RhoA inactivation enhances endothelial barrier function

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RhoA inactivation enhances endothelial barrier function. Am. J. Physiol. 277 (Cell Physiol. 46): C955–C964, 1999.—The modulation of endothelial barrier function is thought to be a function of contractile tension mediated by the cell cytoskeleton, which consists of actomyosin stress fibers (SF) linked to focal adhesions (FA). We tested this hypothesis by dissociating SF/FA with Clostridium botulinum exoenzyme C3 transferase (C3), an inhibitor of the small GTP-binding protein RhoA. Bovine pulmonary artery endothelial cell (EC) monolayers given C3, C3 + thrombin, thrombin, or no treatment were examined using a size-selective permeability assay and quantitative digital imaging measurements of SF/FA. C3 treatment disassembled SF/FA, stimulated diffuse myosin II immunostaining, and reduced the phosphorysorine (PY) content of paxillin and 130- to 140-kDa proteins that included p125FAK. C3-treated monolayers displayed a 60–85% decline in F-actin content and a 170–300% increase in EC surface area with enhanced endothelial barrier function. This activity correlated with reorganization of F-actin and PY protein(s) to β-catenin-containing cell-cell junctions. Because C3 prevented the thrombin-induced formation of myosin ribbons, SF/FA, and the increased PY content of proteins, these characteristics were Rho dependent. Our data show that C3 inhibition of Rho proteins leads to CAMP-like characteristics of reduced SF/FA and enhanced endothelial barrier function.

Clostridium botulinum exoenzyme C3 transferase; immunofluorescent digital imaging; stress fibers; myosin; β-catenin

Small GTP-binding proteins act as triggered amplifiers of signal transduction. Rho proteins are members of the Ras superfamily of small GTP-binding proteins, which modulate the formation of SF/FA in many cell types (3, 5, 13, 15). The activation of Rho proteins appears to stimulate multiple signaling mechanisms linked to the modulation of the F-actin cytoskeleton. GTP-bound Rho activates phosphatidylinositol 4-phosphate 5-kinase and initiates the synthesis of 4,5-bisphosphate, a substrate that regulates a variety of actin-binding proteins, causing the polymerization of F-actin and the formation of FA (4). The rapid transformation of RhoA from its inactive GDP-bound form to its active GTP-bound form allows for the transient activation of Rho kinase (3). Rho kinase causes SF/FA formation by initiating myosin light chain (MLC) phosphorylation (MLC-P) due to two mechanisms: direct phosphorylation of MLC at serine-19 and inhibition of myosin phosphatase activity via phosphorylation of the regulatory myosin binding subunit of myosin phosphatase (3, 15). Phosphorylation of MLC reveals myosin’s actin-binding site, initiating myosin II aggregation into spots and ribbons along actin filaments, forming SF/FA (5, 9, 10). FA, which consist of a number of phosphorysorine (PY)-containing proteins, including paxillin and FA kinase (p125FAK), are seen as streaklike plaques located at the ends of SF (for review see Ref. 5). These integrin-mediated adhesion sites serve as anchoring points between actomyosin filaments and the extracellular matrix, permitting the generation of contractile forces associated with directed cell migration (29). The bacterial toxin Clostridium botulinum exoenzyme C3 transferase (C3) specifically inhibits the small G proteins of the Rho subfamily by ADP-ribosylating the GTP-binding site (19). Inhibition of RhoA leads to a reduction in MLC-P and loss of SF in neurites and human umbilical vein endothelial cells (HUVEC) (8, 13, 29) as well as SF/FA disassembly in Swiss 3T3 cells (20). Signals that cause an elevation in the intracellular free Ca2+ concentration initiate the formation of MLC-P and increased contractile force (for reviews see Refs. 1 and 11). This process appears to be activated by the serine protease thrombin, which stimulates increased MLC-P, leading to myosin ribbon formation and SF/FA (10). These events mediate increases in centripetal tension and endothelial barrier failure (9, 17, 24). It has been proposed that the thrombin-induced development of SF/FA and endothelial barrier dysfunction may be Ca2+/protein kinase C (PKC) dependent (9, 17). However, the thrombin-induced increase in intracellular free Ca2+ concentration returns to baseline levels long before SF/FA and endothelial barrier dysfunction subside. In addition, thrombin-induced SF are unaffected by Ca2+ chelators and PKC inhibitors (27). These data suggest that the thrombin-induced forma-
tion of SF/FA may be modulated by other second messenger systems. The purpose of the present study was to test the role of Rho proteins in the modulation of the EC cytoskeletal and endothelial barrier function. We used C3 to inhibit Rho protein-dependent formation of SF/FA, then used measurements of the size-selective permeability of endothelial monolayers and quantitative immunofluorescence digital imaging microscopy to characterize the EC cytoskeletal morphology with EC barrier function. We show that C3 inhibition of RhoA caused the disassembly of SF/FA and reorganized F-actin and PY-containing proteins to β-catenin-containing cell–cell junctions, a process that enhanced endothelial barrier function. In addition, C3 prevented thrombin-induced myosin ribbons and SF/FA formation, indicating that these processes are Rho dependent. However, thrombin-stimulated endothelial barrier failure was not prevented by C3. These data show that Rho plays a major role in the modulation of SF/FA and endothelial barrier function and imply a partial role for noncytoskeletal forces in thrombin-induced barrier failure.

MATERIALS AND METHODS

Cell culture. EC, isolated from bovine pulmonary arteries as previously described (23), were grown in DMEM containing 10% fetal bovine serum (HyClone, Ogden, UT). Experiments were performed with cells between passages 5 and 11 at 4–6 days postconfluence.

[^32P]ADP-ribosylation of Rho. EC grown in 60-mm dishes were treated with or without 10 µg/ml of C3 ADP-ribosyltransferase for 16 h (Upstate Biotechnology, Lake Placid, NY or Calbiochem, San Diego, CA). The cells were lysed in ice-cold buffer containing 1% NP-40 and 50 mM Tris·HCl, pH 8.0, with 10 µg/ml each of leupeptin, aprotinin, and pepstatin A. The cells were scraped from the culture dish, passed through a 26-gauge needle, and centrifuged at 12,400 g for 5 min at 4°C. Cell lysates (10 µl) were incubated with 12.5 µl of ADP and PY-containing proteins to β-catenin-containing cell–cell junctions, a process that enhanced endothelial barrier function. We show that C3 inhibition of RhoA caused the disassembly of SF/FA and reorganized F-actin and PY-containing proteins to β-catenin-containing cell–cell junctions, a process that enhanced endothelial barrier function. In addition, C3 prevented thrombin-induced myosin ribbons and SF/FA formation, indicating that these processes are Rho dependent. However, thrombin-stimulated endothelial barrier failure was not prevented by C3. These data show that Rho plays a major role in the modulation of SF/FA and endothelial barrier function and imply a partial role for noncytoskeletal forces in thrombin-induced barrier failure.

Immunoprecipitation and immunoblotting. EC grown in six-well plates were treated as described above and lysed by the addition of 1 ml of 4°C extraction buffer containing 20 mM Tris·HCl, pH 7.6, 1% Triton X-100, 5 mM EDTA, 1 mM Na3VO4, 0.1 mM NaN3, 200 µM phenylarsine oxide, 3 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 µg/ml each of aprotinin, leupeptin, and pepstatin. Equivalent amounts of supernatant protein (135 µg) were precleared with 20 µl of recombinant protein A-agarose (Oncogene Sciences, Cambridge, MA) and immunoprecipitated overnight with 2–4 µg of the affinity-purified rabbit antibodies to Rhoc, mouse anti-PY (PY99) IgG (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti-paxillin antibodies at 4°C, then subjected to capture with 20 µl of protein A-agarose. The washed pellets were diluted using 2× SDS sample buffer, with 10% 2-mercaptoethanol, boiled for 5 min, and separated by SDS-PAGE in 12% or 7.5% slab gels with a Laemmli buffer system. The proteins were transferred to 0.2-µm nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in Towbin's solution at 4°C. The membranes were immunoblotted with antibodies to Rhoc (Santa Cruz Biotechnology), paxillin, p125FAK (Transduction Laboratories), and anti-PY (4G10, Upstate Biotechnology) with enhanced chemiluminescence plus detection (Amersham, Arlington Heights, IL). X-ray films were developed at various time intervals to obtain an exposure within the linear range of the film. Densitometry was performed using MetaMorph software.

Endothelial monolayer barrier function. The techniques listed here have been described in detail elsewhere (23–25). Briefly, the size-dependent passage of fluorescein isothiocyanate-labeled hydroxy ethyl starch macromolecules across bovine pulmonary artery EC monolayers was used to detect the formation or absence of a small “pore” barrier. At 4 days postconfluence, each monolayer was incubated with 10 or 25 µg/ml of C3 for 36 h in conditioned media. Monolayer barrier function in the presence or absence of 10 U/ml of thrombin was performed exactly as previously described (24).

Digital imaging of immunofluorescent microscopy. An Olympus IMT-2 microscope was used with the following attachments: 1) an Olympus ×60, 1.4 NA immersion objective, 2) an Olympus x60 lamp and an achromatic focusing lens (Optonique, Highland Mills, NY), 3) an eight-position Metatek filter wheel and shutter containing 490 ± 10, 555 ± 15, and 635 ± 15 nm excitation filters (Chroma Technology, Brattleboro, VT), 4) a quad filter set polychromatic beam splitter/emitter (series 84, Chroma Technology), and 5) a z-axis controller (Ludl Electronic Products, Hawthorne, NY). Epi-fluorescent digital images were collected with a charge-coupled device camera (model PXL, Photometrics, Tucson, AZ). MetaMorph software (version 2.5, Universal Imaging) was used to color encode the separately obtained fluorescein (fluorescein, tetramethylrhodamine, and CY-5) images of each cell.

Labeling of cytoskeletal structures. EC grown to 4 days postconfluence on gelatin/fibronectin-coated eight-well plastic (Nunc, Naperville, IL) slides or Transwell membranes were stained with the following monoclonal antibodies: 1) paxillin (monoclonal antibody to paxillin [paxillin] (3G4, Chemicon International), 2) β-catenin (no. 13-8400, Zymed Laboratories), and 3) affinity-purified rabbit antibodies to nonmuscle myosin II (BT-561, myosin II, Biomedical Technologies, Stoughton, MA), anti-RhoA, or anti-PY (4G10 (Upstate Biotechnology, Lake Placid, NY), Oregon Green 488-phalloidin (Molecular Probes, Eugene, OR) was used to label F-actin. The cells were fixed and permeabilized with 4% formaldehyde in PBS for 1 min, then for 30 min with 2% formaldehyde. 0.2% Triton X-100, and 0.5% deoxycholate, as described by Goekeler and Wysolmerski (10). Nonspecific binding was minimized by incubation with PBS containing 1% BSA and 0.05% polyethylenimine for 15 min at room temperature. EC were stained with anti-paxillin (1:200 dilution), anti-β-catenin (1:50 dilution), or 4G10 (1:50 dilution) in 0.1% BSA-PBS for 60 min, washed, and incubated with anti-myosin II (1:25 dilution) or Rhoc (1:50 dilution) for 60 min. After they were washed and incubated with 5% goat serum, the cells were incubated with TRITC-labeled anti-rabbit IgG (1:25 dilution) and goat-Cy5 anti-mouse IgG (1:25 dilution; Jackson ImmunoResearch Labs, West Grove, PA) at room temperature for 45 min. After the cells were washed again, F-actin was stained with Oregon Green 488-phalloidin. The slide was covered with 30 µl of Vectashield mounting medium (Vector Laboratories, Burlington, CA) and a no. 1 coverslip and sealed with nail polish. We did not observe changes in these staining patterns in the presence or absence of mouse or rabbit IgG.
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Fig. 1. Clostridium botulinum exoenzyme C3 transferase (C3)-induced $^{32}$P ADP-ribosylation and Western blotting of Rho in bovine pulmonary artery endothelial cells (EC). Autoradiogram shows C3-induced $^{32}$P ADP-ribosylation of Rho in EC (lane 1) that was prevented by pretreatment with C3 (10 µg/ml for 16 h; lane 2). Western blotting of RhoA detected a ~22-kDa protein band in EC lysates (lane 3) that was also detected by immunoprecipitation with RhoA polyclonal antibody (lane 4). MW, molecular weight.

Quantification of endothelial surface area and F-actin content. After endothelial barrier function measurements, each Transwell membrane was fixed and double stained for F-actin (tetramethylrhodamine isothiocyanate-phalloidin) and β-catenin, as described above. Blinded digital images for both fluorochromes were collected simultaneously (10 images/treatment) and at random for each Transwell membrane of each treatment group. MetaMorph software was used to trace the β-catenin outline of each cell and compute EC surface area ($\mu$m$^2$). The F-actin content was computed for the identical cells as the total tetramethylrhodamine isothiocyanate fluorescence intensity divided by the surface area of each EC. We divided each random image into two distinct populations of C3-treated EC on the basis of the following morphological criteria: 1) the most responsive (MR) C3-treated EC were large EC that lacked F-actin SF, but a thin rim of F-actin was present at cell-cell junctions; and 2) the least responsive (LR) C3-treated EC exhibited a linear shape and displayed thinner, less prominent actin filaments than control cells, as well as reorganization of F-actin to a thin junctional rim. The percentages of the MR and LR EC were computed from the total number of cells counted.

Statistical analysis. Values are means ± SE. The 2$\alpha$ = 0.05 level was selected for statistical significance. A paired Student's t-test and an ANOVA (Hewlett-Packard 41CV Stat Pack) were used to compare each test interval with baseline values for selected measurements.

RESULTS

Immunoprecipitation and $^{32}$P ADP-ribosylation. RhoA was detected as an ~22-kDa protein by Western blotting of EC lysates with or without immunoprecipitation (Fig. 1, lanes 4 and 3, respectively). C3-dependent $^{32}$P ADP-ribosylation of EC lysates revealed a protein band with an apparent molecular mass of ~25 kDa (Fig 1, lane 1). The appearance of this $^{32}$P ADP-ribosylated protein was prevented by pretreatment with C3 (Fig 1, lane 2).

Effects of C3, C3 + thrombin, and thrombin on endothelial barrier function. We used a high-performance size exclusion chromatography technique (25) to quantitatively measure the size and number of holes formed across endothelial monolayers by analyzing their size-selective solute permeability characteristics. Restricted diffusion, displayed by control monolayers (Fig 2A) and created by predominantly small "pores" (~250-A pore radius), is characterized by a decline in the size-selective permeability (P)-to-free diffusion coefficient (D$_0$) ratio with increasing solute molecular radius (a$_e$) (23). In contrast, the formation of large holes (~>2,000-A pore radius) is characterized by an increase in P/D$_0$ for solutes of increased a$_e$ (24). C3 treatment (25 µg/ml for 36 h) significantly (α = P < 0.05; Fig. 2A) enhanced this endothelial barrier function characteristic, an effect that was not seen when 10 µg/ml of C3 was used (data not shown). In contrast, endothelial monolayers exposed to 10 U/ml thrombin exhibited barrier failure, as characterized by a shift in the P/D$_0$ vs. a$_e$ curve upward and to the right in comparison to control (Fig 2B). Pretreatment of endothelial monolayers with C3 (25 µg/ml for 36 h) did not inhibit the barrier failure characteristic of thrombin alone (Fig 2B).

![Fig. 1. Clostridium botulinum exoenzyme C3 transferase (C3)-induced $^{32}$P ADP-ribosylation and Western blotting of Rho in bovine pulmonary artery endothelial cells (EC). Autoradiogram shows C3-induced $^{32}$P ADP-ribosylation of Rho in EC (lane 1) that was prevented by pretreatment with C3 (10 µg/ml for 16 h; lane 2). Western blotting of RhoA detected a ~22-kDa protein band in EC lysates (lane 3) that was also detected by immunoprecipitation with RhoA polyclonal antibody (lane 4). MW, molecular weight.](http://apcell.physiology.org/)

![Fig. 2. C3 enhances endothelial monolayer barrier function. Ratios of permeability to free diffusion coefficient (D$_0$) vs. solute molecular radius curves are shown. A: control monolayers exhibited restricted diffusion, characterized by a polynomial decline in this curve. Pretreatment with C3 (25 µg/ml for 36 h) produced significantly enhanced barrier function for all solute molecular radii (*P < 0.05). B: monolayers exposed to thrombin (T, 10 U/ml) exhibited solute convection characterized by upward and rightward shift in curve, a property of large paracellular hole formation (24). C3 (25 µg/ml for 36 h) followed by thrombin (10 U/ml, C3-T) did not prevent this effect. There was no significant difference between thrombin and C3 + thrombin treatments. Values are means ± SE of 6 separate determinations.](http://apcell.physiology.org/)
Heterogeneous F-actin morphology of C3-treated EC with or without thrombin. 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-catenins (6). We previously showed enhanced EC barrier function in association with reorganization of F-actin to a thin junctional rim after treatment with cAMP (22). To test whether C3-induced reorganization of F-actin to a thin rim colocalized at adherens junctions, Transwell membranes from the barrier function assays listed above were fixed and double stained for F-actin and β-catenin. Control cells displayed actin-containing SF and prominent dense peripheral bands of F-actin (Fig. 3a). Two morphologically distinct populations of C3-treated EC were observed. The MR C3-treated EC were large cells that exhibited no distinct F-actin SF, but a thin rim of F-actin was observed at β-catenin-containing cell-cell junctions (Fig. 3, b and f). In contrast, the LR cells in our C3-treated population exhibited reorganization of F-actin to the cell border in conjunction with thinly attenuated actin filaments. In the MR and LR C3-treated EC, the rim of F-actin (arrows, Fig. 3, b and c) colocalized with β-catenin (arrows, Fig. 3, f and g). In addition, the β-catenin fingerlike staining pattern in control (arrow, Fig. 3e) and thrombin-treated EC (arrows, Fig. 3h) appeared linked to F-actin filaments (Fig. 3a; arrow, Fig. 3d). Thrombin stimulated the formation of actin filament bundles that was prevented by C3 pretreatment, although these cells displayed large paracellular holes that did not show β-catenin staining. Thrombin-induced hole formation was unaffected by C3 pretreatment (Fig. 3, c and d; and Fig. 3, d and h).

Heterogeneous effects of C3 on F-actin content and EC surface area. The MR C3-treated EC (18.5 ± 3%) showed a 300% increase in EC surface area compared with the controls (1,018 ± 84 and 329 ± 14 µm², respectively; Fig. 4A). In contrast, the LR C3-treated EC displayed a 170% increase in EC surface area (549 ± 29 µm², P < 0.0001) compared with the control or thrombin-treated groups. The same characteristics were seen in the group treated with C3 + thrombin. EC surface area after thrombin treatment was not significantly different from that of the control group. Measurements of F-actin intensity per unit cell surface area (µm²) showed that C3 treatment initiated a decline in F-actin content (Fig. 4B). The LR and MR C3-treated EC showed 60 and 85% declines in F-actin content, respectively, compared with the control group. This effect was not significantly different from the group treated with C3 + thrombin. The F-actin content of the thrombin treatment group was below that of the control group.

C3 stimulates diffuse myosin II immunostaining and prevents thrombin-induced myosin ribbon formation. Activated Rho kinase and thrombin are known to stimulate the phosphorylation of MLC on serine-19 (MLC-P), a biochemical process reduced by C3 (2, 8, 29). Because MLC-P initiates the reorganization of myosin spots into ribbons along actin filament bundles (10, 29), we tested whether thrombin-induced myosin spots and ribbons were inhibitable by C3. In addition, we determined whether C3 treatment alone caused a diffuse pattern of myosin II immunostaining, an indirect characteristic of reduced MLC-P (10). Thrombin stimulated the creation of myosin ribbons that traversed in a focal plane below the nucleus across the base of most cells (Fig. 5b, arrows and inset). In contrast, control EC showed random myosin spots (Fig. 5a, arrows and inset). The MR EC stimulated with C3 and C3 + thrombin displayed a diffuse, perinuclear, myosin staining pattern (Fig. 5, c and d, respectively). Because each image was collected at the focal plane of F-actin at the base of the cell, the appearance of the nucleus with perinuclear myosin staining in these C3-treated EC indicates that these cells had flattened. Similar reorganization of the myosin focal plane was seen in the LR and MR EC; however, the loss of myosin spots was less marked in the LR C3-stimulated EC (data not shown).

C3 disassembles SF and FA, reorganizes F-actin to a thin junctional rim, and prevents thrombin-induced SF/FA. Because the formation of SF/FA appears to be Rho dependent (13), we tested whether C3 disassembled SF/FA and prevented thrombin-induced SF/FA formation by triple immunostaining EC monolayers for F-actin (green), paxillin (red), and nonmuscle myosin II (blue; Fig. 6). Control EC showed prominent dense peripheral bands of F-actin (Fig. 6a, double arrows) that appear to be above the focal plane. Colocalized myosin II and F-actin appear as a few blue-green filaments that cross the base of the cell (Fig. 6a, arrow). Paxillin-containing FA (arrowheads) are seen at the ends of these filaments as red and orange streaklike plaques. Thrombin stimulated the formation of thick blue-green-appearing actomyosin SF (arrow, Fig. 6c) that spanned the entire base of the cell and terminated in paxillin-containing FA. These FA colocalized paxillin with F-actin, as indicated by the color transition from green to yellow to red (arrowhead, Fig. 6c) at the ends of these filaments. The dense peripheral bands of F-actin (double arrows, Fig. 6c) that appear out of focus.
Above the base of this EC indicate the rounded, "contractile" nature of the thrombin-treated EC. These morphological characteristics were not displayed in the C3-treated EC. The MR C3 and C3 + thrombin-treated EC displayed disintegrated F-actin SF and redistribution of F-actin (green) to a thin rim at the perimeter of these cells (arrow, Fig. 6, b and d). The image plane of these EC displayed a prominent nucleus with a diffuse myosin II staining pattern and small disorganized streaks of paxillin in the nuclear region, characteristic of cell flattening. A few paxillin-containing streaks (arrowhead, Fig. 6d) were associated with thin, blue-green actomyosin filaments.

C3 stimulates reorganization of PY-containing proteins to cell-cell junctions. Because FA appear as streak-like plaques containing a variety of PY-containing proteins, we tested whether treatment with C3, C3 + thrombin, and thrombin altered the immunofluorescence-staining pattern of these PY-containing proteins compared with controls (Fig. 7). Control EC displayed PY-containing proteins at random locations around the perimeter of the cell (arrows) and a few streaklike plaques localized at the ends of SF (arrowheads, Fig. 7a). In contrast, thrombin stimulated a marked increase in the number of streaklike PY-containing proteins at the base of the cell (arrowheads, Fig. 7b and inset) and at the cell edge (arrows). This pattern corresponds to the paxillin-containing plaques seen at the ends of SF (arrowhead, Fig. 6c). In the MR and LR C3-treated EC, there was complete loss of these streaklike plaques, and PY-containing proteins were reorganized to the cell-cell junctions (arrows, Fig. 7c). A similar effect was observed in EC treated with C3 + thrombin (arrows, Fig. 7d).

C3 inhibits tyrosine phosphorylation of the FA protein paxillin. We next identified the PY-containing proteins in control EC and EC stimulated with C3, C3 + thrombin, and thrombin by immunoprecipitation with anti-paxillin and anti-PY (PY99) antibodies followed by Western blot analyses with anti-PY (4G10) and anti-paxillin IgG (Fig. 8). Although C3 did not affect the quantity of immunoprecipitated paxillin (Fig. 8B), this inhibitor stimulated a decline in the PY content of two protein bands, paxillin and a 130- to 140-kDa band, with or without thrombin treatment (Fig. 8, A, C, and D). Densitometry of the thrombin-stimulated PY-containing proteins revealed an increase in the PY content of paxillin and the 130- to 140-kDa protein band (Fig. 8, B, C, and D), which included p125Fak (data not shown). Because C3 prevented these changes in the presence or absence of thrombin, these tyrosine phosphorylation events were Rho dependent.

DISCUSSION

The major finding of this study is that C3-dependent ADP-ribosylation of Rho proteins caused SF/FA disassembly and enhanced endothelial barrier function. This process reflects a reduction in basal cytoskeletal tension, as indicated by the loss of actin SF and punctate myosin staining, a reduction in F-actin content, and an increase in EC surface area, which mediated a decline in the size-selective permeability of endothelial monolayers. Hallmarks of these cAMP-like events were reorganization of F-actin and PY-containing proteins to a thin band that colocalized with β-catenin-containing cell-cell junctions and reduction in size of the paracellular space. Because the thrombin-stimulated formation of myosin ribbons and assembly of SF/FA were inhibitable by C3, these events appear to be Rho dependent. However, C3 did not inhibit thrombin-induced barrier dysfunction, implying that this process is partially mediated by noncytoskeletal forces.

We used recombinant C3 to test the role of Rho proteins in the SF/FA-dependent modulation of endothelial barrier function. The identity of the 22- to 25-kDa protein that we detected in our EC is probably RhoA for the following reasons: 1) this protein was detected by a polyclonal antibody to RhoA at an apparent molecular mass similar to that described in a recent report (2), 2)
this band was [32P]ADP-ribosylated by C3, and 3) detection of this activity was inhibited by exposure to C3 for 24 h.

Previous reports suggest that the effects of C3 on cellular functions may be limited by the slow uptake of C3 into cells, which results in the heterogeneous F-actin SF morphology (2, 12). In this regard, Aepelebacher et al. (2) reported that although many C3-treated HUVEC showed no SF, a significant percentage of these cells displayed substantial F-actin filaments. They attributed this effect to inefficient endocytosis of C3 by the SF-containing subpopulation. In the present study, our C3-treated monolayers displayed similar heterogeneous F-actin morphologies. However, all C3-treated EC exhibited diffuse myosin II staining, decreased F-actin content, reduced PY of FA plaques, and increased surface area. These data suggest that the activity of C3 is greater in EC than that revealed by F-actin staining alone.

Activated Rho proteins have been reported to modulate the direct and indirect phosphorylation of serine-19 of MLC (3, 15), a process that reveals myosin's actin-binding site and initiates myosin II aggregation from a diffuse cloud into spots and ribbons along actin filaments, forming SF/FA (5, 10, 29). These findings are confirmed by recent studies in EC that show that C3 reduces MLC-P and prevents thrombin from generating this product (8, 29). This activity is similar to that initiated by cAMP or the MLC kinase inhibitor KT-5926 (13, 21, 22). Our morphological data provide indirect evidence of this C3 activity. We show that this exoenzyme stimulates a diffuse myosin-staining pattern with reorganization of F-actin and PY-containing proteins to cell-cell adherens junctions, flattening of EC, and increased surface area with enhanced monolayer barrier function. These findings indicate that C3 reduces MLC-P, leading to diffuse myosin, EC flattening, and an increase in EC surface area.

How inhibition of active Rho proteins affects monolayer barrier function remains unclear. Using Clostridium difficile toxin B to UDP-glucosylate and inhibit Rho proteins, Hippensiel and colleagues (12) reported an 80% decline in F-actin and complete barrier failure in porcine aortic EC monolayers. cAMP-increasing agents did not prevent this barrier defect, indicating a noncontractile mechanism. C. difficile toxin B appears to affect multiple GTPase-linked processes because of its inhibition of a variety of small G proteins of the Rho superfamily, including Rho, Rac, and CDC42 (26). In the present study, C3 stimulated an increase in the size selectivity of EC monolayers, which indicates an enhancement of endothelial barrier function. These data are similar to those of two recent reports in which C3 initiated a decline in HUVEC monolayer permeability (8, 28). Thus the selective inhibition of Rho proteins by C3 initiates a decline in F-actin and reduced endothelial monolayer permeability to small solutes, whereas inactivation of Rho, Rac, and CDC42 appears to initiate monolayer barrier failure.

RhoA appears to regulate the formation of integrin-dependent FA sites in lymphocytes and EC (2, 16). In the present study we show that C3 prevents the thrombin-induced increase in the PY content of paxillin and PY-containing FA. In addition, C3 alone caused EC to lose paxillin and PY-containing proteins at FA and reorganized tyrosine phosphorylated proteins to intercellular junctions. This process was associated with a decline in the tyrosine phosphorylation of paxillin and a 130- to 140-kDa band that included p125FAK in C3-treated EC with and without exposure to thrombin. Similar tyrosine phosphorylation signals were observed when EC were treated with C3 in the presence...
or absence of cyclic strain (30). In these experiments, cyclic strain caused increased tyrosine phosphorylation of FA, possibly by activation of p125FAK. C3 pretreatment prevented these tyrosine phosphorylation events and reduced the PY content of paxillin and p125FAK. Together, these data suggest that Rho plays an important but perhaps indirect role in the modulation of the PY content of FA (3).

C3-induced changes in cell shape resulted in the formation of tyrosine-phosphorylated proteins at endothelial cell-cell attachment sites. This effect has been seen in vivo in the endothelium of guinea pig aorta, where significant shear stress and cell stretch occur (14). In addition, we previously showed that an expansion of EC surface area by cAMP-increasing agents or KT-5926 initiates similar characteristics (22). These data are consistent with the notion that the C3-induced reorganization of tyrosine-phosphorylated proteins to cell-cell junctions appears linked to an increase in endothelial surface area, producing increased cell-cell apposition.

Thrombin is known to stimulate actomyosin-based SF contractile forces, causing cell retraction and rounding in neurites and EC (9, 12, 21). These effects were prevented by C3 pretreatment of thrombin-stimulated HUVEC or neurites (8, 13). Because C3 treatment of neurites did not prevent thrombin-stimulated Ca²⁺ release, these data suggest that C3 does not inhibit thrombin-induced signal transduction (13). In the present study we extend this view, showing that inhibition of RhoA prevents thrombin-induced formation of myo-

Fig. 6. Effects of C3 and thrombin on stress fibers/focal adhesions. EC monolayers exposed to conditions listed below were fixed, extracted, and triple stained for F-actin (green), nonmuscle myosin II (blue), and paxillin (red). a: control cells exhibit prominent dense peripheral bands of F-actin (double arrows) as well as stress fibers (arrow) linked to paxillin-containing focal adhesions (arrowheads). b: MR (see MATERIALS AND METHODS) C3-treated EC (10 µg/ml for 16 h) exhibit stress fiber disassembly and translocation of F-actin to cell-cell junctions (arrow). c: thrombin-treated EC (10 U/ml for 0.5 h) show paxillin-containing focal contacts (arrowhead) at ends of increased actomyosin stress fibers (arrow). d: MR EC treated with C3 (10 µg/ml for 16 h) and thrombin (10 U/ml for 0.5 h) exhibit stress fiber disassembly. A rim of F-actin is seen at cell-cell junctions (arrow) in association with paxillin remnants (arrowhead). Scale bars, 5 µm.
sin ribbons and PY-containing SF/FA, since these effects were not seen in the EC treated with C3 + thrombin. Thus thrombin-induced formation of SF/FA is RhoA dependent.

Moy et al. (18) proposed that endothelial barrier failure is determined by centripetal cytoskeletal forces generated by MLC-P-dependent actomyosin filaments, and these stresses are opposed by centrifugal forces generated by cell-cell adhesion. Whether Rho-dependent phosphorylation of MLC-P and SF formation cause thrombin-induced endothelial monolayer hyperpermeability remains controversial. In HUVEC, thrombin-induced increases in permeability were prevented or attenuated by pretreatment with C3 for 24 h (8, 28). Our data contrast these findings. Although C3 pretreatment of EC monolayers blocked thrombin-induced SF/FA, C3 did not prevent thrombin-induced barrier dysfunction in bovine pulmonary artery EC. These data suggest that thrombin-induced monolayer hyperpermeability is independent of MLC-P and SF/FA formation. Similar results were reported with use of serine-threonine phosphatase inhibitors to modulate mono-

![Image](https://example.com/image1.png)

**Fig. 7.** C3 and thrombin reorganize phosphotyrosine (PY)-containing proteins. EC were treated as described below, fixed, and immunostained with anti-phosphotyrosine antibody (4G10) in control (a), 10 U/ml of thrombin for 0.5 h (b), 10 µg/ml of C3 for 16 h (c), and 10 µg/ml of C3 for 16 h followed by 10 U/ml of thrombin for 0.5 h (d). a: Control EC displayed a diffuse PY-staining pattern with few streaklike plaques, consistent with focal contact formation (arrowheads). b: Thrombin-stimulated EC displayed increased streaklike plaques (arrowheads), indicating increased PY-containing focal contacts. c: MR C3-treated EC exhibited prominent PY staining at cell-cell junctions (arrows) and no streaklike stains in cytosolic region. d: MR EC treated with C3 and thrombin appeared similar to those treated with C3 alone. Scale bars, 5 µm; inset: 200% enlargement.

![Image](https://example.com/image2.png)

**Fig. 8.** C3 reduces paxillin tyrosine phosphorylation. EC monolayers were pretreated with C3 (25 µg/ml for 48 h) with or without thrombin (10 U/ml for 30 min) or were not treated (IgG and control). EC lysates were immunoprecipitated (IP) with paxillin or anti-PY (PY99) antibody and Western blotted (IB) for paxillin or PY-containing proteins with use of 4G10 antibodies (A–C). Densitometry revealed that relative PY content of paxillin and a 130- to 140-kDa band that included p125FAK was increased by thrombin and reduced in C3-treated EC compared with control (D). A representative blot of 2 separate experiments is shown.
layer barrier function (7). Blockade of these phosphatases initiated maximal MLC-P, which, in turn, was inhibited by the specific MLC kinase inhibitor KT-5926. Although this inhibitor prevented MLC-P, it did not block monolayer hyperpermeability, suggesting that changes in cell-cell adhesion may be involved in this process. These data suggest that thrombin-induced monolayer dysfunction involves MLC-P and cell-cell adhesive mechanisms.

In conclusion, our data indicate that Rho GTPases modulate the thrombin-induced formation of myosin ribbons, SF, and tyrosine-phosphorylated FA in EC. Enhanced barrier function stimulated by C3 appears to be due to the disassembly of SF/FA, reorganization of F-actin and tyrosine-phosphorylated proteins to cell-cell junctions, and increased EC surface area.

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