3 Involvement of Rho kinase in endothelial barrier maintenance.

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Abstract

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. Here, we investigated the contribution of basal Rho kinase activity to the regulation of endothelial barrier integrity.

Using a phospho-specific antibody against the myosin phosphatase targeting subunit (Thr^{696}-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.

Rho kinase has opposing activities in regulation of the 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 anti-vascular leak therapy.
Introduction

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. Vascular leakage may also cause circulatory collapse in sepsis and contribute to intra-vitreous scar formation in diabetic retinopathy leading to blindness. The permeability of tumor vessels is well documented in tumor models and in human cancer, having implications for metastasis. 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 sub-endothelial 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) Ca$^{2+}$-dependent activation of myosin light chain kinase, (2) a RhoA/Rho kinase-signaling pathway, (3) a protein tyrosine kinase/phosphatase pathway that enhances disruption of intercellular junctions and (4) a new pathway that involves protein kinase C zeta.

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 acto-myosin contractility, which facilitates the breakdown of intercellular junctions causing barrier dysfunction. 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. 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. More recently, the barrier-stabilizing properties of cAMP-activated small GTPase Rap1 were
discovered. 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 the 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. Myosin phosphatase (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 upon 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 sub-cellular distribution of Rho kinase activity under basal conditions was compared to 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 & Methods

Sources of reagents are listed in the expanded Materials and Methods section in the online data supplement section (http://atvb.ahajournals.org or
CD chapter 3). Human umbilical vein endothelial cells (HUVECs) were cultured as previously described. HUVECs 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 horse radish peroxidase (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 Marianas™ 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’s 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 Thr696 of MYPT1 (phosphoMYPT1) 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 HUVEC was detected by Western blotting, demonstrating a double band of ~130kDa (please see supplemental Figure I, CD chapter 3), 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 phosphoMYPT1 after stimulation with thrombin for 30 minutes (please see supplemental Figure II, CD chapter 3). 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 phosphoMYPT1 serves as a proper surrogate marker for Rho kinase inhibition.

To determine the sub-cellular localization of phosphoMYPT1 in HUVEC in detail, we used wide-field 3D-deconvolution fluorescence microscopy. High power magnification demonstrated under control conditions a punctate
cytoplasmic distribution pattern enriched in peri-nuclear areas that did not co-localize with the fine cytoplasmic F-actin meshwork in ECs (Fig. 1A and enlargement of its white box in Fig. 1C; the accompanying line intensity scan is presented at the bottom of panel C). In thrombin-stimulated cells phosphoMYPT1 decorated F-actin SFs (Fig. 1B). This suggests that inhibition of MP in thrombin-stimulated cells contributes to contractile properties of SFs.

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, phosphoMYPT1 was visible as a fine peripheral lining (enlargements of the yellow boxes in Fig. 1E). As can be derived from the line intensity scan (see Fig. 1E) cortical phosphoMYPT1 perfectly co-localized with F-actin. To our surprise and in contrast to the global cellular increase in phosphoMYPT1 by thrombin (please see supplemental Figure II, CD chapter 3), phosphoMYPT1 staining was lower in junctional areas in thrombin-stimulated cells (see enlargement of the yellow box in Fig. 1F). Quantification confirmed that thrombin reduced phosphorylation of MYPT1 at cell-cell contacts significantly (please see supplemental Figure III, CD chapter 3). Of note, thrombin did not influence the total amount of MYPT1 in these areas.
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Figure 1. Subcellular distribution of phospho-MYPT1 in control and thrombin-stimulated HUVECs.

A, B) Double staining for phosphoMYPT1 (green) and F-actin (red) of a control EC shown in panel A and ECs stimulated with 1 U/mL thrombin for 30 min shown in panel B. Nuclei were stained with DAPI (blue). Yellow boxes represent junctional areas and white boxes cytoplasmic areas. Boxes are enlarged in panel C-F. Images were obtained by the 3D mode of the microscope; 70 slices were taken with 0.1 μm-increments in the z-axis. From these stacks, a single optical section with the junctions in focus is shown. Bar, 10 μm. C, D) PhosphoMYPT1 in junctional areas. Images represent enlargements of the indicated white boxes in panels A and B. First row of pictures in panel C and D are raw images straight from the camera, second row represent same images but after application of deconvolution technique to reduce out-of-focus light and improve signal-to-noise ratio. Graphs at the bottom represent line intensities of the indicated lines for F-actin (red) and phosphoMYPT1 signals (green). E, F) PhosphoMYPT1 in cytoplasmic areas. Images represent enlargements of the indicated white boxes in panels A and
B. For sake of clarity only deconvolved images are presented. Graphs at the bottom represent line intensities of the indicated lines for F-actin (red) and phosphoMYPT1 signals (green).

To verify these findings in intact vessels, rat renal arterioles were isolated, cannulated and perfused with thrombin. Staining of ECs in situ confirmed the co-localization of phosphoMYPT1 with cortical F-actin in control vessels (Fig. 2 arrow heads). In addition, enhanced MYPT1 phosphorylation associated with central F-actin filaments was observed after exposure of intact vessels to thrombin (Fig. 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 phosphoMYPT1 was quantitated (please see supplemental Figure IV, CD chapter 3). Treatment with Y-27632 markedly reduced junctional phosphoMYPT1. 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 (please see supplemental Figure V, CD chapter 3), and reduced upon treatment with Y-27632. Thrombin stimulation enhanced phospho-MLC-2 mainly at SFs, but reduced cortical phospho-MLC-2.

In conclusion, visualization of Rho kinase activity at the sub-cellular level reveals regional differences in Rho kinase activity. In post-confluent ECs basal Rho kinase activity colocalized with the cortical rim of F-actin, but did not co-localize 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 SFs, whereas cortical Rho kinase activity was decreased in thrombin-stimulated ECs.
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Figure 2. MYPT1 phosphorylation in ECs of intact rat arterioles.

Immuno-cytochemical staining for phosphoMYPT1 (upper row in green) and F-actin (second row in red) or both (third row; yellow indicates colocalization) in ECs in intact arterioles. Nuclei are stained with DAPI (blue). Vessels were stimulated with thrombin (right column) or left untreated (left column). Bar, 10 μm. For identification purposes, outlines of individual ECs are presented in the lower row.

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 post-confluent 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.

Figure 3. Inhibition of Rho kinase has opposing effects on basal and thrombin-enhanced HRP passage across HUVEC monolayers.

A) Schematic representation of experimental protocol: HUVEC monolayers grown on porous filters were preincubated with 10 μM Y-27632 for 30 min, 24 hours and 96 hours as indicated in panel B and subsequently a HRP transfer assay was performed in control and thrombin-stimulated monolayers in the absence or presence of Y-27632. HRP passage during a 1-hour period was measured. B) Inhibition of Rho kinase increases basal HRP passage and reduces thrombin-enhanced HRP passage. HUVEC monolayers were incubated for the indicated time periods with 10 μM Y-27632 and HRP passage during a 1-hour period was subsequently measured under basal conditions (filled bars) and after stimulation with 1 U/mL thrombin (hatched bars). Delta represents the thrombin-induced HRP passage that remains after inhibition of Rho kinase with Y-27632 (6 determinations in 2 different cultures). * p<0.05 basal HRP passage of monolayers pretreated with Y-27632 for 24 or 96 hours versus basal HRP passage of control monolayers. # p<0.05 thrombin-enhanced HRP passage of monolayers pretreated with Y-27632 for 24 or 96 hours versus thrombin-enhanced HRP passage of control monolayers.
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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 (please see supplemental Figure VI, CD chapter 3). 24 Hour pretreatment with the structurally unrelated Rho kinase-inhibitors fasudil (10 μM) and H-1152 (1 μM) 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 (Fig. 4, inset). Targeting both Rho kinase isoforms by siRNA significantly reduced TEER (Fig. 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 Fig. 3B). The remaining increase in HRP passage reflects the Rho kinase-independent hyperpermeability response of thrombin (indicated with ∆ in Fig. 3B). The Rho kinase-independent increase in endothelial permeability upon 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 vs. 0.109 ± 0.061, control vs. 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 vs. 1.26 ± 0.25, control vs. Y-2763-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, and altered eNOS activity results in alterations of barrier function. However, eNOS expression
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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 TUNEL assay (1.9 ± 1.3% vs. 1.3 ± 0.7%, control vs. 24 hr. 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 (please see supplemental Figure VIIA left panel, CD chapter 3). 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 (please see supplemental Figure VIIA right panel). These findings were not observed after inhibition of Rho kinase for 30 minutes (Figure VIIA middle panel). Quantitative analysis confirmed that less VE-cadherin was accumulated in junctional areas after Rho kinase inhibition for 24 hours (Fig. 5), or after targeting both Rho kinase isoforms by siRNA (please see supplemental Fig VIIA right panel, CD chapter 3).

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; Fig. 5). Targeting both Rho kinase isoforms by siRNA similarly reduced VE-cadherin expression, whereas targeting the single isoforms had no effect (please see 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 (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).\textsuperscript{21}

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

The major findings of the present study are that for the first time we demonstrate that Rho kinase has a dual role in the regulation of endothelial barrier function with opposite effects: Rho kinase has (1) an intrinsic activity...
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at cell margins that is essential for proper barrier integrity, and (2) an induced activity at SFs 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 ROCK-I/II 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, that 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.

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 isoforms. Remarkably, single targeting of ROCK-II even improved barrier function, suggesting that this is the isoform that is mainly responsible for the barrier disruptive effects. Indeed, it was shown in epithelial that ROCK-II, but not ROCK-I mediates disassembly of the junctions. In ECs ROCK-II, but not ROCK-I has been implicated in microparticle generation. These specific functions require further investigation.
In epithelial cells Rho kinase was necessary for the local concentration of E-cadherin in cell-cell contacts. 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 endothelial 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 cytoskeleton properly to the plasma membrane. A proper plasma membrane anchorage is essential to develop acto-myosin tension, which is required for correct recruitment of adherens junction 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 kinase has been implicated in endosomal trafficking. Therefore, 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, this data provides a warning for the single use of quantitative Western blotting to measure Rho kinase activity by surrogate markers like phosphoMYPT1, as concurrent opposite subcellular activities are masked. Second, it indicates that timing and sub-cellular 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 response, 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.
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In conclusion, those data reveal a dual role for Rho kinase-mediated MP inactivation in the regulation of barrier integrity.

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