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Rho modulates hepatic sinusoidal endothelial fenestrae via regulation of the actin cytoskeleton in rat endothelial cells

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The presence of actin-like microfilaments in the vicinity of sinusoidal endothelial fenestrae (SEF) indicates that the cytoskeleton of sinusoidal endothelial cells (SEC) plays an important role in the modulation of SEF. Rho has emerged as an important regulator of the actin cytoskeleton, and consequently cell morphology. The present study aimed to examine how a Rho stimulator; lysophosphatidic acid (LPA), and a Rho inhibitor; bacterial toxin C3 transferase (C3-transferase), affect the morphology of SEF. Monolayers of SEC culture were established by infusing a rat liver with collagenase for 30 min and then culturing in RMPI medium for 24 h. The cells were separated into three groups; control, LPA-treated (15 μM), and C3-transferase-treated (15 μg/ml) groups. SEF morphology was observed by scanning electron microscopy. Formation of F-actin stress fibers was observed by confocal microscopy. Rho A and phosphorylated myosin light-chain kinase were analyzed by Western blotting. Active Rho was measured by Ren's modification. Treatment of SECs with LPA contracted the SEF, concomitant with increases in F-actin stress fiber and actin microfilament, and high expression of phosphorylated myosin light-chain kinase. Following treatment with C3-transferase, SEF dilated and fused, concomitant with a loss of F-actin and microfilament, and low expression of phosphorylated myosin light chain. Rho A expression does not change by both treatments. In conclusion, these results indicate that Rho modulates fenestral changes in SEC via regulation of the actin cytoskeleton.
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Various morphological mechanisms regulate the distribution of blood flow to and from the sinusoids in the hepatic microvascular system. In the sinusoid, endothelial cells and hepatic stellate cells have been identified as the putative cellular elements in the regulation of resistance.[1] Hepatic sinusoidal endothelial cells (SEC) possess fenestrae mostly arranged in sieve plate-like pores. These sinusoidal endothelial fenestrae (SEF) lack a diaphragm and a basal lamina and therefore are open connection between the lumen of the sinusoid and the space of Disse.[2] They are not single and independent pores but are interconnected labyrinth-like structures.[3]

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Members of the Rho-like GTPase family; including Cdc 42, Rac 1 and Rho A, have been recognized as key regulators of signal transduction pathways that mediate the distinct actin cytoskeleton changes required for cell motility and adhesion.[4] Rho regulates the cytoskeleton by triggering the assembly of cytoplasmic stress fibers composed of filamentous actin, and focal adhesions involving a complex of vinculin.[5] Rho signaling has been investigated extensively. *Clostridium botulinum* C3-like transferase (C3-transferase) is an inhibitor of Rho proteins. Its targets are all Rho subfamily proteins (eg, Rho, Rac, and Cdc42), while other low molecular mass GTP-binding proteins including Ras, Rab, arf, or Ran subfamilies, or heterotrimetric G proteins are not modified.[6,7] Lysophosphatidic acid (LPA) is a Rho promoter. LPA binds to surface G-protein-coupled receptors and its biological activities are mediated in part by the cytosolic small GTPase Rho.[8] Among the target molecules of Rho, Rho-kinase (ROCK) has

been recently shown to participate in the induction of focal adhesions and stress fibers in Swiss 3T3 cells and contraction of smooth muscle by mediating calcium sensitization.[9] It has been assumed that Ca^{2+} -signaling pathways mediate the contractile force generated by smooth muscle, where contraction is powered by Ca^{2+}-dependent myosin activation.[10] Contraction of smooth muscle is thus governed principally by a Ca^{2+}-signaling pathway, and Rho A signaling pathways play a secondary role.[11] In contrast, recent studies indicate that contraction of nonmuscle cell types, such as fibroblasts, is mediated primary by Rho A signaling pathways, with Ca^{2+} signaling pathways playing a subordinate role.[12] In hepatic stellate cells, Rho directs activation-associated morphological changes via regulation of the actin cytoskeleton. Rho signaling pathways may play an important role in stellate cell activation and response to liver injury.[13] Actin microfilaments and calmodulin are closely associated with the endothelial plasma membrane, suggesting that in the presence of calcium ions, calmodulin and actin microfilaments may be involved in the contraction and dilatation of endothelial cell fenestrae.[3,14] It is not yet known which signaling pathway plays a greater role in regulating SEF contraction.

The present study was designed to elucidate the role of Rho and actin cytoskeleton in morphological and functional alterations of SEF.

Material and methods

Experimental Animal

Male Wistar strain rats weighing 150–180 g were used in this study. The rats were housed in individual cages and allowed free access to chow and water. All animal procedures in the present experiments were performed in compliance with the standard guidelines for experiments of the School of Medicine, Keio University. In this study, we investigated 20 animals.

Isolation, Purification, and Culture of Sinusoidal Endothelial Cells

SEC were isolated by a modified method of Braet *et al*.[15] Briefly, the liver of a male Wistar rat was perfused with Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution followed by 0.5% collagenase A (Sigma type 1) via a polyethylene catheter inserted into the portal vein trunk. After incubation of the tissue fragments in the same solution at 37°C for 20 min, the resulting cell suspension was centrifuged at 100*g* for 10 min to remove the parenchymal cells. The supernatant containing a mixture of sinusoidal liver cells was then layered on top of a two-step Percoll gradient (25–50%) and centrifuged for 20 min at 900*g*. The intermediate zone located between the two density layers was enriched in SEC. The purity of SEC was further increased by selective adherence of Kupffer cells and spreading of the SEC on collagen. SEC were seeded on collagen coverglass (Becton Dickinson, Biocoat 4426) placed in six-well (Falcon 3046; Falcon, Lincoln Park, NJ, USA) or two-well dishes (Falcon 3001). Serum-free SEC culture medium was RPMI-1640 medium supplemented with 2 mM L-glutamine and 100 μg/ml gentamycin. The dishes were incubated in a CO_2 gas incubator at 37°C for 24 h.

Treatment of Cultured Cells with Bacterial Toxin, C3 Transferase and Lysophosphatidic Acid

The 12-h cultures of hepatic SEC were washed with RPMI-1640 culture medium (37°C) and divided into three groups. C3-transferase and LPA were dissolved in the culture medium. The control group contained culture medium and received no treatment. The C3-transferase-treated group was pretreated with 15 μg/ml of C3 transferase (Wako Pure Chemical Industries. Ltd, Osaka, Japan) for 30 min.[16] The LPA-treated group was pretreated with 15 μM LPA (Sigma Chemical Co., St Louis, MO, USA) for 30 min.[4] We investigated 50 dishes for each group.

Scanning Electron Microscopy

Sinusoidal endothelial cells cultured on a glass coverslip were fixed with 1.2% glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4°C, and postfixed with 1% osmium tetroxide in cacodylate buffer (pH 7.4) for 1 h at 4°C. After dehydration in a graded series of ethanol solution, the cultured cells were dried in a critical point apparatus (Hitachi HCR-2, Tokyo, Japan)[3] and coated with gold in a Hitachi vacuum coating unit. The cell surfaces were observed under a scanning electron microscope (Hitachi, Tokyo, Japan) at 15-kV acceleration voltage.

Confocal Immunofluorescence Microscopy

F-actin staining
SECs were fixed with 4% formaldehyde in PBS at room temperature for 10 min and then permeabilized for 10 min with 0.1%. Triton X-100 in PBS containing 1% BSA. The cells were then incubated with rhodamine–phalloidine (Molecular Probes, Inc., Eugene, OR, USA) for 30 min. Each dish was washed three times with PBS. Then, a confocal laser scanning microscope (FV 300; Olympus, Tokyo, Japan) equipped with argon/krypton laser capable of dual excitation and detection was used to visualize the distribution of F-actin.

Conventional Transmission Electron Microscopy

The cells were fixed in 1.2% glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4°C, and then postfixed with 1% osmium tetroxide in cacodylate buffer (pH 7.4) for 1 h at 4°C. After staining with 1% aqueous uranyl acetate solution for 10 min at room temperature, the cells were dehydrated in a graded series of ethanol solutions. For transmission electron microscopy, the blocks were embedded in Epon after dehydration. Ultrathin sections were cut with a diamond knife on a LKB ultramicrotome (BROMMA), stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (JEM-1200 EX, JEOL, Tokyo, Japan) at 80 kV acceleration voltage.

Heavy Meromyosin-Decorated Reaction

Transmission electron microscopy with the heavy meromyosin binding technique[17] was used to identify actin microfilaments around SEF. Sinusoidal endothelial cells cultured on the bottom of a Petri dish were permeabilized with 0.1% Triton X in PHEM buffer for 5 s. Then the cells were treated with 15% glycerinated PHEM buffer containing 2 mg/ml of heavy meromyosin (Wako Pure Chemical Co, Tokyo, Japan) in PHEM buffer (pH 6.9) at 4°C for 10 min. After rinsing with PHEM buffer, the cells were directly fixed with 1.2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 60 min at room temperature, followed by postfixation with 1% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.4) and dehydration in a graded series of ethanol. The cells were embedded in Epon. Ultrathin sections were cut with a diamond knife on a LKB ultramicrotome, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (JEM-1200 EX) at 80-kV acceleration voltage. Actin filaments were identified by the characteristic 'arrowhead decoration' of thin filaments.

Western Blotting of Phosphomyosin Light-Chain Kinase and Rho A in Isolated SEC

The SEC were lysed with a solution containing 200 mM octyl-α-D glucopyranoside, 100 mM Tris-HCl, and 1 mM phenylmethylsulfonyl fluoride for 1 h at 4°C and centrifuged twice at 2000 rpm for 10 min each. The protein concentrations were determined by the Bradford assay. The samples (30 μg protein/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing condition on 7.5% acrylamide gel, and electrophoretically transferred to a PVD7 (Millipore, Bedford, MA, USA). Filters were blocked with 1% dry milk and 1% bovine serum albumin in PBS with 0.02% Tween for 2 h, probed with antiphosphomyosin light-chain kinase antibody (1:500) and anti-Rho A antibody (1:100) for 1 h, rinsed in PBS, and then probed with a horseradish peroxidase conjugated goat anti-mouse antibody (1:2000) for 30 min. After rinsing in PBS, color was developed with the ECL system (Super signal West Femto, PIRCE).

Measurement of Rho A Activity

Rho A in its inactive state is bound to GDP, and is activated when GDP is exchanged for GTP. To determine if there was an increase in active Rho A, that is, Rho A-GTP, we used the Rhotekin binding domain affinity precipitation assay for Rho A-GTP described by Ren *et al*[18] and O'Connor *et al*.[19] Sinusoidal endothelial cells were grown in a 15-cm diameter dish overnight to 75–85% confluence. The cells were rinsed briefly with PBS for 2–5 min at room temperature to remove serum proteins, and then lysed with 500 μl of 1 × lysis buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, 1% Triton X-100). A cell scraper was used to help dissociate the cells from the plate. After clarifying by centrifugation at 8000 rpm and 4°C for 5 min in a microcentrifuge, the cell lysate (500 μl; 0.5–1.0 μg/μl protein) was added to 20 μl (30 μg) of Rhotekin-RBD beads (Cytoskeleton, Inc., Denver, Co.) and centrifuged at 4000 rpm, 4°C for 3 min. The beads were then washed twice with a washing buffer. Rho A bound to the beads was solubilized in Laemmli's SDS sample buffer and boiled for 5 min. Protein (17 μg of protein) from each extract was subjected to SDS-PAGE in 12% acrylamide gel and transferred to nitrocellulose membrane (Millipore), Rho A was detected by incubation with 1:500 dilution of anti-Rho antibody (supplied with the kit) in TBST overnight at 4°C and a goat anti-mouse horseradish peroxidase-conjugated IgG at 1:50 000 dilution in TBST for 1 h at room temperature. Bands were detected with enhanced chemiluminescence (Pierce Super Signal West Dura Extended Duration Substrate, Pierce, Rockford).

Results

Scanning Electron Micrographs of Sinusoidal Endothelial Fenestrae

In the control hepatic SEC, there were two types of fenestration. One type appeared as sieved plate-like small pores and the other type showed relatively isolated scattered pores (Figure 1a). In C3-transferase-treated SEC, the fenestrae were dilated and fused, forming large pores compared to the control cells, large pores were found around the nucleus, and surrounded by flat, fenestrated cytoplasmic extensions (Figure 1b). In the LPA-treated SEC, the diameters of SEF were decreased. There were very

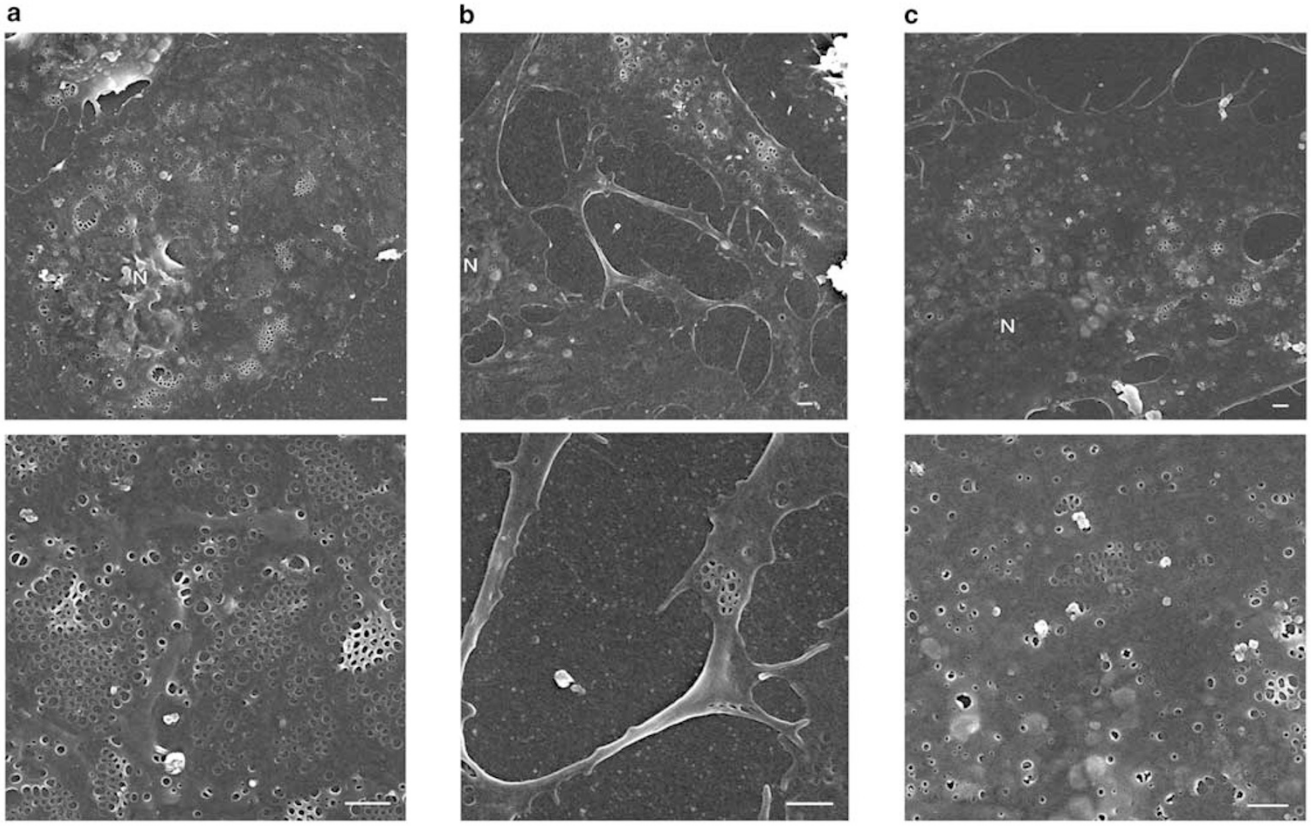

Figure 1 Scanning electron micrographs of sinusoidal endothelial fenestrae (SEF). Counterstained with lead citrate. Upper micrographs; low magnification, Lower micrographs; high magnification. Bar denotes 1 μm. (**a**) Control hepatic sinusoidal endothelial cell (SEC); there are two types of fenestration; sieved plate-like small pores and relatively isolated scattered pores. (**b**) C3 transferase-treated SEC; the fenestrae are clustered, dilated, and fused, forming larger pores compared to control cells. Large pores are found around the nucleus, and surrounded by flat, fenestrated cytoplasmic extensions. (**c**) LPA-treated SEC; diameters of SEF are decreased. There are very few fenestrated areas especially around the nucleus, compared to the control cells. N denotes nucleus.

few fenestrated areas especially around the nucleus, compared to the control SEC (Figure 1c).

Confocal Microscopy

To elucidate the underlying mechanism of C3-transferase and LPA-induced morphological change, we stained actin stress fibers with rhodamine–phalloidine. Staining of non-treated control SECs show a few thin and linear actin stress fibers within each cell (Figure 2a). Treatment of the SECs with C3-transferase for 30 min induced stress fiber disassembly, with a loss of stress fibers compared to the control SECs (Figure 2b). Treatment of the SECs with LPA for 30 min induced formation of dense thick actin stress fibers (Figure 2c).

Transmission Electron Micrographs of Sinusoidal Endothelial Fenestrae

Conventional transmission electron micrograph showed membranous structures running along the SEF (Figure 3a). In SEC treated for 30 min with C3 -transferase, the SEF were dilated, forming large pores, and no filamentous structure is found in the hyaloplasm (Figure 3b). In LPA-treated cells, dense actin filament bundles increased compared to non-treated SEC (Figure 3c).

Transmission electron micrograph of heavy meromyosin-decorated reaction showed decorated filaments orienting close to the SEF (Figure 3d). In SEC treated for 30 min with C3 -transferase, the SEF were dilated and fused, forming large pores, and no filamentous structure was seen in the hyaloplasm (Figure 3e). In LPA-treated cells, dense decorated filaments bundles were increased compared to nontreated SEC (Figure 3f).

Western Blot of Rho A and Phosphorylated Myosin Light-Chain Kinase in Isolated SEC

To confirm the light and electron microscopic results and to further investigate the role of Rho in SECs, we performed Western blotting for Rho and phosphorylated myosin light-chain kinase in untreated, LPA-treated, and C3-transferase-treated SECs. The level of myosine light-chain phosphorylation is regulated in part by myosin phosphatase,

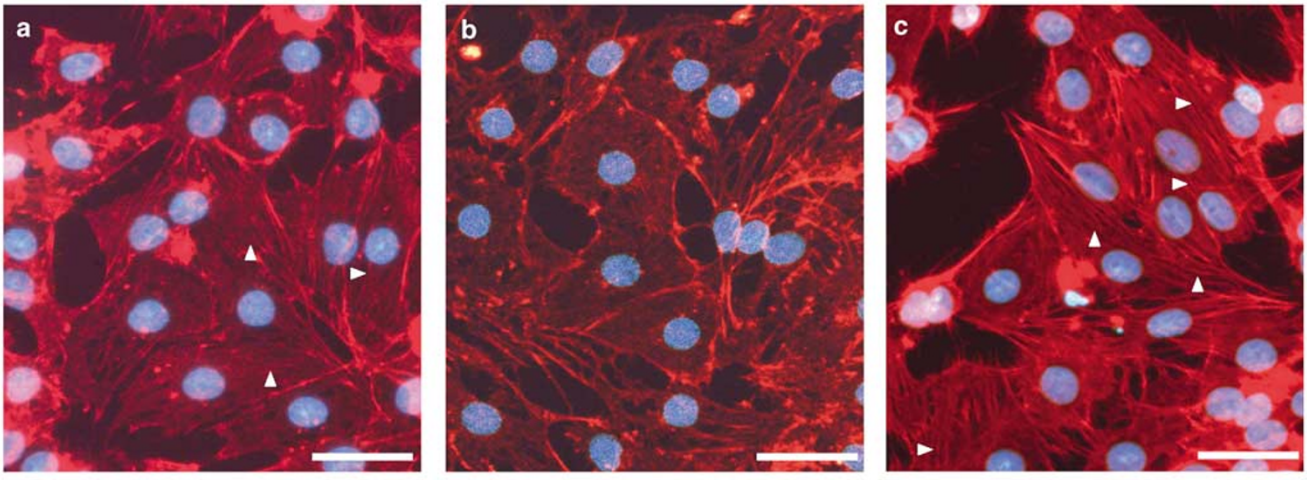

Figure 2 F-actin staining by rhodamine-phalloidine examined by confocal immunofluorescence microscopy. Nuclei were counterstained with DAPI. Scale bar denotes 20 μm. (**a**) Control SEC; thin stress fibers are observed in each cell. (**b**) C3 transferase-treated SEC; there is a loss of stress fibers in the treated cells compared with the control. (**c**) LPA-treated cells; there is an increase of stress fibers compared with the control. Arrowheads denote stress fibers.

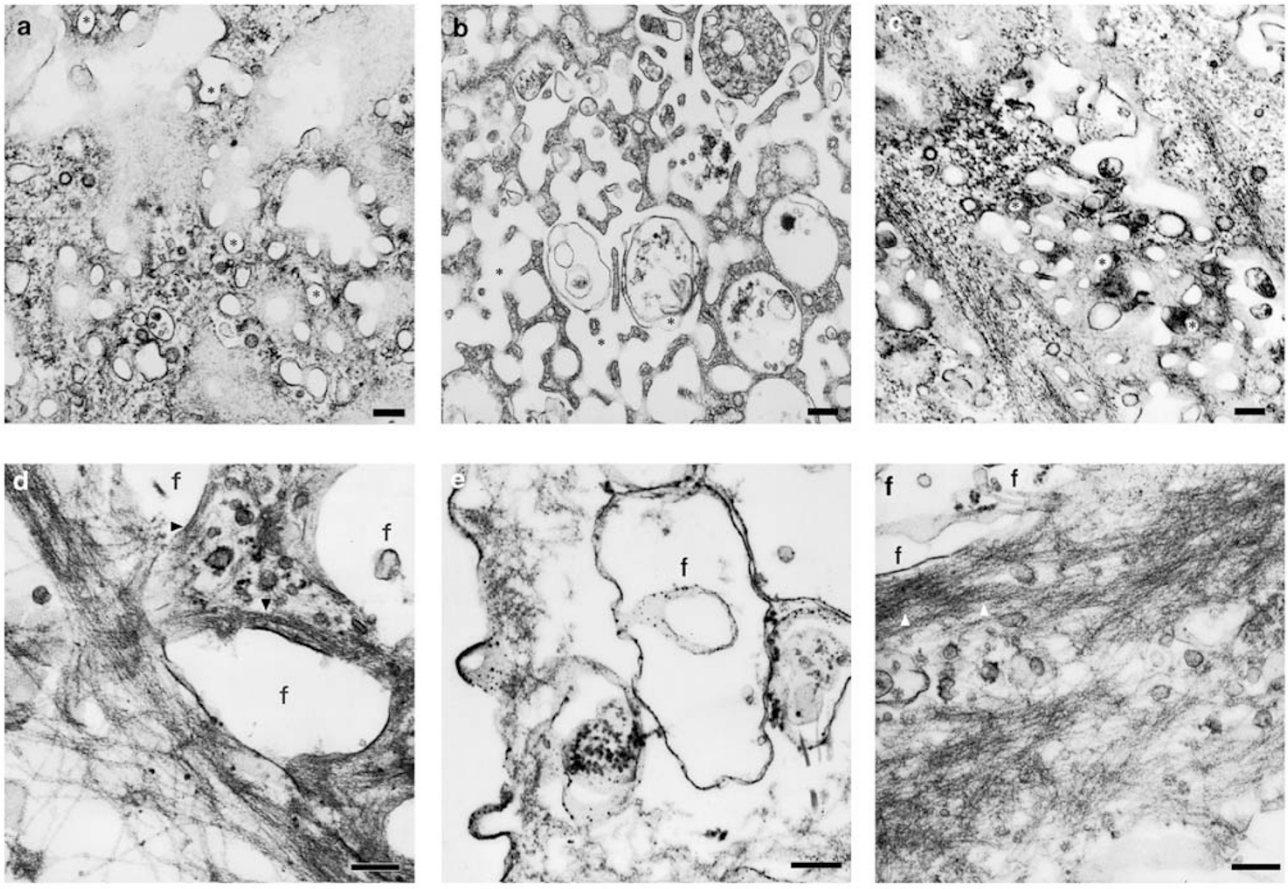

Figure 3 Transmission electron micrographs of sinusoidal endothelial fenestrae. (**a**)–(**c**) Conventional electron microscopy. Bar denotes 200 nm. (**a**) Control SEC; microfilaments are occasionally noted around the labyrinth-like structures of the SEF. (**b**) C3 transferase-treated SEC; microfilaments are hardly noted in the hyaloplasm around the fused and dilated SEF. (**c**) LPA-treated cells; densely packed filament-like structures are seen in the hyaloplasm around the SEF with reduced diameter compared to control. (**d**)–(**f**) Heavy meromyosin-decorated reaction. Bar denotes 100 nm. (**d**) Control SEC; decorated filaments run close to the SEF. (**e**) C3 transferase-treated SEC; fenestrae are dilated and fused, forming large pores. No filamentous structure can be seen in the hyaloplasm. (**f**) LPA-treated cells; bundles of dense decorated filaments are more abundant than those in control cells. Asterisk denotes SEF. f denotes sinusoidal endothelial fenestrae (SEF), and arrowheads denote actin filaments.

which is controlled by ROCK. In the Western blot, phosphorylated myosin light-chain protein expression was high in LPA-treated SCE, but very low in C3-transferase-treated cells (Figure 4), while Rho A expression levels were similar in the three groups

Rho A Activation Assay

SEC were treated with LPA or C3 -transferase for 30 min, lysed, and assayed for active Rho A by RBD binding. Treatment with LPA for 20 min increased the amount of activated Rho A, while there was no increase in control cells or C3-transferase-treated cells (Figure 5).

Discussion

Ultrastructual studies have shown that SECs contain a cytoskeletal framework of filamentous actin,[3] and that the presence of actin in the form of a calmodulin–actomyosin complex is responsible for regulation of the diameter of fenestrae.[3]

In human umbilical vein endothelial cells, Rho-mediated stress fiber assembly induced by thrombin and histamine led to increased tension exerted on junctional lesions, and this could contribute to junction disassembly.[20] Extensive cytoskeletal remodeling of endothelial cells occurred after *in vitro* infection with *Bordetella bacilliformis* (a Rho stimulator). The cells became spindle shaped and contained arrays of actin stress fibers orientated parallel to the long axis of the cell.[21]

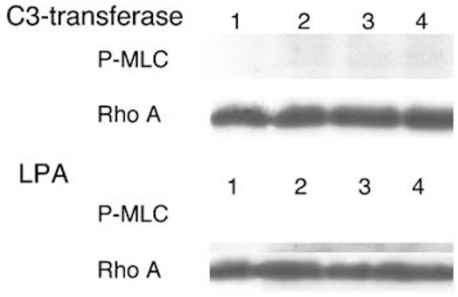

Figure 4 Western blot of Rho A and phosphorylated myosin light chain (P-MLC) in isolated SEC. Samples containing 30 μg of protein were subjected to SDS/PAGE (4–20% gels) and analyzed by Western blotting. Lane 1: 0 min, lane 2: 5 min after treatment, lane 3: 15 min after treatment, lane 4: 30 min after treatment. P-MLC expression is high in LPA-treated cells from 5 to 30 min, but very low in C3-transferase-treated cells. Rho A expression is similar in control, C3 transferase-treated, and LPA-treated cells.

Figure 5 Rho A activation assay in sinusoidal endothelial cells. The cells were stimulated with LPA or C3 transferase for 30 min, lysed, and subjected to Rho A activity assay by RBD binding. Western blot of active Rho is shown. LPA treatment increases the amount of active Rho A compared to untreated control and C3 transferase-treated SECs.

In the present study, treatment with LPA induced the formation of dense thick actin stress fibers in SEC. In the control of nonmuscle cell contractility, there are two regulatory systems of actomyosin contraction. One type of contraction is rapid and lasts for a short duration. Such contraction may be induced by the Ca^{2+}/calmodulin myosin light-chain kinase (MLCK) system and terminated by the Ca^{2+}/calmodulin-MLCK system.[21,22] Thus, contraction triggered by Ca^{2+} may be rapid, short-lived, and localized. Another type of contraction is sustained and may be achieved more readily by controlling the level of myosin phosphate activity, which is a function of ROCK.[22] Moreover, ROCK controls not only MLC phosphorylation (by direct phosphorylation) but also MLC dephosphorylation (by inhibiting myosin phosphatase).[23] This type of contraction would be difficult to regulate by the Ca^{2+}-dependent system alone, as it will be necessary to fine tune Ca^{2+} concentrations inside the cell.

As regards the Ca^{2+}-dependent system, we also demonstrated that attenuation of fenestral plasma membrane Ca^{2+}–Mg^{2+}-ATPase activity by endothelin, which acts on a cell surface receptor coupled to a heterotrimetric G-protein, induced both a rise in intracellular free calcium concentration and activation of Rho.[24] This would cause an elevation of intracellular Ca^{2+} and possibly inhibit extrusion of free Ca^{2+}, resulting in contraction of the SEF.[24]

During cell morphogenesis and motility, cells undergo extensive remodeling of the actin cytoskeleton, a phenomenon mediated by various actin binding proteins.[25] The small guanosine triphosphatase (GTPase) Rho is converted from the inactive GDP-bound form to the active GTP-bound form in response to stimuli such as serum LPA, and induces various morphological events such as cell adhesion and motility.[4] Among the Rho effectors isolated, the ROCK family of Rho-associated serine–threonine protein kinases is implicated in Rho-mediated cell adhesion and smooth muscle contraction.[4] This results in an increase in MLC phosphorylation and consequently an increase in actomyosin-based contractility, events that contribute to Rho-mediated stress fiber formation and smooth muscle contraction.[26]

Many of the effectors of Rho A act via the actomyosin cytoskeleton, but only Rho-associated kinase has been demonstrated to control the level of MLC phosphorylation.[27] We therefore investigated the role of Rho-associated kinase using a bacterial toxin, C3 transferase. Consistent with previous findings,[27] we found that inhibition of Rho A by C3 transferase reduced MLC phosphorylation. We also found that downstream inhibition of ROCK by the inhibitor Y-27632 [(*R*)-(+)-*trans*-4-(1-aminoethl)-

N-(-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate] produced the same results (data not shown).

A third type of contraction is the generation of general cytoplasmic tension. Inhibition of ROCK activity induces stress fiber disassembly and loss of cytoplasmic tension.[28] It is not clear why loss of Rho activity induces stress fiber disassembly and SEF dilatation. We speculate that cytoplasmic tension may in part maintain fenestral structure. Confocal microscopy and transmission electron microscopy showed stress fibers orientating close to the SEF. The SEF, a contractile ring, has an actin-based structure.[29] SEF formation requires *de novo* actin polymerization, and cytoplasmic division occurs by myosin-based contractility generated in this structure.

Stress fibers are commonly thought of as cytoskeletal structures found along the basal plasma membrane. Many basal stress fibers respond to various inhibitors. In previous studies, stress fibers were reported to orient generally parallel to the long axis of SEC.[29] When SEC were treated with cytochalasin B, disrupted stress fibers were observed.[29] In the present SEM study, untreated SEC showed good preservation of characteristic surface ultrastructure, with a central, bulging nucleus, surrounded by flat and fenestrated cytoplasmic extensions. Remarkably, within 1 h of C3 transferase treatment, defenestrated area and large clustered sieve plates were observed around the nucleus. However, there were no clustered fenestrae in the periphery of the cell. The reason for cells to use Rho kinase systems differentially to control peripheral and central stress fibers is not clear. A recent study using human foreskin fibroblasts has reported that the central stress fiber is more sensitive to ROCK inhibitors than to MLCK and calmodulin inhibitors, whereas the peripheral stress fiber is more susceptible to the same set of MLCK and calmodulin inhibitors than to ROCK inhibitors.[22] In the present study, very small fenestrated areas remained after half an hour of LPA treatment. In fenestral contraction, it is known that ROCK is responsible for Rho-mediated enhancement of myosin-based contractility.

The Rho switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state.[26] Understanding the mechanisms that regulate activation and inactivation of the GTPases has obvious biological significance, and is a subject of intense investigation. The fact that many Rho family effector proteins specifically recognize the GTP-bound form of the protein[30] has been exploited experimentally to develop a powerful affinity purification assay that monitors Rho activation.[18] The assay uses the Rho binding domain (also called the RBD) of the Rho effector protein, Rhotekin. The RBD protein motif has been shown to bind specifically to the GTP-bound form of Rho.

The present study successfully applied a pull-down assay specific for GTP-Rho A to intact sinusoidal endothelial cell, using a recombinant GST-Rhotekin fusion protein. Determination of membrane-bound Rho A was previously performed to evaluate cellular Rho A activity,[31] because it was generally thought that membrane-bound Rho A represented the GTP-bound, active form of Rho A. However, a recent study has shown that translocation of Rho A to membranes is also affected by the phosphorylation state of Rho A.[32] We also demonstrated that an increase in Rho A activation plays a role in SEF contraction.

We speculate that Rho modulates fenestral contraction, because SEF pores were contracted by LPA treatment, while SEF clustered, dilated, and fused to form large pores by C3 transferase treatment.

The results provide further evidence for the role of excitatory agonist of Rho in inducing sensitization of MLC phosphorylation to Ca^{2+}, which acts in concert with phospholipase C signaling to result in a full contractile response. Furthermore, the contraction and dilatation of the SEF as well as of the sinusoids would be involved in the regulation of hepatic microcirculation.[3] These findings suggest the crucial role of the calcium–calmodulin–actomyosin complex in the regulation of the fenestral diameter. Therefore, Rho is implicated in physiological and pathological functions associated with cytoskeletal rearrangements such as SEF morphology. These results suggest that Rho A is one of the key regulators of SEF contractility and that the clinical application of Rho A and ROCK should be considered as molecular targets for the treatment of portal hypertension in cirrhosis. Recently, a synthetic ROCK inhibitor (Y-27632) has been shown to be effective for portal hypertension in animal model.[33] This type of inhibitors may have therapeutic potential.

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