Activation of RhoA by Thrombin in Endothelial Hyperpermeability
Role of Rho Kinase and Protein Tyrosine Kinases

Geerten P. van Nieuw Amerongen, Sanne van Delft, Mario A. Vermeer, John G. Collard, Victor W.M. van Hinsbergh

Abstract—Endothelial cells (ECs) actively regulate the extravasation of blood constituents. On stimulation by vasoactive agents and thrombin, ECs change their cytoskeletal architecture and small gaps are formed between neighboring cells. These changes partly depend on a rise in \([Ca^{2+}]_i\), and activation of the \(Ca^{2+}/calmodulin\)-dependent myosin light chain kinase. In this study, mechanisms that contribute to the thrombin-enhanced endothelial permeability were further investigated. We provide direct evidence that thrombin induces a rapid and transient activation of RhoA in human umbilical vein ECs. Under the same conditions, the activity of the related protein Rac was not affected. This was accompanied by an increase in myosin light chain phosphorylation, the generation of F-actin stress fibers, and a prolonged increase in endothelial permeability. Inhibition of the RhoA target Rho kinase with the specific inhibitor Y-27632 reduced all of these effects markedly. In the presence of Y-27632, the thrombin-enhanced permeability was additionally reduced by chelation of \([Ca^{2+}]_i\) by BAPTA. These data indicate that RhoA/Rho kinase and \(Ca^{2+}\) represent 2 pathways that act on endothelial permeability. In addition, the protein tyrosine kinase inhibitor genistein reduced thrombin-induced endothelial permeability without affecting activation of RhoA by thrombin. Our data support a model of thrombin-induced endothelial permeability that is regulated by 3 cellular signal transduction pathways. (Circ Res. 2000;87:335-340.)

Key Words: human endothelial cells, RhoA, protein tyrosine kinases, calcium, phosphorylation

The endothelium is the main barrier for blood constituents and actively regulates the extravasation of blood components to the surrounding tissues. Formation of minute gaps between endothelial cells (ECs), for instance during inflammation, leads to extravasation of fluid and macromolecules and may cause life-threatening edema. Analogous to smooth muscle cell contraction, phosphorylation of the myosin light chain (MLC) by the \(Ca^{2+}/calmodulin\)-dependent kinase I, the classic MLC kinase, directs the actin-myosin–based contraction process in ECs and is dependent on calcium ions and calmodulin. In addition to a \(Ca^{2+}/calmodulin\)-dependent transient increase in permeability induced by histamine and substance P, thrombin induces a prolonged disturbance of endothelial barrier function. This is associated with a reorientation of the F-actin cytoskeleton, a prolonged MLC phosphorylation, and the formation of intercellular gaps. In contrast to smooth muscle cells, MLC phosphorylation in ECs in vivo does not result in a general contraction of the cells, but a contraction process does occur at the margins of a cell. In the last few years it has become clear that MLC phosphorylation is a highly regulated process in which small G proteins of the Rho family play a crucial role. Most attention has been paid to the small GTPase RhoA. In smooth muscle cells, activation of RhoA results in “calcium sensitization”; ie, independently of changes in \([Ca^{2+}]_i\), MLC phosphate levels increase by inhibition of the smooth muscle myosin phosphatase, resulting in force generation. Other proteins of the Rho family of small GTPases also could be involved in MLC phosphorylation. Activation of the p21-activated kinase (Pak), an enzyme that is activated by the small G proteins Cdc42 and Rac, was shown to affect MLC phosphorylation. In HeLa cells, overexpression of Pak reduces MLC kinase activity. In contrast, in ECs Pak appears to increase MLC phosphorylation by being an MLC kinase itself.

It is a matter of debate which Rho-like small GTPases are involved in endothelial permeability. In human umbilical vein ECs (HUVECs), the thrombin-induced endothelial hyperpermeability was reduced by C3 transferase from Clostridium.
Materials and Methods

Materials
Cell culture reagents were obtained as described previously. Bovine thrombin was from Leo Pharmaceutical Products. Horseradish peroxidase (HRP) was obtained from Sigma Chemical Company. BAPTA-acetoxymethyl ester and rhodamine-phalloidin were from Molecular Probes. Genistein was from Alexis GmbH & Co. Antibody against Rho kinase was supplied by Yoshitomi Pharmaceutical Industries. [32P]Orthophosphoric acid and Tran-35S (a mixture of 35S-labeled amino acids) were from ICN Pharmaceuticals, Inc. Antibody against Rho kinase was provided by Dr. L. Lim (Institute for Molecular and Cell Biology, Singapore). Antibody against RhoA was from Santa Cruz Biotechnology, Inc. Antibody against Rac was from Transduction Laboratories. Anti–platelet myosin Ig (nonmuscle) was from Sanbio. Secondary antibodies were from Dakopatts.

Cell Culture and Evaluation of Barrier Function
HUVECs were isolated and cultured as previously indicated. Barrier function was evaluated by the transfer of HRP across HUVEC monolayers grown on fibronectin-coated polycarbonate filters of the Transwell system (Costar).

MLC Phosphorylation
MLC phosphorylation was measured by a double labeling technique. To that end, HUVECs were incubated for 24 hours with 150 μCi/mL Tran-35S label and for 2 hours with 150 μCi/mL [32P]Orthophosphoric acid in phosphate-free buffer before stimulation of the cells. Details have been given previously.

Rho Activity Assay
Rhotekin binding assays were essentially performed as described. Briefly, 20 cm² confluent HUVECs were preincubated for 1 hour in medium 199+1% human serum albumin (HSA). Cells were stimulated and lysed. Lysates were cleared by centrifugation and incubated with GST-Pak-Cdc42/rac1 interactive binding domain (CRIB, ie, the GTPase binding domain) immobilized on glutathione-coupled Sepharose beads for 30 minutes at 4°C. Beads were washed, eluted in Laemmli sample buffer, and analyzed by Western blotting using an antibody specific for RhoA antibody.

Rac Activity Assay
Pak binding assays were performed as described. Confluent HUVECs (20 cm²) were preincubated for 1 hour in medium 199+1% HSA. Cells were stimulated and lysed. Lysates were cleared by centrifugation and incubated with GST-Pak-Cdc42/rac1 fusion protein, which is reflected in the pull-down fraction of Figure 1 (top). Under control conditions hardly any RhoA-GTP could be detected. The amount of RhoA-GTP in the pull-down fraction was greatly enhanced 30 seconds after stimulation with 1 U/mL thrombin and diminished after 5 minutes. The overall amount of Rac did not change (Figure 1, bottom). In contrast, the activity of Rac, which was measured by use of a GST-Pak fusion protein, did not change or even slightly decreased after thrombin stimulation (Figure 1, middle). With the same reagents, activation of Rac by bradykinin in rat PC12 cells was demonstrated previously (see Reference 15). Thus, thrombin rapidly and transiently activates RhoA but not Rac in HUVECs.

Statistical Analysis
Data are reported as mean±SEM. Comparisons among >2 groups were made by 1-way ANOVA, followed by the Bonferroni-adjusted χ² test. Comparisons of time curves of 2 groups were made using repeated-measures ANOVA. Individual group comparisons were done using a Student t test for post hoc comparisons of the means. Differences were considered significant at P<0.05.

Results
Activation of RhoA but Not Rac by Thrombin
To evaluate whether thrombin directly activates RhoA and Rac in ECs, we used 2 newly developed assays for RhoA and Rac activity. The assay for RhoA activity is based on binding of activated RhoA (RhoA-GTP) to a GST-Rhotekin fusion protein, which is reflected in the pull-down fraction of Figure 1 (top). Under control conditions hardly any RhoA-GTP could be detected. The amount of RhoA-GTP in the pull-down fraction was greatly enhanced 30 seconds after stimulation with 1 U/mL thrombin and diminished after 5 minutes. The overall amount of RhoA did not change (Figure 1, bottom). In contrast, the activity of Rac, which was measured by use of a GST-Pak fusion protein, did not change or even slightly decreased after thrombin stimulation (Figure 1, middle). With the same reagents, activation of Rac by bradykinin in rat PC12 cells was demonstrated previously (see Reference 15). Thus, thrombin rapidly and transiently activates RhoA but not Rac in HUVECs.
Rho Kinase Is Involved in the Thrombin-Enhanced Permeability

To investigate whether RhoA acts in endothelial permeability via Rho kinase, the cell-permeant Rho kinase inhibitor Y-27632 was used at a high concentration of 10 μmol/L that was shown to completely inhibit Rho kinase.19 We first checked by Western blotting whether Rho kinase was present in ECs and found 1 single band at the expected molecular mass of 160 kDa in HUVECs (Figure 2A, inset) and other types of human macro- and microvascular ECs (glomerular and foreskin microvascular ECs, iliac artery and vein ECs, and aorta ECs; data not shown). The Rho kinase inhibitor Y-27632 was used to study the role of Rho kinase in the thrombin-induced endothelial barrier dysfunction. Preincubation for 1 hour with 10 μmol/L Y-2763219 had no effect on basal permeability (Figure 2A) but significantly attenuated the thrombin-enhanced endothelial permeability, as evidenced by a decreased passage of the marker molecule HRP through the endothelial monolayer. This attenuation was partial, even when Y-27632 was used at a higher concentration (up to 100 μmol/L) or when its preincubation time was prolonged to 3 hours (data not shown).

Inhibition of Rho kinase by Y-27632, which was maximal at 10 μmol/L, together with chelation of intracellular Ca2+ ions by BAPTA (Figure 2B), nearly completely blocked the thrombin-enhanced HRP passage, suggesting that RhoA/Rho kinase- and Ca2+- dependent processes act on EC permeability by separate pathways.

Inhibition of Rho Kinase Reduces MLC Phosphorylation and Cytoskeletal Reorganization

Alterations in endothelial permeability are accompanied by actin nonmuscle myosin interaction, which is regulated by MLC phosphorylation. MLC phosphorylation, as determined by 32P incorporation in MLC, was increased on activation of ECs by thrombin in agreement with previous observations (Figure 3).3,4,9,23 Preincubation with the Rho kinase inhibitor Y-27632 significantly reduced MLC phosphorylation under both basal and thrombin-stimulated conditions (Figure 3). Formation of both thrombin-induced F-actin stress fibers and

Figure 2. Involvement of Rho kinase in thrombin-enhanced endothelial permeability. A, Effect of the Rho kinase inhibitor Y-27632 on the passage of HRP through HUVEC monolayers under basal conditions (○, □) and after exposure to 1 U/mL thrombin (●, ▼), Cells were preincubated for 1 hour in medium 199+1% HSA without (○, □) or with 10 μmol/L Y-27632 (●, ▼), and HRP passage was measured as described in Materials and Methods. Values are mean±SEM of 9 cultures in 3 independent experiments. The interaction between time and Y-27632 treatment was significant (P<0.001) for thrombin-stimulated cells. Treatment with Y-27632 resulted in lower HRP passage compared with no treatment. *P<0.05, Y-27632-pretreated vs nonpretreated cells that were stimulated with thrombin. Inset, Detection of Rho kinase by Western blot in HUVEC extracts. Estimated molecular mass of the protein was 160 kDa. B, Effect of BAPTA and Y-27632 on basal (open bars) and thrombin-induced HRP passage (solid bars) across HUVEC monolayers. Cells were pretreated for 1 hour with 3 μmol/L BAPTA, 10 μmol/L Y-27632, or both. HRP passage was measured 2 hours after sham treatment or exposure to 1 U/mL thrombin. Values are mean±SD of 6 cultures in 2 experiments. *P<0.05, cells pretreated with BAPTA or Y-27632 vs nonpretreated cells. #P<0.05, cells pretreated with a combination of BAPTA and Y-27632 vs pretreatment with each compound alone.

Figure 3. MLC phosphorylation is inhibited by Y-27632. A, Autoradiograph of MLCs immunoprecipitated from cells under basal conditions (−) and 10 minutes after stimulation with 1 U/mL thrombin (+). Cells were labeled with 32P and 35S as described previously4 and preincubated with 10 μmol/L Y-27632 for 1 hour. MLC phosphorylation was measured as described in Materials and Methods. Top, Exposure in which a filter was present to block 35S signal. Bottom, Exposure without filter. B, Quantification of MLC phosphorylation. Shown is effect of 10 μmol/L Y-27632 on basal (open bars) and thrombin-enhanced (solid bars) MLC phosphorylation. The level of 32P incorporation into MLCs was calculated relative to the amount of 35S incorporation into MLCs of the same sample, as was described previously.4 Values are mean±SEM of 3 different cultures in duplicate. *P<0.05 Y-27632-pretreated vs nonpretreated cells.
Inhibition of PTKs Reduces Thrombin-Induced Permeability but Not RhoA Activation

To investigate whether the PTKs involved in endothelial permeability act upstream of Rho activation, ECs were preincubated for 1 hour with 30 μg/mL of the PTK inhibitor genistein. This dose of genistein is a maximally effective dose for inhibiting endothelial barrier dysfunction. Genistein did not affect the thrombin-induced activation of RhoA (Figure 5, inset). In parallel cultures genistein, but not its inactive analogue daidzein (data not shown; see also Reference 4), inhibited the thrombin-enhanced passage of HRP through the endothelial monolayer (Figure 5). This indicates that the genistein-sensitive PTKs, which are involved in endothelial barrier dysfunction, do not act upstream of RhoA activation.

Subsequently, endothelial monolayers were coincubated with genistein and Y-27632 to test whether PTKs act downstream of Rho kinase in inducing endothelial barrier dysfunction. The inhibition of the thrombin-induced HRP passage by genistein was in addition to the inhibition by Y-27632 (Figure 5). This indicates that a PTK-dependent process also acts on a RhoA/Rho kinase-independent pathway that affects EC permeability.

Discussion

In the present study, we provide direct evidence that thrombin potently activates RhoA in ECs but does not affect the activity of Rac. Activity of the PTKs involved in endothelial hyperpermeability was not required for the thrombin-induced activation of RhoA. Furthermore, it was shown that thrombin increases endothelial permeability via Rho kinase independently from a rise in [Ca2+]i, and that PTKs and Rho kinase have additive effects on endothelial hyperpermeability.

To our knowledge, we provide the first direct demonstration that RhoA is activated by thrombin in confluent endothelial monolayers. Its rapid onset was comparable with the rise in [Ca2+]i and that PTKs and Rho kinase have additive effects on endothelial hyperpermeability.

In addition to RhoA, thrombin-enhanced endothelial permeability requires Ca2+-ions. Similarly, as previously found with the Rho inhibitor C3 transferase, inhibition of Rho kinase inhibited the increased permeability, but only partly at
the maximally effective dose of Y-27632. In combination with the chelation of Ca\(^{2+}\) ions, Y-27632 almost completely prevented the thrombin-induced HRP passage. This indicates not only that both Ca\(^{2+}\) ions and the RhoA/Rho kinase pathway are necessary for the full thrombin response but also that RhoA/Rho kinase signaling and Ca\(^{2+}\)-dependent processes additionally contribute barrier dysfunction.

MLC phosphorylation plays a pivotal role in initiating actin-myosin interaction and in the development of barrier dysfunction.\(^9\) Here, we extend our previous observation on RhoA\(^4\) and show that Rho kinase contributes to MLC phosphorylation in ECs. This is comparable with the situation in smooth muscle cells\(^{25}\) and blood platelets,\(^{26–28}\) in which Rho kinase has recently been shown to be involved in MLC phosphorylation. Rho kinase can increase MLC phosphorylation by inhibiting the myosin phosphatase.\(^29\) It is likely that the same mechanism of Rho kinase–induced MLC phosphorylation acts in ECs, as it was shown that in ECs the myosin phosphatase is inhibited by thrombin\(^{30,31}\) and that the inhibition of Rho by C3 transferase prevents the inhibition of the phosphatase induced by thrombin.\(^9\) In addition to an effect on MLC phosphorylation, Rho kinase also phosphorylates other proteins. Several of these proteins are involved in stress fiber formation and may act on EC barrier function, including LIM kinase,\(^32\) adducin, and members of the ERM (ezrin/radixin/moesin) family.\(^33\) Their involvement in endothelial permeability remains to be demonstrated. The contribution of additional factors activated by Rho cannot be excluded. Phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) may be involved, as intracellular levels of PIP\(_2\) can be increased after activation of Rho\(^34\) and PIP\(_2\) is known to interfere with the actin cytoskeleton.\(^35\)

We have shown previously that, in addition to a rise in intracellular Ca\(^{2+}\) ions, PTKs are involved in the thrombin-enhanced endothelial barrier dysfunction.\(^4\) Other investigators demonstrated that a genistein-sensitive PTK acts upstream of lysophosphatidic acid–induced RhoA activation in ECs.\(^18\) The present study shows that inhibition of PTKs with genistein does not influence activation of RhoA by thrombin, indicating that PTKs do not act upstream of the activation of RhoA in thrombin-enhanced endothelial permeability. This suggests differences between activation of RhoA by either lysophosphatidic acid or thrombin, which may be the involvement of G\(_{13}\). G\(_{13}\) is involved in the activation of RhoA by lysophosphatidic acid but has not been demonstrated to be involved in thrombin-induced signal transduction.\(^36\)

Inhibition of PTKs with genistein reduced the thrombin-enhanced barrier dysfunction in addition to the effect of Y-27632. This indicates that PTKs and Rho kinase at least act by separate pathways. Interestingly, this study and our previous study show that chelation of Ca\(^{2+}\) by BAPTA acts in addition to the inhibition of both PTKs and Rho kinase. This suggests that the Ca\(^{2+}\), RhoA, and PTK-mediated pathways induced by thrombin reflect separate pathways and that all converge in increased permeability. For the RhoA- and Ca\(^{2+}\)-dependent pathways, this convergence point is probably the MLC phosphorylation (compare Figure 6, left).

Inhibition of PTKs may act on endothelial permeability by various mechanisms. PTK inhibitors attenuate agonist-induced increases in [Ca\(^{2+}\)]\(_i\).\(^37,38\) In accordance with this, Garcia et al\(^10\) recently reported that activity of an EC-specific MLC kinase is regulated by tyrosine phosphorylation in a RhoA-dependent manner. However, additional mechanisms must exist, as PTKs are involved in endothelial barrier dysfunction independent of changes in [Ca\(^{2+}\)]\(_i\),\(^4\) and RhoA signaling (present study). A likely mechanism is the destabilization or disruption of junctions by tyrosine phosphorylation of junctional proteins. Tyrosine phosphorylation of occludin and ZO-1 can occur under conditions of hyperpermeability.\(^39\) The agonist-induced disruption of adherens junctions is accompanied by tyrosine phosphorylation of vascular endothelial cadherin (VE-cadherin) and the associated catenins, resulting in the dissociation of VE-cadherin/catenins.\(^40,41\) Disintegration of junctional complexes and the actin-nonmuscle myosin interaction in the periphery of ECs may thus act in concert in prolonged thrombin-induced endothelial permeability (Figure 6).

Our data point to an important role for RhoA and Rho kinase in the regulation of endothelial permeability. Future studies have to demonstrate whether and when these factors are involved in altered endothelial barrier function in vivo. In large-vessel ECs, in particular in areas with altered shear forces, stress fibers are found and Rho-mediated processes are likely to be involved. The recent finding of Essler et al,\(^42\) that mildly oxidized LDL activates Rho/Rho kinase signaling in ECs, suggests the involvement of RhoA and Rho kinase in vascular leakage during the development of atherosclerosis. No information is presently available on microvascular ECs in vivo. However, it should be noticed that Rho kinase plays a role in cell migration and that prolonged permeability might be a reflection of the altered behavior of ECs during cell migration and angiogenesis, which occur in wound healing and pathological conditions.
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References
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