ROCK mediates thrombin’s endothelial barrier dysfunction

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Carabajal, José M., Max L. Gratrix, Chung-Ho Yu, and Richard C. Schaeffer, Jr. ROCK mediates thrombin’s endothelial barrier dysfunction. Am J Physiol Cell Physiol 279: C195–C204, 2000.—Thrombin-induced endothelial monolayer hyperpermeability is thought to result from increased F-actin stress fiber-related contractile tension, a process regulated by the small GTP-binding protein Rho. We tested whether this process was dependent on the Rho-associated protein kinase, ROCK, using a specific ROCK inhibitor, Y-27632. The effects of Y-27632 on thrombin-induced myosin light chain phosphorylation (MLCP) and tyrosine phosphorylation of p125 focal adhesion kinase (p125FAK) and paxillin were measured by Western blotting. F-actin organization and content were analyzed by digital imaging, and endothelial monolayer permeability was measured in bovine pulmonary artery endothelial cell (EC) monolayers using a size-selective permeability assay. Y-27632 enhanced EC monolayer barrier function due to a decline in small-pore number that was associated with increased EC surface area, reduced F-actin content, and reorganization of F-actin to β-catenin-containing cell-cell adherens junctions. Although Y-27632 prevented thrombin-induced MLCP, stress fiber formation, and the increased phosphotyrosine content of paxillin and p125FAK, it attenuated but did not prevent the thrombin-induced formation of large paracellular holes. These data indicate that thrombin-induced stress fiber formation is ROCK dependent. In contrast, thrombin-induced paracellular hole formation occurs in a ROCK-independent manner, whereas thrombin-induced monolayer hyperpermeability appears to be partially ROCK dependent.

nonmuscle myosin; myosin light chain phosphorylation; paxillin; Y-27632; immunofluorescent digital imaging; Rho-associated coiled-coil forming kinase

HYPERPERMEABILITY of the blood vessel wall permits leakage of excess fluids and protein into the interstitial space. This acute inflammatory event is frequently assisted with tissue ischemia and acute organ dysfunction. Thrombin formed at sites of activated endothelial cells (EC) initiates this microvessel barrier dysfunction due to the formation of large paracellular holes between adjacent EC. This process features changes in EC shape due to myosin light chain phosphorylation (MLCP) that initiates the development of F-actin-dependent cytoskeletal contractile tension (1, 10, 12, 17).

The signaling mechanism of this contractile process involves the proteolytic cleavage and activation of the thrombin receptor. This receptor is coupled to heterotrimeric G proteins of the Gq family that stimulate phospholipase Cβ to release D-myo-inositol 1,4,5-trisphosphate, mobilizing Ca2+ from intracellular stores (10, 17). The subsequent rise in intracellular Ca2+ concentration activates Ca2+-calmodulin-dependent MLC kinase, which phosphorylates serine-19 and threonine-18 of MLC (11). MLCP initiates myosin Mg2+-ATPase activity, causing the binding of myosin to F-actin and subsequent actomyosin stress fiber formation (20). The phosphorylation of MLC converts the soluble folded 10S form of nonmuscle myosin II to the insoluble unfolded 6S form. This process is characterized by reorganization of myosin from a diffuse intracellular cloud to punctate spots and ribbons associated with large bundles of F-actin (28). The phosphorylation of MLC is also adjusted by the activity of the MLC-associated phosphatase (PP1M). PP1M is composed of three components: a 130-kDa regulatory subunit, a 37-kDa catalytic subunit, and a 20-kDa subunit. Recently, it has been shown that the Rho-associated serine/threonine kinase β/p160, or Rho-associated coiled-coil forming kinase (ROCK) inactivates PP1M through the phosphorylation of the 130-kDa PP1M subunit (19). Thus RhoA-activated ROCK inhibits PP1M activity and elevates MLC phosphorylation, myosin ribbon and stress fiber formation, and endothelial contractile tension (3, 4, 15).

The RhoA, B, and C small GTP-binding proteins have been implicated in the modulation of the F-actin cytoskeleton and cell shape (21). Rho proteins are active in their GTP-bound form and inactive when bound to GDP. Guanine nucleotide exchange factors and GTPase activating proteins regulate this control switch. Constitutively active V14RhoA initiates the formation of actomyosin stress fibers and focal adhesions, causing tyrosine phosphorylation of the focal adhesion proteins paxillin and p125 focal adhesion kinase (p125FAK) and

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MATERIALS AND METHODS

Directly initiates polymerization of the actin cytoskeleton (PI 3-kinase), PI 5-kinase, mDia (a mammalian diaphanous homologue), and ROCK modulate the F-actin cytoskeleton (5, 18, 29). Whereas mDia directly stimulates the formation of F-actin by activation of the actin-binding protein profilin, activated ROCK indirectly initiates polymerization of the actin cytoskeleton by two mechanisms: 1) increasing MLCP, and 2) inhibiting the activity of cofilin, an actin-severing protein. The latter is modulated by ROCK-dependent activation of LIM kinase (3, 9, 18). Together, these findings show that activated ROCK modulates the formation of F-actin stress fibers both by promotion of actin polymerization and prevention of actin depolymerization. Recent findings have shown that a specific inhibitor of ROCK, Y-27632, prevents smooth muscle contraction and the formation of F-actin stress fibers, indicating that Rho proteins modulate these processes (18, 27). These data also suggest that Y-27632 may be useful to assess the role of endothelial contraction in thrombin-induced EC monolayer hyperpermeability.

Thrombin-induced endothelial hyperpermeability may also be mediated by changes in cell-cell adhesion (7). Endothelial cell-cell adhesion is determined primarily by the function of vascular endothelial cadherin (cadherin 5), a Ca^{2+}-dependent cell-cell adhesion molecule that forms adherens junctions. Cadherin 5 function is regulated from the cytoplasmic side through association with the accessory proteins β-catenin, plakoglobin (γ-catenin), and p120 that are linked, in turn, to α-catenin (homologous to vinculin) and the F-actin cytoskeleton. Whether the Rho family of GTPases modulates these processes is not known.

In the present study, we tested the hypothesis that thrombin-induced stress fiber formation and thrombin-induced paracellular hole formation are ROCK-dependent processes. Y-27632 inhibited thrombin-induced MLCP and stress fiber formation, indicating that these processes are ROCK dependent. Because Y-27632 attenuated but did not avert thrombin-induced paracellular hole formation, the mechanisms of thrombin-induced endothelial hyperpermeability appear to be due to a combination of ROCK-dependent increases in EC contractile tension and ROCK-independent events.

MATERIALS AND METHODS

Cell culture. Our personal stock of low-passage (passages 5–11) EC, isolated from bovine pulmonary arteries as previously described (24), was grown in DMEM containing 10% FCS (Hyclone, Ogden, UT) and used between 3 and 4 days postconfluence.

Immunoprecipitation and immunoblotting. EC grown in gelatin-coated 60-mm dishes were treated with or without 1–50 μM Y-27632 (Yoshitomi Pharmaceutical Industries, Saitama, Japan) in the presence or absence of human α-thrombin (10 U/ml; Sigma, St. Louis, MO). After the appropriate treatments, EC were lysed in extraction buffer and immunoprecipitated for phosphotyrosine (PY)-containing proteins using 2 μg of monoclonal antibodies, PY-99 (Santa Cruz Biotechnology, Santa Cruz, CA), or paxillin using anti-paxillin IgG (Transduction Laboratories, Lexington, KY) as previously described (4). After capture with protein G agarose, the pellets were washed, boiled with 2× SDS sample buffer under reducing conditions for 5 min, and separated by SDS-PAGE in 7.5% vertical slab gels using a Laemmli buffer system. Proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) and the membranes immunoblotted with antibodies to anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) or monoclonal IgG to p125FAK or paxillin, followed by Enhanced Chemiluminescence (ECL) Plus detection (Amersham, Arlington Heights, IL). X-ray films developed at various intervals were used to detect each protein within the linear range of the film.

Endothelial monolayer barrier function. The techniques listed herein have been described in detail elsewhere (22, 24–26). In brief, the size-dependent passage of fluorescein isothiocyanate-labeled hydroxethyl starch (FITC-HES) macromolecules across bovine pulmonary artery EC monolayers was used to measure the presence or absence of a small-pore barrier. FITC-HES samples from both sides of the monolayer/filter support barrier were analyzed by high-pressure size-exclusion liquid chromatography (HPLC) and quantified with a fluorescence spectrophotometer-computer detection system (26).

EC monolayers were prepared by seeding 75 × 10^4 cells onto gelatin/fibronectin-coated Transwell inserts (2,000 Å pore radius filter supports, 0.33 cm² surface area; Costar, Cambridge, MA) in a 24-well plate. At 4–5 days postconfluence, each monolayer was incubated with Y-27632 for 1 h as indicated in the text. Barrier function studies were performed by washing each Transwell with 37°C serum-free HEPES-buffered (pH = 7.4) MEM. The experiment was initiated by the addition of 100 μl of FITC-HES (3 mg/ml) to the top chamber (Transwell insert) and 650 μl of serum-free HEPES-MEM to the bottom chamber (24-well plate) followed by Y-27632 and/or 10 U/ml of thrombin. Samples (30 μl) from the bottom and top chambers were removed after 1.5 h. Computation of permeability/free diffusion coefficients was performed exactly as previously described (25). Monolayer experiments that did not achieve restricted diffusion in controls were excluded from this study.

Digital imaging immunofluorescence microscopic workstation. The digital imaging microscopic workstation used in these studies has been previously described (4). In brief, an Olympus IMT-2 microscope with an Olympus ×60 magnification, 1.4 numerical aperture oil-immersion objective, a 150-W xenon lamp, a MetalTek filter wheel, and a Chroma Technology 84 series quad filter set polychromatic beamsplitter/Emitter (Chroma Technology, Brattleboro, VT) were used to simultaneously collect Alexa 488, tetramethylrhodamine (TRITC), or Cy5 staining pattern for each sample. Epifluorescent digital images were collected with online background subtraction and shading correction at 12-bit depth using a Photometrics (Tucson, AZ) PXL charge-coupled device camera containing a Kodak 1400 chip that was thermally cycled to −25°C to reduce dark current noise and controlled with MetaMorph software (Universal Imaging, Version 4.0). MetaMorph was used to identify regions of colocalization by color coding the separate immunofluorescent images of each cell.

Labeling of cytoskeletal structures. EC grown to 4 days postconfluence in Transwell membranes or eight-well slides were treated and stained as described in the text. Primary monoclonal antibodies to β-catenin (no. 13–8400, 1:100 dilution; Zymed Laboratories) or rabbit IgG to α-catenin (no. 71–1200, 1:50 dilution) were used to detect the adherens junctions, mouse anti-paxillin IgG (1:200 dilution; Chemicon, Temecula, CA) was used to detect focal adhesions, and affin-
ity-purified rabbit antibodies to nonmuscle myosin II (BT-561, 1:25 dilution; Biomedical Technologies) were used to label myosin II. After washing, secondary goat TRITC or Cy5-labeled anti-rabbit IgG (1:25 dilution) and goat-Cy5 antit_mouse IgG (1:25 dilution; Jackson Immunoresearch Labs, Westgrove, PA) were used to label primary IgG. Alexa 488 or TRITC-phalloidin (Molecular Probes, Eugene, OR) was used to label F-actin. The Transwell membranes were removed and placed on a glass slide, and the slide was covered with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). A no. 1 coverslip was applied, and the edges were sealed with nail polish.

MLCP. The phosphorylation of MLC was measured by urea/glycerol PAGE separation of the mono- and diphosphorylated forms as previously described (9, 20). EC grown in 60-mm dishes were stimulated with thrombin in the presence or absence of Y-27632 as indicated, and the reaction was stopped by the addition of 1 ml of ice-cold 10% perchloric acid. Cells were scraped and centrifuged for 10 min at 20,000 g at 4°C. The pellets were washed with 1 ml of ice-cold water followed by 1 ml of ice-cold ethanol, and the pellet was resuspended in sample buffer (6.7 M urea, 10 mM Tris, 22 mM glycine, and 270 mM sucrose, pH 9.0). Equal amounts of protein were added to each lane of a 40% glycerol/10% acrylamide gel and run at 400 V for ~1 h. The protein samples were transferred to nitrocellulose in 0.25 mM phosphate buffer (pH 7.6) for ~1 h. The unphosphorylated (P0), monophosphorylated (P1), and diphosphorylated (P2) forms of MLC were detected by Western blotting using a polyclonal MLC antibody (1:1,000; James Stull, Dallas, Texas) followed by detection using ECL Plus (Amersham) and a Storm phosphorimager (Molecular Dynamics). The stoichiometry of MLC (moles of phosphate per moles of MLC) was determined using Imagequant software and calculated using the formula P1 + 2 × P2/P0 + P1 + P2 as previously described (9).

Statistical analysis of EC surface area and F-actin content. Digital images of the F-actin and b-catenin staining patterns were collected (10 images/treatment) at random from Transwell membranes exposed to thrombin with or without Y-27632 pretreatment as indicated in the text. EC surface area and F-actin content were determined as previously described (4). The Student’s t-test (paired) was used for the statistical analysis of the results. These values are expressed as means ± SE.

RESULTS

Y-27632 enhances monolayer barrier function and attenuates thrombin-induced endothelial monolayer hyperpermeability. Rho proteins have been reported to adjust thrombin-induced MLCP and F-actin stress fiber formation in endothelial cells, leading to the loss of EC barrier function (2, 9). We quantitatively measured the size and number of paracellular holes formed across thrombin-stimulated EC monolayers in the presence or absence of the specific ROCK inhibitor Y-27632 by analyzing their size-selective solute permeability characteristics using an HPSEC technique (26). Restricted diffusion, created by predominantly small “pores” (<250 Å pore radius), is characterized by a decline in the size-selective permeability/free diffusion coefficient (P/D0) with increasing solute molecular radius (aS) (24). In contrast, the formation of large holes (>2,000 Å pore radius) is displayed by an increase in P/D0 for solutes with large molecular radii (25). Control EC monolayers showed significant restricted diffusion, a property that was enhanced by pretreatment with 5, 10, and 50 μM Y-27632 (Fig. 1A). In contrast, thrombin-stimulated monolayers displayed large hole barrier hyperpermeability as illustrated by a shift in the P/D0 vs. aS curve up and to the right. Although Y-27632-thrombin-treated monolayers displayed P/D0 values that were substantially lower than those for monolayers exposed to thrombin alone, these values were significantly greater than those for Y-27632 alone as well as above the control P/D0 at aS > 60 Å (Fig. 1B). Similar effects were seen when 5 or 50 μM Y-27632 was used in the presence or absence of thrombin (data not shown).
Y-27632 reorganizes the F-actin cytoskeleton to β-catenin-containing adherens junctions but does not avert thrombin-induced paracellular hole formation. Adherens junctions, characterized by homophilic binding of Ca2+ -dependent adhesion (cadherins) molecules, are linked to the F-actin cytoskeleton by the accessory proteins α-, β-, and γ-catenin (7). To test whether Y-27632 stimulated the reorganization of F-actin and prevented thrombin-induced large hole formation, Transwell membranes from the barrier function assays listed above were fixed and double stained for F-actin and the adherens junctional protein β-catenin. Control EC displayed a few F-actin stress fibers (Fig. 2, A and C, arrows) and some F-actin at the β-catenin-containing cell-cell junctions (Fig. 2, B and C, arrowheads). Thrombin stimulated the formation of large F-actin stress fibers (Fig. 2, D and F, arrows) and large paracellular holes that did not show β-catenin staining (Fig. 2, D–F, asterisks). A fingerlike β-catenin staining pattern appeared linked to large F-actin filament bundles (Fig. 2, E and F, arrowheads). Y-27632-treated (10 μM) EC displayed no F-actin stress fibers because most F-actin was colocalized with β-catenin at cell-cell junctions (Fig. 3, A–C, arrowheads), characteristics that remained in 10 μM Y-27632-thrombin-treated monolayers (Fig. 3, D–F). Thrombin-induced paracellular hole formation remained present in the 10 μM Y-27632-thrombin-treated monolayers (Fig. 3, D–F, asterisks). Similar effects were observed in 5 and 50 μM Y-27632-thrombin-treated monolayers (data not shown).

Y-27632 decreases F-actin content, increases EC surface area, and prevents thrombin-induced increases in F-actin content. The role of the F-actin cytoskeleton in the modulation of endothelial barrier function remains controversial. Bacterial toxins that decrease F-actin content have been shown to enhance or degrade endothelial monolayer barriers (2, 13). In the present report, measurements of F-actin content (F-actin intensity per unit cell surface area, FI/μm²) showed that Y-27632 initiated up to a 30% decline in F-actin content that was most marked at 5 μM (Fig. 4A). Higher (10–50 μM) amounts of Y-27632 displayed less difference in FI/μm² compared with the control group. This effect was associated with the increased appearance of amorphous F-actin pools in these EC (data not shown). Although the F-actin content of the thrombin group was significantly above the control group, the Y-27632-thrombin treatments showed a 20–30% decline in F-actin content values compared with the effects of thrombin alone.
Y-27632-treated EC exhibited a 13–18% increase in EC surface area compared with the controls (Fig. 4B; 50 μM Y-27632 = 564 ± 35 μm² vs. 476 ± 24 μm², respectively). The Y-27632-thrombin-treated EC displayed an 11% increase in EC surface area (50 μM Y-27632 = 569 ± 25 μm²) that was not significantly different from the effect of 50 μM Y-27632 alone. EC surface area after thrombin treatment alone was not significantly different from that of the control group.

Y-27632 prevents thrombin-induced MLCP. Thrombin is known to stimulate the rapid phosphorylation of MLC, achieving a maximum within 2 min and initiating the EC contractile forces associated with EC monolayer hyperpermeability (10, 11). We tested the concentration-dependent effects of Y-27632 to prevent the thrombin-induced phosphorylation of MLC (Fig. 5). EC monolayers were pretreated with Y-27632 (1–50 μM, 1 h), followed by stimulation with 10 U/ml of thrombin for 2 min. The P₀, P₁, and P₂ forms of MLC were separated (Fig. 5A), quantified by densitometry, and expressed as moles of phosphate per moles of MLC (Fig. 5B) as described in MATERIALS AND METHODS. Thrombin stimulated the rapid incorporation of phosphate into MLC that was 1.8-fold above control after 2 min. Pretreatment with 5–10 μM Y-27632 prevented this thrombin effect, whereas 50 μM reduced MLCP below control levels.

Y-27632 reorganizes nonmuscle myosin II and prevents thrombin-induced myosin ribbon formation. Thrombin-induced formation of MLCP initiates the reorganization of myosin from spots into ribbons along large actin filament bundles (11, 28). We tested whether Y-27632 prevented thrombin-induced myosin ribbon formation by immunostaining Transwell membranes with antibodies to nonmuscle myosin from the permeability experiments listed above. Control EC exhibited a few myosin spots (Fig. 6A, arrowheads, inset). Thrombin initiated the formation of multiple myosin ribbons across EC and at the cell borders (Fig. 6B, arrowheads, inset). These effects were not seen in Y-27632-pretreated EC, which exhibited a diffuse myosin immunostaining pattern in the presence and absence of thrombin (Fig. 6, C and D).

Y-27632 inhibits tyrosine phosphorylation of the focal adhesion proteins paxillin and p125FAK. Thrombin stimulates the formation of focal adhesions at the ends of F-actin stress fibers and initiates the tyrosine phosphorylation of the focal adhesion proteins paxillin and p125FAK, events that are sensitive to the Rho protein inhibitor C3 exoenzyme and the MLC kinase inhibitor
We tested whether Y-27632 prevented tyrosine phosphorylation of specific focal adhesion proteins in thrombin-stimulated EC by immunoprecipitation with anti-paxillin and anti-phosphotyrosine (PY-99) antibodies followed by Western blot analyses with anti-phosphotyrosine (4G10), anti-p125 FAK, and anti-paxillin IgG (Fig. 7, A–D). Although Y-27632 did not affect the quantity of immunoprecipitated paxillin (Fig. 7B), this inhibitor stimulated a decline in the PY content of two protein bands, paxillin and p125 FAK, with or without thrombin treatment (Fig. 7, A, C, and D). Densitometry of the thrombin-stimulated PY-containing proteins revealed an increase in the PY content of paxillin and p125 FAK (Fig. 7, C and D). Because Y-27632 prevented these changes in the presence or absence of thrombin, these tyrosine phosphorylation events appear to be ROCK dependent.

Y-27632 inhibits thrombin-induced formation of paxillin-containing focal adhesions. Finally, we tested whether Y-27632 prevented thrombin-induced formation of focal adhesions. EC monolayers, pretreated with Y-27632 (10 μM, 1 h) followed by the presence or absence of thrombin (10 U/ml, 0.5 h), were triple stained for F-actin, paxillin, and α-catenin (Fig. 8). Figure 8, A–D, shows a control EC that displays dense peripheral bands of F-actin (A, arrows) with few focal adhesions (B, arrowheads) and α-catenin-containing adherens junctions (C, arrows) that are merged into a tricolor image (D). In contrast, thrombin stimulated the formation of F-actin stress fibers (Fig. 8, E and H, arrows) linked to paxillin-containing focal adhesions (Fig. 8, F and H, arrowheads and insets). Y-27632 in the presence or absence of thrombin reorganized F-actin (arrows) and paxillin (arrowheads) to α-catenin-containing cell-cell adhesions (Fig. 8, I–P, arrows).

DISCUSSION

With the use of pulmonary artery EC monolayers as an in vitro analog of blood vessel barrier function and Y-27632 as a specific ROCK inhibitor, we tested the hypothesis that thrombin-induced EC barrier hyperpermeability was due to ROCK-dependent formation of actomyosin stress fibers. Y-27632-treated EC displayed the disassembly of actomyosin stress fibers with reorganization of F-actin to α- or β-catenin-containing adherens junctions. These effects, in combination with an increase in EC surface area and decline in F-actin...
content, characterize the Y-27632-dependent enhancement of EC monolayer barrier function. Although this inhibitor prevented the thrombin-induced phosphorylation of MLC, myosin ribbon formation, and increased F-actin content, Y-27632 treatment attenuated but did not avert thrombin-induced monolayer barrier hyperpermeability or the loss of β-catenin staining at sites of paracellular hole formation. These data show that ROCK regulation of the endothelial F-actin cytoskeleton plays an important role in the modulation of endothelial monolayer barrier function. Inhibition of ROCK improves monolayer barrier function by a mechanism that elevates EC surface area in association with a decline in the number of small paracellular pores. In addition, ROCK mediates thrombin-induced development of actomyosin stress fibers linked to focal adhesions. However, Y-27632 inhibition of ROCK does not completely prevent paracellular hole formation, implying that thrombin-induced monolayer hyperpermeability is partially mediated by ROCK-independent noncytoskeletal forces.

Activated Rho proteins regulate the formation of the F-actin cytoskeleton by activation of PI 3-kinase, PI 5-kinase, mDia, and ROCK (5, 18, 29). Whereas the PI kinases and mDia initiate actin polymerization, ROCK inhibits the depolymerization of F-actin. In addition, ROCK indirectly stimulates the formation of F-actin stress fibers by inhibiting myosin

![Fig. 6. Y-27632 stimulates diffuse nonmuscle myosin II immunostaining and prevents thrombin-induced myosin ribbon formation. Transwell EC monolayers from the experiments listed in Fig. 1 were fixed, extracted, and immunostained for nonmuscle myosin II as described in MATERIALS AND METHODS. A: control EC displayed punctate myosin spots (A, arrowhead and inset) at the base of the cell. B: thrombin (10 U/ml, 1.5 h) stimulated myosin ribbon formation throughout the cell (B, arrowheads and inset). C-D: Y-27632-treated EC (10 μM, 1 h) with (D) or without (C) thrombin (10 U/ml, 1.5 h) displayed a diffuse myosin staining pattern (insets). Bar = 5 μm; inset bar = 1.25 μm.]

![Fig. 7. Y-27632 reduces paxillin and p125 FAK tyrosine phosphorylation. EC monolayers were pretreated with Y-27632 (Y, 10 μM, 1 h) with or without thrombin (T, 10 U/ml, 2 min) or received no treatment (IgG or control). EC lysates were immunoprecipitated (IP) with paxillin or anti-phosphotyrosine (PY-99) antibodies and Western blotted (IB) for paxillin, p125 FAK, or PY-containing proteins (A–C, 4G10). Densitometry revealed that relative phosphotyrosine content of paxillin and p125 FAK was increased by T and reduced in Y-27632 and Y-T-treated EC compared with the control (D). A representative blot of 2 separate experiments is shown.]

![A]

![B]

![C]

![D]
phosphatase (18). Y-27632 appears to inhibit these processes in a ROCK-dependent manner because Y-27632 shows a 2,000 times greater affinity for ROCK than for protein kinase C (PKC) using purified enzyme substrates (27). These data, in combination with the observation that 10 μM Y-27632 maximally inhibits ROCK-dependent smooth muscle cell contraction and higher amounts nonselectively inhibit both PKC and cAMP-dependent protein kinase, indicate that 10 μM Y-27632 selectively suppresses ROCK-dependent formation of F-actin as shown in LIM kinase 1-expressing HeLa cells (18, 27). Consistent with these data, we report that Y-27632 reduces F-actin stress fibers, F-actin content, and MLCP and initiates a diffuse myosin staining pattern at concentrations (5–10 μM) that are selective for ROCK in other cell types. These events were associated with increased EC surface area and reduced monolayer size-selective solute permeability. The Y-27632-induced reorganization of F-actin to α- or β-catenin-containing adherens junctions marks the cytoskeletal events leading to a substantial improvement in monolayer barrier function. These morphological characteristics are also identical to those observed after ADP ribosylation of RhoA with C3 exoenzyme or treatment with the MLC kinase inhibitor KT-5926 (4, 9, 14, 20, 23). Thus the increased F-actin observed at cell-cell junctions in C3- and Y-27632-treated EC suggests that this pool of F-actin is formed when RhoA or ROCK is inhibited.

Rho proteins have been reported to indirectly modulate the formation of integrin-dependent focal adhesion sites at the ends of large F-actin filament bundles (2, 16). In the present study, Y-27632 caused EC to lose paxillin-containing focal adhesions and prevented the thrombin-induced increase in the PY content of paxillin. Y-27632 stimulated a decline in the tyrosine phosphorylation of both paxillin and p125FAK with or with-
out exposure to thrombin, indicating that the thrombin-induced activation of p125<sup>FAK</sup> may lead, in part, to increased tyrosine phosphorylation of focal adhesions. Taken together, these data suggest that by adjusting the F-actin cytoskeleton, ROCK plays an indirect role in the modulation of focal adhesions and their PY-containing proteins.

Thrombin stimulates actomyosin-based stress fiber contractile forces, causing cell retraction and rounding in EC and neurites (10, 14). In the present study, we tested whether thrombin-induced monolayer barrier dysfunction was due to a contractile mechanism. Y-27632 (10 μM) completely inhibited the biochemical events of thrombin-induced EC contraction by arresting the phosphorylation of MLC and the formation of myosin ribbons linked to F-actin stress fibers. Although this treatment significantly attenuated thrombin-induced monolayer hyperpermeability, Y-27632 did not completely prevent thrombin-induced large hole formation. Thus, thrombin-induced formation of stress fibers and focal adhesions is ROCK dependent, whereas the initiation of large paracellular hole formation appears to be ROCK independent. In addition, thrombin-induced monolayer hyperpermeability is only partially dependent on MLCP and stress fiber formation. A recent study demonstrated that serine/threonine phosphatase inhibitors stimulated monolayer hyperpermeability that appeared to be independent of MLCP (8). Although the MLC kinase inhibitor KT-5926 prevented MLCP, it did not block monolayer hyperpermeability, suggesting that changes in cell-cell adhesion may be involved in the process. Together, these data suggest that thrombin-induced monolayer barrier dysfunction involves both the phosphorylation of MLC leading to EC contraction and cell-cell adhesive mechanisms.

In conclusion, our results confirm recent reports in which Rho proteins have been shown to be important...
mediators of the cytoskeletal events associated with thrombin. Y-27632 alone stimulated enhanced monolayer barrier function due to an increase in EC surface area resulting from the disassembly of stress fibers and the reorganization of F-actin to cell-cell junctions. In addition, ROCK modulates the thrombin-induced phosphorylation of MLCK as well as the formation of myosin ribbons and F-actin stress fibers in EC. However, although thrombin-induced monolayer hyperpermeability is partially dependent on ROCK-stimulated F-actin stress fiber formation, thrombin-induced paracellular hole formation appears to be ROCK independent.

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