo Pharmaceutical Products (Weesp, The Netherlands). FITC- and HRP-labeled secondary antibodies were from Dako (Ostrup, Denmark). Rhodamine phalloidin was from Molecular Probes (Eugene, Oregon). Anti panMYPT1 antibody was a kind gift from Prof. Y Takuwa, Kanazawa, Japan. Anti-phosphoMYPT1 (T696) antibody was from Upstate (Cambridge, UK). Anti-eNOS and anti-phospho-MLC (S18/T19) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). VE-cadherin and β-catenin antibodies were from Sigma. Y-27632 was obtained from Calbiochem (Amsterdam, The Netherlands).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated, cultured, and characterized as previously described.2,3 ECs were cultured on fibronectin- or gelatin-coated dishes in Medium 199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% human serum, 10% heat-inactivated NBCS, 150 μg/ml crude ECGF, 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. Before all experiments cells were washed once with Medium 199 and preincubated for 1 hour in Medium 199 + 1% HSA.

HUVEC transfections

For studies of RNA interference, transfections were performed with ROCK-I– and ROCK-II–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). At 48 hours after siRNA transfection,
endothelial barrier function was evaluated or cells were harvested to determine the level of ROCK-I and ROCK-II proteins by immunoblotting.

**Western blotting**

Proteins were separated by gel electrophoresis, blotted onto a nitrocellulose membrane, and stained with the indicated primary antibodies. Proteins were detected by chemiluminescence 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).

**3D Digital imaging microscopy**

EC were fixed with 2% formaldehyde in PBS for 10 minutes at room temperature, and stained for VE-cadherin and MYPT1. The presence of F-actin was visualized by direct staining with rhodamine-phalloidin. Immunostained 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. 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 Slidebook™ software (Intelligent Imaging Innovations, Denver, CO). Images were taken with a custom 40x air and 63X oil lens (ZEISS). The data acquisition protocol included confocal optical planes to obtain 3D definition, followed by a constrained iterative deconvolution operation of the images. For quantification of MYPT1 phosphorylation, green pixel intensity was expressed as percentage green pixels of total pixels in a region of interest using the mask tool of the Slidebook™ software.
Junctional protein accumulation assay

F-actin/MYPT1/VE-cadherin accumulation at cell contacts was measured using the line scan function in Slidebook™ software to quantitate the respective fluorescence intensities at cell-cell contacts. In short, a line perpendicular to the cell periphery was drawn through each contact, and the average pixel intensity along the line was determined. Area under the curve was determined using AIDA software.

Evaluation of the barrier function

For the evaluation of the barrier function, confluent monolayers of HUVEC (first and second passage) were released with trypsin-EDTA and seeded in high density on fibronectin-coated polycarbonate filters of the Transwell™ system 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. Preceding the HRP passage, monolayers were preincubated with the Rho kinase inhibitor Y-27632 for the indicated time periods. Alternatively, transendothelial electrical resistance (TEER) was measured as described previously.

Assay of RhoA and Rac1 activity

Activity of RhoA was measured with a RhoA G-LISA and activity of Rac1 with a pulldown Rac activation assay biochem kit according to the manufacturer’s protocol (Cytoskeleton Inc., Denver Colorado, USA).

Quantification of apoptosis

The cells were grown in a 24-well chamber on gelatin-coated glass cover slips. Cells were fixed in 4% formaldehyde and then permeabilized with 0.2% triton X-100. Cells were stained for the nucleus with Vectashield®. Apoptosis on each glass cover slip was quantified using a DeadEnd™ Fluorometric TdT-mediated dUTP Nick End Labeling (TUNEL) System from Promega Corporation (Madison, Wisconsin, USA) with 3D fluorescence microscopy and Slidebook software. The number of apoptotic nuclei (TUNEL-positive) and the total number of nuclei (DAPI-positive) were counted in three nonoverlapping microscope fields/glass cover slip, on 10x enlargement and averaged. The number of apoptotic nuclei was expressed as percentage of the total number of nuclei.
Rat renal arteries

The investigation conforms to the Guide for the Care and Use of laboratory animals published by the US National institutes of health (NIH Publication # 85-23). The ethics committee for animal experiments (DEC) at the Vrije Universiteit of Amsterdam approved the procedures.

Wistar rats were anesthetized with sodium pentobarbital (70 mg/kg i.p.). Similar-sized first-order side branches of the right renal artery, pointing cranially and caudally, respectively, were dissected free and a segment (1.5–2 mm in length, 0.3 mm inner diameter) was cut from each vessel. The arteries were dissected at 4°C in MOPS buffer consisting of (in mM) 145 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgSO$_4$, 1 Na$_2$H$_2$PO$_4$, 5 D-glucose, 2 pyruvate, 0.02 EDTA and 3 mM 3-(N-morpholino)propanesulphonic acid.

Both ends of the segments were tied around glass cannulas (outer diameter approximately 0.6 mm) in a pressure myograph consisting of a temperature-controlled, glass-covered chamber, a thermistor and a heating coil for temperature control. One cannula was connected to a reservoir to pressurize the vessel. The vessels were filled with MOPS and superfused with a physiological Krebs solution consisting of (in mM) 110 NaCl, 5 KCl, 2.5 CaCl$_2$, 1 MgSO$_4$, 24 NaHCO$_3$, 1 KH$_2$PO$_4$, 0.02 EDTA and 10 dextrose which was equilibrated with 95% air, 5% CO$_2$ at 37°C to yield pH of 7.4, pO$_2$ of 150 mmHg and pCO$_2$ of 35 mmHg. Throughout the protocol, the transmural (inside minus outside) pressure was maintained at 100 cm H$_2$O (75 mm Hg) and flow through the vessel was zero. Segments were equilibrated at their in vivo length for 30 min before starting the experimental protocols. At the end of the experiment, the vessels were perfusion fixed for 30 min at a pressure of 100 cm H$_2$O with ice-cold 2% formaldehyde (in MOPS), subsequently filled with Con A (20 µg/ml) + 1% BSA and incubated for 5 min (according to Barber and Antonetti 2003), and then perfused with Triton X-100 (0.05% in MOPS) for 30 seconds to permeabilize the endothelial cell membrane. The permeabilized endothelium was stained for 1 h with 0.264 microM Rhodamine Phalloidin (R-415, Molecular Probes Europe BV, Leiden, The Netherlands) and then washed again with MOPS. The fixation and staining were performed at room temperature.

To visualize the stained endothelial cells, the vessels were cut open longitudinally and mounted in Vectashield on a glass cover slip with the endothelial side facing upward. The portions of the vessel segment near the ties (about 10% of the length) were not used in the analysis because of possible damage of the endothelium.
Statistics

Data are reported as mean ± SD. Experiments were repeated for the times indicated. For basic statistical analysis SPSS software was used. Data were compared by a Student's t-test. P-values of less than 0.05 were considered to be significant.
Legends supplementary figures

Figure I MYPT1 in HUVECs
Expression of MYPT1 in HUVECs. Detection of MYPT1 by Western blotting. The estimated molecular mass of the double band was 130 kD.

Figure II Thrombin induces threonine phosphorylation of MYPT1 in a Rho kinase-dependent manner.
Quantitative analysis of MYPT1 phosphorylation. Monolayers were pretreated for 30 minutes with 10 microM Y-27632 (black bars) or vehicle (grey bars), and subsequently stimulated with 1 U/mL thrombin for 15 min when indicated. MYPT1-phosphorylation was measured as indicated in Materials & Methods. Values are the mean ± SD of 10 determinations in 2 different cultures. * p<0.05

Figure III Quantitative analysis of MYPT1 phosphorylation in junctional areas.
ECs were double-stained for F-actin and either panMYPT1 or phosphoMYPT1. The amount of junctional (phospho)MYPT1 was determined as the peak value of a line intensity scan as presented in figure 3 C-F, expressed as the ratio of the peak values of (phospho)MYPT1/F-actin. Phospho-MYPT1/F-actin of control ECs was arbitrary set at 1.0. Values are the mean ± SD of 6 determinations in 3 different cultures. * p<0.05

Figure IV MYPT1 phosphorylation in junctional areas is reduced by inhibition of Rho kinase.
A Inhibition of Rho kinase for 24 hr reduced MYPT1 phosphorylation in junctional areas. EC were incubated with 10 microM Y-27632 for 24 hours (left panel) or left untreated (right panel), fixated and stained phospho-MYPT1 (green).
B Quantitative analysis of MYPT1 phosphorylation in junctional areas was performed as indicated in the Materials & Methods section. Values are the mean ± SD of 6 determinations in 2 different cultures. * p<0.05

Figure V MLC-2 phosphorylation in junctional areas is reduced by inhibition of Rho kinase.
Panel A-C: EC were left untreated (panel A), incubated with 10 microM Y-27632 for 24 hours (panel B) or stimulated with thrombin (1U/mL) for 30 minutes (panel C), fixated and stained for phospho-MYPT1 (green) and nuclei (blue). Panel D: control for non-specific binding of secondary antibody.
**Figure VI** Prolonged inhibition of Rho kinase reduces trans endothelial electrical resistance (TEER)

Effect of treatment with Rho kinase inhibitors Y-27632 (10 microM), Fasudil (10 microM), and H-1152 (1 microM) for 24 hours on basal TEER.

**Figure VII** Inhibition of Rho kinase interferes with VE-cadherin at cell-cell contacts.

A: ECs were incubated for 30 minutes (middle panel), 24 hours with Y-27632 (right panel) or left untreated (left panel) and subsequently stained for VE-cadherin (green) and nuclei (blue). Representative lines for quantitative analysis are shown. Arrow in right panel points to small gap between to neighboring ECs. Bar, 10 μm.

B: MYPT1 does not form a stable complex with the junctional proteins VE-Cadherin or β-catenin. A representative immunoblot is shown of MYPT1, VE-cadherin and β-catenin of HUVECs (n=3). Cells were preincubated for 1 hour in medium 199 + 1% HSA. VE-cadherin or β-catenin was immunoprecipitated (IP) from whole cell lysates (WCL). MYPT1 expression in the lysates was measured by western blotting with MYPT1 antibody (IB: MYPT1, upper panel). VE-cadherin expression in the lysates was measured by western blotting with VE-cadherin antibody (IB: VE-cadherin, lower panel). β-catenin expression was measured by β-catenin antibody. Note that VE-cadherin co-immunoprecipitates with β-catenin and vice versa, indicating that they form a stable complex, but that MYPT1 is not detectable in this complex. Normal mouse IgG (NS) was used as a negative control for nonspecific IP of MYPT1, VE-cadherin and β-catenin. 1/10 of control whole cell lysate (WCL) was used as a positive control for immunoblotting.

**Figure VIII** Targeting of Rho kinase by siRNAs interferes with VE-cadherin at cell-cell contacts.

A: ECs were transfected with ROCK siRNAs. After 48 hrs, cells were fixated and stained for VE-cadherin (green) and nuclei (blue).

B: Representative Western blot showing in the lower panel reduced VE-cadherin protein levels after transfection with ROCK-I and –II siRNAs. Upper and middle panel; the same blot was reprobed with ROCK-I (upper) and ROCK-II (middle) antibody to verify reduced ROCK expression upon siRNA transfection.
Reference List


