Thrombin-induced endothelial barrier disruption in intact microvessels: role of RhoA/Rho kinase-myosin phosphatase axis

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van Nieuw Amerongen GP, Musters RJ, Eringa EC, Sipkema P, van Hinsbergh VW. Thrombin-induced endothelial barrier disruption in intact microvessels: role of RhoA/Rho kinase-myosin phosphatase axis. Am J Physiol Cell Physiol 294: C1234–C1241, 2008. First published March 19, 2008; doi:10.1152/ajpcell.00551.2007.—Endothelial hyperpermeability is regulated by a myosin light chain-2 (MLC2) phosphorylation-dependent contractile mechanism. Thrombin is a potent inducer of hyperpermeability of cultured monolayers of endothelial cells (ECs) via Rho kinase-mediated MLC2-phosphorylation. The aim of the present study was to investigate the effects of thrombin on in situ endothelial morphology and barrier integrity. Cytoskeletal dynamics, regions of paracellular flux, and MLC2-phosphorylation of ECs were visualized by digital three-dimensional imaging microscopy of pressurized rat kidney arterioles. Myosin phosphatase targeting subunit (MYPT1)-phosphorylation was used as a surrogate marker for Rho kinase activity. Thrombin induced the formation of F-actin filaments in ECs in situ and rounding of the ECs in the absence of obvious formation of gaps between ECs. These changes were accompanied by an increase in MLC2 phosphorylation and a decrease in barrier integrity. In vitro analysis revealed that Rho kinase activity on F-actin filaments was associated with a contractile response that enhanced opening of the barrier. Rho kinase activity was not detectable on F-actin filaments induced by histamine, an inducer of a more transient hyperpermeability response. Inhibition of the myosin phosphatase mimicked the effects of thrombin on barrier function. The thrombin-induced changes in in situ MLC2 phosphorylation and barrier function were Rho kinase dependent. These data demonstrate a direct effect of thrombin on EC morphology and barrier integrity in intact microvessels. Furthermore, they establish an important contribution of enhanced Rho kinase activity to the development of prolonged but not transient types of endothelial barrier dysfunction.

vascular biology; three-dimensional digital imaging; cytoskeleton; stress fibers

INCREASED ENDOTHELIAL PERMEABILITY is a vascular reaction to many inflammatory and angiogenic stimuli, resulting in extravasation of fluid, solutes, and macromolecules. Increased vascular leakage contributes to the pathogenesis of numerous, often life-threatening, disorders. For instance, excessive plasma extravasation may aggravate acute life-threatening obstruction of respiratory airways during asthma and related pulmonary disorders (for a review, see Ref. 16). Vascular leakage may also cause circulatory collapse in sepsis. It may contribute to intravitreal scar formation in diabetic retinopathy leading to blindness (33).

Endothelial barrier function is maintained principally by cytoskeletal elements that determine cell shape, facilitate cell adhesion to subendothelial matrix, and participate in the formation of junctional complexes. A major cause of vascular leakage under inflammatory conditions is the loss of endothelial cell (EC) junction integrity, which is accompanied by the formation of small gaps between ECs and disturbed barrier function of the endothelial monolayer. In vitro studies on thrombin-induced endothelial hyperpermeability have identified at least four independent signaling pathways that contribute to barrier dysfunction: 1) Ca2+-dependent activation of myosin light chain kinase kinase (44); 2) a RhoA/Rho kinase-signaling pathway in a Ca2+-independent manner (12, 38); 3) a net increase in protein tyrosine kinase activities at the cell margin accompanied by disruption of intercellular junctions (21a, 37); and 4) a new pathway that involves the atypical protein kinase C isoform PKCζ (22).

A striking feature of activation by thrombin is the formation of cytoplasmic stress fibers in cultured cells. Similarly, the formation of stress fibers is enhanced by shear forces exerted on ECs by flowing blood (13). Stress fibers are long cytoskeletal cables of F-actin bundles and myosin II/nonmuscle myosin filaments that can contract and exert tension and are linked to the plasma membrane at focal adhesions (20). Myosin-II is believed to be involved in the generation of contractile forces. Its activity is mainly controlled by its light chain (MLC2) phosphorylation, which is regulated by two classes of enzymes: MLC kinases and myosin phosphatases. MLCK, and Rho kinase are the two major MLC kinases, but others exist as well.

A type 1 myosin-associated phosphatase activity has been implicated in 1995 in the regulation of EC gap formation in vitro (40), and pharmacological inhibitor studies suggested its importance in endothelial contractility (21). Smooth muscle MLC2 phosphatase is a holoenzyme 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 two isoforms M130/133 exist and a 20-kDa small subunit of unknown function (17). MYPT1 is the major regulatory subunit, because it binds both PP1c and phosphorylated myosin-II, thus targeting the substrate to the catalytic core. Several kinases phosphorylate MYPT1 and inactivate the myosin phosphatase, including P21-activated kinase and Rho kinase (43). The expected consequence is greater MLC2 phosphorylation and thereby increased contractility. Recently, MYPT1 phosphorylation has been demonstrated to occur upon stimulation with thrombin in cultured pulmonary ECs (4), in agreement with an earlier report of transient decreased myosin phosphatase activity (12).
Despite the wealth on in vitro data available about the mechanisms of thrombin-enhanced endothelial permeability, remarkably little is known about the in vivo hyperpermeability response by thrombin. Initial data indicated that thrombin evokes pulmonary vascular leakage in awake sheep (23). Similarly, in isolated mouse lungs thrombin induces lung edema (32). Furthermore, it has been reported that a high dose of antithrombin III can reduce endotoxin-induced lung hyperpermeability and vascular leakage caused by ischemia-reperfusion injury in animals (11, 29). This suggests an important role for thrombin in specific forms of altered barrier function. Recently, we reported that thrombin enhanced MYPT1 phosphorylation in intact vessels, indicating that thrombin might exert direct effects on vascular barrier function (36). However, the in vivo observations on thrombin-induced vascular leakage has not been linked fully to the in vitro thrombin effects (42).

In the present study, we investigated the effects of thrombin on in situ endothelial morphology and barrier integrity. To clarify the contribution of the morphology changes, we compared the effects of two hyperpermeability inducers histamine and thrombin with different effects on the F-actin cytoskeleton and related this to their distinct hyperpermeability responses. Subsequently, phosphorylation of myosin phosphatase was used as a surrogate marker for Rho kinase activity to investigate subcellular distribution of Rho kinase activity under thrombin-stimulated conditions. Finally, we verified the effects of thrombin-induced Rho kinase activity on barrier function of intact vessels using a specific Rho kinase inhibitor.

MATERIALS AND METHODS

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 was prepared from bovine hypothalamus as described by Maciag et al. (25). 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 Sanguin CLB (Amsterdam, The Netherlands). Trypsin was purchased from Gibco, heparin, histamine, and thrombin from Leo Pharmaceutical Products (Weesp, The Netherlands). FITC- and horseradish peroxidase-labeled secondary antibodies were from Dako (Glostrup, Denmark). Rhodamine phalloidin was from Molecular Probes, and Alexa Fluor 488 conjugated-concanavalin A (ConA) was from Molecular Probes (Eugene, OR). Anti-phospho-MYPT1 (T696) antibody was from Upstate (Cambridge, UK). Anti-RhoA and anti-phospho-MLC (S18/T19) were from Santa Cruz. Y-27632 was from Tocris (Bristol, UK).

Rat renal arteries. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23). The ethics committee for animal experiments (DEC) at the Vrije Universiteit of Amsterdam approved the procedures.

Wistar rats were anesthetized with pentobarbital sodium (70 mg/kg ip). 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, as described before (41). The arteries were dissected at 4°C in MOPS buffer consisting of (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄, 5 d-glucose, 2 pyruvate, 0.02 EDTA, and 3 mM 3-(N-morpholino)propanesulfonic acid (pH 7.4).

Both ends of the segments were tied around glass cannulas (outer diameter ~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 CaCl₂, 1 MgSO₄, 24 NaHCO₃, 1 KH₂PO₄, 0.02 EDTA, and 10 dextrose, which was equilibrated with 95% air-5% CO₂ at 37°C to yield pH of 7.4, PO₂ of 150 mmHg and PCO₂ of 35 mmHg. Throughout the protocol, the transmural (inside – outside) pressure was maintained at 100 cmH₂O (75 mmHg), and flow through the vessel was zero. Segments were equilibrated at their in vivo length for 30 min before the start of the experimental protocols. At the end of the experiment, the vessels were perfusion fixed for 30 min at a pressure of 100 cmH₂O with ice-cold 2% formaldehyde (in MOPS), subsequently filled with ConA (20 μg/ml) + 1% bovine serum albumin and incubated for 5 min (according to Barber and Antonetti in Ref. 3), washed and then perfused with Triton X-100 (0.05% in MOPS) for 30 s to permeabilize the endothelial cell membrane. The permeabilized endothelium was stained for 1 h with 0.264 μM rhodamine phalloidin (R-415, Molecular Probes Europe, Leiden, The Netherlands) and then washed again with MOPS. The fixation and staining were done at room temperature.

To visualize the stained endothelial cells, the vessels were cut open longitudinally and mounted in Vectashield containing 4,6-diamidino-2-phenylindole on a glass coverslip with the endothelial side facing upward. Small variations in the flatness of the specimen in some cases resulted in not all of the cells being in focus in one photomicrograph. The portions of the vessel segment near the ties (~10% of the length) were not used in the analysis because of possible damage of the endothelium.

To validate the use of ConA as a marker for dysfunction of the endothelial barrier, the endothelium was damaged by gentle perfusion of an air bubble. At sites where the endothelium was removed, as evidenced by absence of F-actin staining, ConA binding increased dramatically (data not shown), whereas intact microvessels had a very low level of ConA binding. Subsequently, we analyzed ConA localization by taking Z stacks of a vessel stimulated with thrombin for 2 min (supplemental Fig. 1). ConA was not observed at the luminal side of the endothelium, but appeared in small spots just below the level of the endothelial nuclei. ConA spots were often closely located near the peripheral F-actin band. ConA did not penetrate deeper into the vessel wall.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated, cultured, and characterized as previously described (18, 37). 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 mg/ml crude endothelial cell growth factor, 2 mmol/l L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C under 5% CO₂-95% air atmosphere. Before all experiments, cells were washed once with medium 199 and reincubated for 1 h in medium 199 + 10% HSA.

Evaluation of the barrier function in vitro. 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 (Amersham). Medium was renewed every other day. Monolayers were used between 4 and 6 days after seeding. Transendothelial electrical resistance (TEER) was measured as described previously (37).

Western blot analysis. Proteins were then separated by gel electrophoresis, blotted onto a nitrocellulose membrane, and stained with the indicated primary antibodies. Proteins were detected with a chemiluminescence kit 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).
Rho activity assay. Rhotekin-binding assays were essentially performed as indicated by the manufacturer (Cytoskeleton, Denver, CO) (38). Briefly, 30-cm² confluent HUVEC were preincubated for 1 h in medium 199 + 1% HSA. Cells were stimulated and lysed. Lysates were cleared by centrifugation and incubated with GST-RBD (where RBD stands for the Rho-binding domain of Rhotekin and GST for glutathione-S-transferase) immobilized on glutathione-coupled Sepharose beads for 30 min at 4°C. Beads were washed, eluted in Laemmli sample buffer, and analyzed by Western blot analysis using a rabbit polyclonal anti-RhoA antibody.

Three-dimensional digital imaging microscopy. Digital imaging microscopy was performed essentially as described before (35). In short, HUVECs were examined with a ZEISS Axiovert 200 Mariana inverted microscope, equipped with a motorized stage (stepper-motor z-axis increments: 0.1 μm). A cooled CCD camera (1280 × 1024 pixels; Cooke Sensicam, Cooke, Tonawanda, NY) recorded images with true 16-bit capability. The camera is linear over its full dynamic range (up to intensities of over 4,000), whereas 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/controlled by Slidebook software (Intelligent Imaging Innovations, Denver, CO). Images were taken with a custom ×40 air and ×63 oil lens (Zeiss). The data acquisition protocol included confocal optical planes to obtain three-dimensional definition, followed by a constrained iterative deconvolution operation of the images. F-actin-MYPT1 colocalization was determined by using the line scan function in Slidebook software to quantitate the respective fluorescence intensities along the indicated lines.

Statistics. Comparisons between two groups were done by Students’ t-test. Data are means ± SD. A value of P < 0.05 was accepted as a significant difference.

RESULTS

Response of intact vessels to thrombin. To investigate whether thrombin affects barrier function in intact vessels, we isolated and cannulated rat renal arterioles and rat gracilis muscle venules and perfused them with thrombin. Vessels were fixated and stained for F-actin.

Endothelial cells of rat renal arterioles in situ were characterized by a dense peripheral band of F-actin and a cytoplasm almost void of F-actin similar to cultured confluent ECs (Fig. 1A). Cells were elongated in the direction of the flow. Occasionally, a few cytoplasmic F-actin bundles were observed. Stimulation with thrombin for 30 min induced a reorganization of the F-actin cytoskeleton. Many F-actin filaments were formed, which in general were less pronounced and shorter than those in cultured ECs stimulated with thrombin (compare top and bottom right of Fig. 1A). In addition, the elongated EC morphology was changed upon stimulation with thrombin (Fig. 1B and supplement Fig. 5).

In contrast to cultured ECs, where thrombin induces the formation of large gaps between ECs at a large scale, only tiny gaps between ECs sporadically were observed in situ after stimulation with thrombin (see also Fig. 1A). Therefore, we wondered whether thrombin induces alterations in barrier function of intact vessels. Vessels were stimulated with thrombin and fixated with paraformaldehyde. Barrier integrity at time of fixation was assessed by using fluorescently labeled ConA, a lectin that binds to the exposed subendothelial matrix when it has passed the endothelial barrier (see also supplemental Fig. 1). Simultaneously, alterations in phosphorylation of...
MLC2 were visualized. In a time course experiment in which rat renal arterioles were stimulated for 2, 10, and 30 min with thrombin, thrombin induced a rapid MLC2 phosphorylation with a maximum at 2 min, which remained elevated for at least 30 min, paralleled by an increase in ConA binding, indicative for a disturbed endothelial barrier (supplemental Fig. 2A). Similarly, MLC2 phosphorylation was elevated by thrombin in rat gracilis muscle venules (supplemental Fig. 2B), whereas low levels of MLC2 phosphorylation were observed in control vessels.

When taken together, these data indicate that thrombin induced a hypermeability response in intact isolated vessels. Specifically, thrombin induced a mild rounding of ECs in situ, which was accompanied by the formation of F-actin filaments, and reduced endothelial barrier integrity.

**Contribution of RhoA/Rho kinase-myosin phosphatase axis to morphology changes of ECs.** The observed rounding of ECs in situ exposed to thrombin accompanied by the formation of F-actin filaments suggested that an actomyosin-based reaction might underlie the thrombin-induced changes in barrier integrity. This was further investigated in HUVEC. The hyperpermeability inducer histamine was included in these experiments for comparison, because histamine, in contrast to thrombin, does not induce isometric tension and actomyosin-based contraction in these cells (14, 26).

Both thrombin and histamine induced the formation of robust long F-actin filaments, so-called stress fibers (supplemental Fig. 3A). The thrombin-induced stress fibers contained a much higher amount of phosphorylated MLC2 (Fig. 2A). This is probably related to the activation of RhoA (Fig. 2B) and Rho kinase (Fig. 2C), which only occurred in thrombin-stimulated cells. In line with these data, the thrombin-induced reduction in endothelial barrier function, as evidenced by a decrease of the transendothelial electrical resistance, was largely prevented by the Rho kinase inhibitor Y-27632 (supplemental Fig. 3B). In contrast, histamine caused only a small transient reduction in barrier function, independent of Rho kinase activity (supplemental Fig. 3B). This suggested that differences in actin-myosin interaction of the formed F-actin filaments underlie the respective hyperpermeability phenomena.

These data indicate that thrombin induced a robust inactivation of the myosin phosphatase on F-actin stress fibers via Rho kinase, resulting in elevated MLC2 phosphorylation on F-actin filaments and a prolonged hyperpermeability response.

**Inhibition of basal MP activity to disrupt endothelial barrier integrity.** To evaluate the effect of pharmacological inhibition of the myosin phosphatase on endothelial barrier function,

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Fig. 2. Differences and similarities between thrombin- and histamine-induced endothelial barrier dysfunction in vitro. A: thrombin-induced F-actin stress fibers have a higher level of myosin light chain II (MLC2) phosphorylation than histamine-induced stress fibers. HUVEC were stimulated with thrombin (left) or histamine (right) for 15 min and subsequently double stained for F-actin (bottom) of phosphorylated MLC2 (top) as indicated in MATERIALS AND METHODS. Bar, 10 μm. Similar effects were observed in 6 independent experiments. B: thrombin, but not histamine, activates RhoA. Top, immunoblot showing activation of RhoA in HUVEC after exposure to thrombin but not histamine for 1 min. Cell lysates were incubated with GST-RBD (where RBD stands for the Rho-binding domain of Rhotekin and GST for glutathione-S-transferase) beads as described in MATERIALS AND METHODS. The beads were washed, and the bound protein was analyzed by Western blot analysis using an antibody specific to RhoA. Bottom, loading control showing that equal amounts of total RhoA were present in all samples. Similar effects were observed in 3 independent experiments. C: Rho kinase is activated on thrombin-, but not histamine-induced F-actin stress fibers. To visualize the activity of Rho kinase on F-actin filaments, we made use of a phosphorylation site-specific antibody of the regulatory subunit of myosin phosphatase (MYPT1). Phosphorylation of T696 of MYPT1 by Rho kinase has been previously reported to inactivate the myosin phosphatase and to serve as a surrogate marker for Rho kinase activity (17). Pictures represent a double staining for phospho-MYPT1 (green) and F-actin (red) in confluent HUVECs. Nuclei are stained with DAPI (blue). Confluent ECs were treated with 1 U/ml thrombin for 30 min (left) or with 10 μM histamine (right). High-power magnification wide-field deconvolution fluorescence microscopy demonstrated that phospho-MYPT1 decorated thrombin-induced F-actin stress fibers, whereas such colocalization could not be observed with histamine-induced F-actin filaments. White boxes are enlarged as indicated by dotted lines. Bar, 10 μm. Graphs at the bottom represent line intensities of line I → II (thrombin) and line III → IV (histamine) for F-actin (red) and phospho-MYPT1 signals (green). Bar, 10 μm. Similar effects were observed in 3 independent experiments.
confluent HUVEC monolayers were grown on porous filters and TEER was measured (supplemental Fig. 4A). Treatment with low concentrations of the semiselective myosin phosphatase inhibitors calyculin A and cantharidin resulted in a decrease in TEER, mimicking the thrombin response. The drop in TEER by cantharidin and calyculin A was paralleled by an increase in MLC2 phosphorylation (supplemental Fig. 4B) and the formation of gaps between neighboring ECs (Fig. 3, asterisks). Similarly, inhibition of the myosin phosphatase by cantharidin in renal arterioles induced a profound reorganization of the F-actin cytoskeleton (Fig. 3), accompanied by enhanced MLC2 phosphorylation.

These data indicate that basal myosin phosphatase activity is essential for proper barrier function. Furthermore, they indicate that general inhibition of the myosin phosphatase disturbs the endothelial barrier, mimicking the response to thrombin.

The thrombin response in intact microvessels is Rho kinase dependent. To verify whether the thrombin response in intact vessels was mediated by Rho kinase, arterioles were pretreated for 30 min with Y-27632 (10 μM) stimulated with thrombin, and vessels were stained for MLC phosphorylation and ConA binding (Fig. 4, A and B). Stimulation with thrombin for 2 min induced a fivefold increase in phospho-MLC2 levels that was almost completely prevented by preincubation with Y-27632 (Fig. 4C). In addition, the thrombin-enhanced ConA binding was largely reduced by Y-27632 (Fig. 4D). Cell rounding after stimulation with 1 U/ml thrombin for 30 min (71 ± 11% of control length-to-width ratio) was completely prevented by pretreatment with 10 μM Y-27632 for 30 min (103 ± 9% of control length/width; 26 determinations in 4 independent experiments. See also supplemental Fig. 5).

**DISCUSSION**

A major finding of the present study is that for the first time we demonstrate that thrombin has a direct permeability-enhancing effect on healthy intact microvessels. Specifically, thrombin induced a rounding of ECs in situ, accompanied by a reorganization of the F-actin cytoskeleton, elevated phosphorylation levels of MLC2, and a loss of endothelial barrier integrity. In addition, we confirm that Rho kinase-mediated contractile events mediate thrombin-induced hyperpermeability responses.

In this study we compared the hyperpermeability response to thrombin of ECs in situ to the well-studied response of cultured ECs. Similar to cultured ECs, ECs in situ residing in freshly isolated intact vessels reacted to thrombin by a hyperpermeability response. This response was mediated by Rho kinase-dependent changes in the F-actin cytoskeleton. In a previous study, in rat mesenteric venules a thrombin response only could be evoked when there had been a previous inflammatory response or injury (9). Therefore, these authors suggested that the direct effects of thrombin on cultured ECs are an artifact reflecting a putative inflammatory-like status of ECs in vitro. Our findings indicate that it is more likely that the response to thrombin is vessel-type dependent, than being an in vitro artifact.

Besides the gross similarities, we observed some remarkable differences between the hyperpermeability response to thrombin of ECs in situ and that of cultured ECs. The most prominent differences that will be discussed below are 1) the hyperpermeability response in the intact vessels occurred in the absence of obvious formation of gaps between ECs, whereas under in vitro conditions gaps do form; 2) the duration of the hyperpermeability response was shorter in intact vessels than that in cultured monolayers; and 3) the barrier dysfunction of intact arterioles was to a larger extent dependent on Rho kinase-mediated signaling than the barrier dysfunction of cultured monolayers.

The profound gap formation induced by thrombin in vitro has led to discussions about the relevance of these studies for

**Fig. 3. Effect of pharmacological inhibition of myosin phosphatase on MLC2 phosphorylation and F-actin organization.** Effects of inhibition of myosin phosphatase on the F-actin of intact arterioles compared with cultured endothelial monolayers are shown. Cultured endothelial cells and intact vessels were stimulated with 1 μM cantharidin for 15 min or left untreated, fixated, and double-stained with rhodamine-phalloidin (in red) and an anti-phospho-MLC2 antibody (in green). Yellow arrows indicate the direction of the flow. *Gaps formed between endothelial cells. Similar results were observed upon stimulation with 10 μM cantharidin.
the in vivo situation. Here, we show that in the absence of such large gaps, thrombin stimulation nevertheless disturbs barrier integrity. Moreover, the finding that the length-to-width ratio of ECs in situ decreases upon stimulation with thrombin strongly suggests that thrombin induces a contractile response that also largely contributes to the hyperpermeability response in vitro. In agreement, Rho kinase activity was associated with F-actin filaments formed in situ, further supporting that cell rounding is caused by increased cellular contraction. Based on these data we conclude that the in vitro observed gap formation is not a mere artifact caused by poor adhesion to glass cover-slips needed for proper imaging but that it is a reflection of the changes in contractile status of the cells.

As evidenced by ConA binding, barrier function of intact vessels after disruption by thrombin was almost recovered within 30 min (supplemental Fig. 2), whereas the thrombin-induced hyperpermeability response of cultured monolayers lasts for at least 1 h (38). Differences in the strength of adhesion of the cells to their respective matrices might cause the time-course differences in the of the hyperpermeability responses. The poor support of artificial coatings for endothelial cells allow the formation of large gaps between ECs, requiring a longer time period to recover. Furthermore, recent data show that hydrostatic pressure, which is almost absent in the in vitro model, dampens hyperpermeability responses, and shear forces enforce the endothelial barrier (13, 27).

Using models of cultured endothelial monolayers, we and others previously established the importance of RhoA-Rho kinase-mediated changes in F-actin cytoskeleton in the development of endothelial barrier dysfunction induced by a variety of mediators other than thrombin, including bacterial toxins, tumor necrosis factor-α (28), transforming growth factor-β (8), activated neutrophils (5), p-cresol (6), Combretastatin A-4-phosphate (19), apolipoprotein(a) (30), and lyso-phosphatidic acid (39). This led to the successful attenuation of vascular leakage by Rho kinase inhibitors in several in vivo models: H₂O₂ induced vascular leakage in rabbit lungs (7), hyperpermeability induced by scalding in rats (45), leukotriene D₄ induced pulmonary edema in guinea pig lungs (34), and vascular endothelial growth factor induced vascular leakage in mice (31) and were all dependent on Rho kinase activity. Clinical findings in patients treated with statins also pointed in the direction of a role of Rho kinase in vascular hyperpermeability (10). However, Adams et al. (1) did not find any

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**Fig. 4.** Thrombin-enhanced MLC2 phosphorylation and concanavalin A (ConA)-binding in intact isolated arterioles is Rho kinase dependent. A: intact vessels were pretreated with Y-27632 when indicated and stimulated with thrombin for 2 min or left untreated. Vessels were fixed and double-stained with rhodamine-phalloidin (left; red) and an anti-phosphoMLC2 antibody (middle; green) or both (right; yellow indicates colocalization). Bar, 10 μm. Yellow arrows indicate the direction of the flow. Similar effects were observed in 3 independent experiments. B: binding of ConA in the same vessels as shown in supplemental Fig. 5A. Bar, 50 μm. Yellow arrows indicate the direction of the flow. C: graph representing the means MLC2 phosphorylation ± SD calculated from 4 independent experiments. D: graph representing the mean ConA binding ± SD calculated from 4 independent experiments.
evidence for a role of RhoA/Rho kinase signaling in platelet-activating factor or bradykinin-induced hyperpermeability in rat mesenteric venules. Neither did Rho kinase inhibition lower histamine- or endotoxin-enhanced vascular permeability in vivo (24, 34).

Our finding that thrombin, but not histamine, induced in vitro hyperpermeability is Rho kinase dependent might explain this apparent discrepancy. It implies that distinct mechanisms underlie the transient versus prolonged hyperpermeability responses as induced by different hyperpermeability inducers. We suggest, therefore, that the different mediators can be classified into distinct groups of hyperpermeability inducers. In agreement with these findings, in vivo studies demonstrate that platelet-activating factor, bradykinin, and histamine (all of them being essentially short-acting mediators) induce a Rho kinase-independent hyperpermeability response (1, 24, 34). This response is dependent on calcium-dependent activation of MLCK (37) as well as on destabilization of junctional complexes via phosphorylation of vascular endothelial-cadherin and related proteins (2).

With the use of isolated stress fibers, it has been proposed that Ca2+-activated MLCK is used to generate rapid contraction, whereas Rho kinase plays a major role in maintaining sustained contraction (20). This might explain why thrombin, in contrast to histamine, induces isometric tension and actomyosin-based contraction in endothelial cells (14, 26). Here, we extend these findings and show that activation of Rho kinase in ECs by thrombin sustains the hyperpermeability response. In accordance with the differences in Rho kinase activity, MLCK phosphorylation was remarkably higher on thrombin-induced F-actin filaments compared with histamine-induced F-actin filaments (Fig. 2, A and C).

To study the effects of thrombin on morphology and barrier integrity in EC in situ, we chose to use a model of isolated intact arterioles. Essential findings were verified in the more fragile venules. A major advantage of the model is that it provides a robust model, in which several of the in vivo factors that may mask the thrombin response are absent. Confounding factors include platelets, circulating thrombin inhibitors, and shear stress. Platelets release barrier-promoting factors upon stimulation with thrombin, countering its barrier disruptive effects. Thrombin inhibitors directly reduce thrombin’s activity, and the shear forces most likely oppose morphology changes by being an active stimulus for elongation of the ECs. Therefore, we anticipate that the barrier-disturbing effects of thrombin under healthy conditions in vivo are less pronounced and underestimated compared with the effects we observed, but that our findings do mostly apply to pathological situations, where massive thrombin generation occurs.

In conclusion, these data verified the relevance of currently used in vitro models to study the biochemical mechanisms of vascular hyperpermeability and point out some of the differences and similarities that exist between in vitro cultured and in situ reside endothelial monolayers.

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C1240 THROMBIN-INDUCED HYPERPERMEABILITY IN SITU


