Microtubule disassembly increases endothelial cell barrier dysfunction: role of MLC phosphorylation

ALEXANDER D. VERIN, ANNA BIRUKOVA, PEIYI WANG, FENG LIU, PATRICE BECKER, KONSTANTIN BIRUKOV, AND JOE G. N. GARCIA. Microtubule disassembly increases endothelial cell barrier dysfunction: role of MLC phosphorylation. Am J Physiol Lung Cell Mol Physiol 281: L565–L574, 2001—Endothelial cell (EC) barrier regulation is critically dependent on cytoskeletal components (microfilaments and microtubules). Because several edemagenic agents induce actomyosin-driven EC contraction tightly linked to myosin light chain (MLC) phosphorylation and microfilament reorganization, we examined the role of microtubule components in bovine EC barrier regulation. Nocodazole or vinblastine, inhibitors of microtubule polymerization, significantly decreased transendothelial electrical resistance in a dose-dependent manner, whereas pretreatment with the microtubule stabilizer paclitaxel significantly attenuated this effect. Decreases in transendothelial electrical resistance induced by microtubule disruption correlated with increases in lung permeability in isolated ferret lung preparations as well as with increases in EC stress fiber content and MLC phosphorylation. The increases in MLC phosphorylation were attributed to decreases in myosin-specific phosphatase activity without significant increases in MLC kinase activity and were attenuated by paclitaxel or by several strategies (C3 exotoxin, toxin B, Rho kinase inhibition) to inhibit Rho GTPase. Together, these results suggest that microtubule disruption initiates specific signaling pathways that cross talk with microfilament networks, resulting in Rho-mediated EC contractility and barrier dysfunction.

transendothelial electrical resistance; nonmuscle contraction; actin rearrangement

THE VASCULAR ENDOTHELIUM ACTS as a selective barrier between the vascular space and underlying tissues. Compromise of endothelial cell (EC) barrier integrity leads to an increase in vascular permeability, a cardinal feature of inflammation resulting in tissue edema, hypoxemia, and, often, increased morbidity and mortality. Substantial work, including work from our own laboratory (23), has verified that the EC barrier is regulated by contractile and tethering mechanisms, the effects of which are critically dependent on EC cytoskeletal components (microfilaments, intermediate filaments, and microtubules). For example, our laboratory has previously shown (21, 58) that specific edemagenic agents such as the serine protease thrombin induce EC barrier dysfunction, primarily via actomyosin-driven contraction initiated by myosin light chain (MLC) phosphorylation and tightly linked to microfilament reorganization. Thrombin increases Ca²⁺ and EC centripetal tension (41, 46), and 20-kDa MLC (MLCₐ) phosphorylation peaks at 2 min and returns nearly to control levels by 60 min, indicating involvement of both MLC kinase (MLCK) and MLC phosphatase (PPase) activity in the thrombin response (21, 57). At least two responsible classes of MLCKs are potentially able to phosphorylate MLC in vivo (4, 21, 29), including the newly cloned endothelial Ca²⁺/calmodulin (CaM)-dependent MLCK (22) and Rho kinase, the activity of which depends on activation of the small G protein Rho.

It has become well established that Ras-related GTPases of the Rho family organize the actin cytoskeleton and regulate focal adhesion formation. Rho GTPases are inactive in the GDP-bound form and are activated by GDP/GTP exchange (17, 56). Rho can be specifically inactivated by bacterial toxins by either ADP-ribosylation (Clostridium botulinum C3 exotoxin) or glucosylation (Clostridium difficile, toxin B), which presumably block the interaction of Rho with downstream targets such as Rac and Rho kinase (1, 2, 50). There is a significant controversy, however, as to the effect of Rho on EC contractile and barrier properties. For example, C3 exotoxin has been suggested to both attenuate EC thrombin-mediated contraction via reduction of the thrombin-induced increase in myosin-specific PPase activity (20) and to disrupt EC barrier properties, with minimal effect on EC contractile properties (61). Our laboratory (26) has recently shown that C3 exotoxin completely abolished thrombin- and diperoxovanadate (DPV)-induced increases in MLC phosphorylation, suggesting the involvement of Rho activation in the EC contraction produced by these agonists. Downstream targets of the Rho/Rho kinase signaling cascade include the regulatory subunit of myosin-specific PPase.
(M 130), the phosphorylation of which by Rho kinase leads to dissociation from the catalytic subunit, thereby decreasing myosin-specific PPase 1 activity and subsequently enhancing the level of MLC phosphorylation (37, 39).

In contrast to the microfilamentous actin cytoskeleton, information about the microtubule network and its linkage to the contractile processes is limited. Disassembly of microtubules caused rapid and substantial strengthening of nonmuscle contractility and led to rapid assembly of microfilament bundles and focal adhesions (7, 15, 28), an effect abolished by microtubule stabilization (15). Isometric contraction in fibroblasts induced by microtubule disassembly correlated well with increases in MLC phosphorylation (40); however, the molecular basis for this finding has not been pursued. Furthermore, the role of the microtubule network in EC shape changes, tension development, and barrier properties is unknown. In this study, we examined the biochemical mechanisms by which microtubule disassembly increases endothelial contraction and barrier dysfunction and elucidated the involvement of increased MLC phosphorylation in this process. These studies confirm that MLC phosphorylation is a common mechanism by which microtubule-disrupting agents and receptor-mediated agonists induce nonmuscle contraction (40) and suggest an important physiological role of the microtubule network in EC barrier regulation.

METHODS

Reagents. EC cultures were maintained in medium 199 (GIBCO BRL, Chagrin Falls, OH) supplemented with 20% (vol/vol) colostrum-free bovine serum (Irvine Scientific, Santa Ana, CA), 15 μg/ml of EC growth supplement (Collaborative Research, Bedford, MA), 1% antibiotic-antimycotic (10,000 U/ml of penicillin, 10 μg/ml of streptomycin, and 25 μg/ml of amphotericin B; K. C. Biologicals, Lenexa, KA), and 0.1 mM nonessential amino acids (GIBCO BRL). Unless specified, reagents were obtained from Sigma (St. Louis, MO). PBS, Hank’s balanced salt solution without phenol red, and LipoceptAMINE were purchased from GIBCO BRL (Grand Island, NY). DPV was kindly provided by Dr. V. Natarajan (Johns Hopkins University, Baltimore, MD). C3 exotoxin, vinblastine, and ML-7 were purchased from Calbiochem (La Jolla, CA). Toxin B was purchased from List Laboratories (Campbell, CA). MLC antibody was produced in rabbit against baculovirus-expressed and purified smooth muscle MLC by the laboratory has previously described in detail (21, 55).

Bovine pulmonary artery EC culture. ECs were obtained frozen at passage 16 from the American Type Culture Collection (CCL 209; Manassas, VA) and were used between passages 19 and 24 as our laboratory has previously described in detail (21, 59).

Endothelial monolayer resistance determinations. The electrical resistance of EC monolayers was measured with the electrical cell impedance sensor technique as previously described (24, 55). In this system (Applied Biophysics, Troy, NY), the cells are cultured on a small gold electrode (10⁻⁴ cm²) in DMEM (GIBCO BRL) supplemented with 20% (vol/ vol) colostrum-free bovine serum, antibiotics, and growth factors as described in Reagents. The cells act as insulating particles, and the total resistance across the monolayers is composed of the resistance between the ventral cell surface and the electrode and the resistance between cells. A 4,000-Hz AC signal with 1-V amplitude was applied to the ECs through a 1-MΩ resistor, creating an approximate constant current source (1 μA). The lock-in amplifier attached to the electrodes detected changes in both magnitude and phase of the voltage appearing across the ECs and was controlled by an IBM-compatible personal computer that was used both to run the experiments and process the data. Electrical resistance increased immediately after the cells attached to and covered the electrodes, and the resistance achieved a steady state when the ECs became confluent. Thus experiments were conducted after the electrical resistance achieved a steady state. Resistance data were normalized to the initial voltage and plotted as a normalized resistance.

Isolated perfused ferret lung preparation. Commercially available ferrets were anesthetized with pentobarbital sodium (30 mg/kg ip), intubated, paralyzed (30 breaths per min, tidal volume 12 ml/kg) via tracheostomy with warmed, humidified gas containing 40% O₂, and then rapidly exsanguinated as previously described (6). After exsanguination, the ventilatory gas mixture was switched to 16% O₂-5% CO₂, the ventilatory rate was adjusted to 10 breaths/min, and an expiratory pressure of 3 mmHg was added. Lungs were isolated by insertion of cannulas into the left atrium via the left ventricle and the pulmonary artery via the right ventricle, and then residual blood was flushed from the lungs with physiological salt solution (PSS) containing 5 mM dextrose, 3 g/dl of porcine albumin, and 2 g/dl of Ficoll (6). Isolated lungs were perfused at constant flow (50 ml·kg⁻¹·min⁻¹) for 60 min with PSS-0.1% ethanol (control) or PSS containing 1 μM vinblastine. Pulmonary arterial, left atrial, and airway pressures were continuously monitored (Grass model 7) with Statham P50 transducers referenced to the left atrium. Glucose concentration and pH were monitored throughout the perfusion period and did not differ among preparations.

After 60 min of extracorporeal perfusion, the pulmonary vasculature was filled with PSS containing washed ferret red blood cells (hematocrit 20%), and the pulmonary arterial and left atrial cannulas were connected to a common reservoir containing the same solution. The reservoir was pressurized to 30 mmHg for 20 min, and samples were rapidly withdrawn from the left atrial cannula for measurement of red blood cell and albumin concentrations as previously described (6). The osmotic reflection coefficient for albumin (σ_ab) was calculated iteratively from the rate of change of albumin concentration relative to red blood cell concentration (6).

MLC phosphorylation. This assay was performed as our laboratory has previously described in detail (21, 55).

Cytotoxicity assay. A cytotoxicity assay was performed using a LIVE/DEAD viability/cytotoxicity kit (Molecular Probes) according to the manufacturer’s protocol. This kit provides a two-color fluorescence cell viability assay that is based on the simultaneous dual determination of two recognized parameters of cell viability: intracellular esterase activity (calcine) and plasma membrane integrity (ethidium homodimer-1). Calcine produces an intense green fluorescence in viable cells, whereas ethidium homodimer-1 enters cells with damaged membranes and binds to nucleic acids, thereby producing a bright red fluorescence in nonviable cells.

Permeabilization of ECs. It is well known that C. botulinum C3 exoenzyme does not easily pass through the cell membrane under native conditions. To penetrate the cell
membrane, EC monolayers (80–100% confluence) grown on 60-mm culture dishes were rinsed with OptiMEM-I medium and LipofectAMINE reagent (GIBCO BRL) added at a final concentration of 20 μg/ml for 1 h followed by the addition of C3 exoenzyme (2.5 μg/ml) for an additional 11 h as we have previously described in detail (9).

Preparation of silicone rubber substrates. To visualize cell contractility, flexible rubber substrates were generated as previously described (8, 31). Approximately 15 μl of silicone monomer (dimethylpolysiloxane; Sigma) were applied to 18-mm glass coverslips and allowed to spread for 30 min. Cells were plated on the top of thin polymerized flexible silicone films, and contractility was assessed by formation of wrinkles on the silicone substrate as observed with time-lapse videomicroscopy.

Western immunoblotting. Protein extracts were separated by SDS-PAGE, transferred to nitrocellulose or polyvinylidene difluoride membranes (30 V for 18 h or 90 V for 2 h), and reacted with specific antibodies of interest. Immunoreactive proteins were detected with the enhanced chemiluminescent detection reagent (ECL) according to the manufacturer's directions (Amersham, Little Chalfont, UK). The relative intensities of the protein in the bands were quantified by scanning densitometry.

Determination of myosin-specific and total serine/threonine PPase activities in endothelium. ECs from D100-mm dishes were treated with vehicle (0.1% methanol or DMSO), 0.2 μM vinblastine, or 10 nM calyculin for 10, 30, or 60 min. To prepare total cell lysates, the cells were rinsed twice with ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 7.6, and 137 mM NaCl) and buffer A (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, and 28 mM mercaptoethanol); then 400 μl of buffer A were added to the dishes, and the cells were quickly frozen at −70°C, scraped, and homogenized by passing the cell suspension several times through a 1-ml tuberculin syringe. Myosin-enriched fractions were prepared with 500 μl of total cell lysate as our laboratory has previously described (59). PPase activity was determined in a final volume of 20 μl with a Malachite Green assay kit (Pierce, Rockford, IL) used according to the manufacturer’s protocol. Basal PPase activity in cell lysates was ~1,500 pmol phosphate·μg total protein−1·min−1.

Immunofluorescent microscopy. ECs were grown to subconfluence on glass coverslips in DMEM. After treatment, cells were fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were then washed three times with PBS, permeabilized with 0.25% Triton X-100 in PBS for 5 min, and blocked with 2% BSA in PBS for 30 min, and then actin was stained with Texas Red-phalloidin (Molecular Probes) for 1 h at room temperature. After three washes with PBS, the coverslips were mounted with a SlowFade antifade kit (Molecular Probes). Analysis of the stained cells was performed with a Nikon Eclipse TE 300 microscope equipped with ×20 to ×100 objective lenses.

Determination of MLCK activity. MLCK activity was determined in non-denaturing MLCK immunoprecipitates with exogenous MLC as a substrate, as our laboratory previously described in detail (58).

Statistics. ANOVAs with a Student-Newman-Keuls test were used to compare the means of kinase and PPase activities, the σalt, and the ratios of un-, mono-, and diphosphorylated MLCs of two or more different treatment groups. Results are expressed as means ± SE. Differences between two groups were considered statistically significant when P < 0.05.

RESULTS

Effect of microtubule alteration on EC barrier function. To examine the effect of microtubule disruption on EC barrier function, we monitored electrical resistance across EC monolayers ([transendothelial electrical resistance (TER)]) after treatment with the microtubule-disrupting agents nocodazole and vinblastine. Both nocodazole (Fig. 1A) and vinblastine (Fig. 1B) decreased TER in a dose-dependent manner, with half-maximal declines in TER after nocodazole (0.3–5.0 μM) and vinblastine (0.2–2.5 μM).
µM occurring in ∼15–30 min. Maximal vinblastine effect was observed at 0.5–2.5 µM; however, incubation of cells with even 200 nM of vinblastine significantly decreased electrical resistance (Fig. 1B). Vinblastine-induced EC permeability was also confirmed in vivo with the use of isolated perfused ferret lungs (Table 1) in which the salb estimated in the control lungs was 0.7, a mean value similar to that previously obtained in uninjured isolated ferret lungs (6). In contrast, after 60 min of perfusion with vinblastine, the salb decreased to 0.35 (Table 1) in association with significant lung weight gain and consistent with vinblastine-induced increased vascular permeability and EC barrier dysfunction. Pulmonary arterial and pulmonary airway pressures remained almost constant (7.0–8.5 and 5.5–6.0 mmHg, respectively) through 60 min of perfusion in the vinblastine preparation and were statistically indistinguishable from control values. Both nocodazole (Fig. 2) and vinblastine (data not shown) perturbed EC barrier properties without cytotoxicity. We next examined the effect of microtubule stabilization on barrier dysfunction induced by microtubule-disrupting agents. Figure 3A demonstrates that the microtubule-stabilizing agent paclitaxel did not affect basal TER but significantly attenuated the decrease in TER induced by nocodazole. Furthermore, paclitaxel reversed the effect of the microtubule-disrupting agents on TER, indicating that the effect of microtubule disruption on EC barrier dysfunction is reversible (Fig. 3B).

Table 1. Effect of microtubule disruption on σsalb in isolated perfused lungs

<table>
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<th>Conditions</th>
<th>σsalb (SE)</th>
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<tr>
<td>Vehicle</td>
<td>0.70 ± 0.07</td>
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<tr>
<td>Vinblastine</td>
<td>0.35 ± 0.04</td>
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Values are means ± SE; n = 3 experiments/group. Osmotic reflection coefficient for albumin (σsalb) was measured in isolated ferret lungs after 60 min of perfusion with vinblastine (1 µM) or vehicle control (0.1% ethanol). After vinblastine, σsalb decreased significantly (P < 0.05 vs. control), consistent with increased pulmonary vascular permeability mediated by vinblastine.

Effect of microtubule disassembly on EC microfilament reorganization. Because our laboratory (23, 25) and others, (47) have demonstrated that agonist-induced EC barrier dysfunction is critically dependent on cytoskeletal changes, we next examined the effect of filamentous actin (F-actin) stabilization by phalloidin on vinblastine-induced EC barrier dysfunction (Fig. 4).
Phalloidin significantly attenuated the decrease in electrical resistance induced by vinblastine, indicating an important role for the actin cytoskeleton in EC activation stimulated by microtubule disruption. To extend these findings, we next studied the direct effect of nocodazole on the EC actin cytoskeleton. Figure 5 shows immunofluorescent images of EC confluent monolayers stained for F-actin with Texas Red-phalloidin. As seen in Fig. 5A, control cells have F-actin organized primarily in a cortical ring, with few actin-associated stress fibers spanning the cell. Nocodazole produces dramatic cortical actin dissolution and a significant increase in stress fibers (Fig. 5B), reflecting contraction. Stress fiber formation induced by nocodazole leads to gap formation, indicating barrier compromise (Fig. 5B).

Effect of microtubule disruption on MLC phosphorylation. Our laboratory (21) and others (66) have previously shown the critical involvement of MLC phosphorylation in agonist-induced barrier dysfunction in macro- and microvascular endothelium. Similar to DPV and thrombin treatments, both of which increase MLCK activity and MLC phosphorylation in ECs (22, 26, 57, 58), pretreatment of ECs with microtubule inhibitors produced significant enhancement of MLC phosphorylation in a time-dependent manner (Fig. 6A). The increases in MLC phosphorylation produced by microtubule disruption (up to 1 mol phosphate/mol MLC, peak at 30–60 min) were less than the levels of DPV-induced MLC phosphorylation (up to 1.8 mol/mol MLC) (26) but similar to the level of thrombin-induced MLC phosphorylation (up to 1.2 mol/mol MLC) (21). Neither vinblastine nor nocodazole at lower (up to 100 nM) concentrations had significant effects on the level of MLC phosphorylation (data not shown). The in-
creased MLC phosphorylation produced by microtubule inhibitors was completely abolished by microtubule stabilization with paclitaxel (Fig. 6B). Increases in MLC phosphorylation correlated well with the time frame of decreased TER produced by microtubule assembly inhibition (shown in Fig. 1), suggesting the involvement of contractile mechanisms in EC barrier dysfunction induced by microtubule disruption. Cell contraction induced by microtubule disruption was confirmed by an increase in the number of wrinkles produced by EC monolayers grown on a thin silicone film (Fig. 7).

**Effect of microtubule disruption on MLCK and myosin PPase activities.** Because MLC phosphorylation status is determined by the balance between MLCK and myosin PPase activities, we next determined the effect of microtubule disruption on EC MLCK activity measured in the MLCK immunoprecipitates in the presence of optimal Ca\(^{2+}\)/CaM availability (maximal activity) in either the presence or absence of the specific MLCK inhibitor ML-7 or the Ca\(^{2+}\) chelator EGTA. The data in Fig. 8A demonstrate that inhibition of MLCK with ML-7 and EGTA completely abolished basal MLCK activity, confirming that MLCK activity assay is valid; however, neither form of microtubule inhibition altered MLCK activity above basal levels in the presence of Ca\(^{2+}\)/CaM. Consistent with these results, ML-7 also failed to alter the MLC phosphorylation induced by nocodazole, whereas thrombin-induced MLC phosphorylation was abolished (Fig. 8B).

To examine the involvement of protein PPases in the increase of MLC phosphorylation induced by microtubule inhibitors, we next determined the effect of vinblastine and calyculin (a potent serine/threonine PPase inhibitor) (33, 59) on PPase activity in cell homogenates (Fig. 9A) and the myosin-specific PPase activity (59) present in the myosin-enriched fraction (Fig. 9B). In contrast to calyculin, vinblastine did not affect total PPase activity but significantly decreased myosin-specific PPase activity, suggesting an involvement of myosin PPase inhibition in the increased levels of MLC phosphorylation induced by microtubule disruption.

**Involvement of Rho in EC barrier dysfunction induced by microtubule disruption.** In smooth muscle and nonmuscle cells including endothelium, myosin PPase activity is mediated by activation of the small GTP-bound protein Rho (20, 39). To examine the role of Rho in MLC phosphorylation induced by microtubule disruption, we first used the specific Rho inhibitor C3 exotoxin, which ADP-ribosylates and thereby inactivates Rho (2, 3). Because C3 exotoxin is not cell per-
meable, we initially permeabilized EC monolayers with LipofectAMINE to enhance C3 exotoxin access and found that the toxin completely abolished increases in MLC phosphorylation induced by either thrombin or vinblastine (Fig. 10A), indicating Rho involvement. Consistent with these data, the cell-permeable toxin B, which glucosylates and inactivates small Rho family G proteins (Rho, CDC 42, and Rac) (2, 13, 36), also completely abolished nocodazole-induced MLC phosphorylation (Fig. 10B). In addition, inhibition of the Rho downstream target Rho kinase by the specific cell-permeant inhibitor Y-27632 significantly attenuated decreases in TER (Fig. 11) and increases in stress fibers (data not shown) and completely abolished nocodazole-induced MLC phosphorylation (Fig. 11, inset), demonstrating the direct involvement of Rho kinase in nocodazole-induced MLC phosphorylation and permeability.

**DISCUSSION**

In this study, we have attempted to clarify the biochemical pathway by which changes in EC permeability are linked to microtubule assembly and disassembly. We used primarily a pharmacological approach to either disrupt or preserve microtubule organization (34) with agents such as nocodazole, a synthetic anti-tubulin agent that reversibly blocks the self-assembly of tubulin and depolymerizes preformed microtubules, and vinblastine, a vinca alkaloid that rapidly and reversibly binds to tubulin causing microtubule depolymerization and inhibition of microtubule assembly. Paclitaxel (taxol), a potent microtubule assembly-promoting and -stabilizing agent, prevented and reversed the effect of microtubule disruptors on tubulin assembly. We observed that both nocodazole and vinblastine caused significant dose-dependent decreases in TER in bovine pulmonary artery ECs, indicating EC barrier dysfunction. Stabilization of the microtubule network by paclitaxel significantly attenuated and reversed the decline in electrical resistance induced by microtubule disruption. Consistent with our results, microtubule disassembly has been noted to decrease electrical resistance across thyroid epithelial cells (65), increase microvascular permeability of the rat small intestine vasculature (51), and increase the permeability and rate of monocyte transendothelial migration across human macro- and microvascular ECs (38, 62). We further demonstrated that EC barrier dysfunction induced by microtubule disruption is linked to decreases in cortical actin and increases in stress fiber formation and endothelial contraction as confirmed by deformation of a silicone substrate (i.e., wrinkling). Microtubule-mediated contractility has recently been demonstrated in smooth muscle (5, 43, 52, 54). In nonmuscle cells such as fibroblasts, microtubule disruption appeared to activate actin polymerization and induce...
inhibitors did not increase MLCK activity, and specific phosphorylation. In contrast to thrombin, microtubule myosin PPase inhibition, and a net increase in MLC with increased Ca\(^{2+}\) and barrier dysfunction are strongly correlated that thrombin-mediated EC cytoskeletal rearrangement (16, 40). This observation contradicts the "tensegrity" model, which suggests that microtubules function as compressive elements that oppose cellular contraction (40). Previous studies have demonstrated that Rho inhibition did not completely abolish the nocodazole-induced decrease in resistance of EC monolayers, consistent with the primary role for activation of Rho pathway in nocodazole-induced EC barrier dysfunction. Results from a representative experiment (n = 3) are shown. Inset: in the same experiment, Rho kinase inhibition completely abolished nocodazole-induced MLC phosphorylation.

Fig. 11. Effect of Rho kinase inhibition on nocodazole-induced decrease in TER. TER was monitored for 5 h. At time indicated by left arrowhead, ECs were pretreated with either vehicle (0.05% DMSO) or the specific Rho kinase inhibitor Y-27632 (10 \(\mu\)M) followed by challenge (right arrowhead) with either vehicle (0.05% DMSO) or nocodazole (2 \(\mu\)M). Inhibition of Rho kinase by Y-27632 significantly attenuated but did not completely abolish the nocodazole-induced decrease in resistance of EC monolayers, consistent with the primary role for activation of Rho pathway in nocodazole-induced EC barrier dysfunction. Results from a representative experiment (n = 3) are shown. Inset: in the same experiment, Rho kinase inhibition completely abolished nocodazole-induced MLC phosphorylation.

stress fibers and focal adhesions (7, 15, 16, 19, 35, 45). Cytoskeletal changes were accompanied by contraction as evidenced by either the measurement of isometric force generated from cells cultured within a collagen lattice (40) or deformation of a silicone substrate (15, 16).

The biochemical events linking the cytoskeletal changes initiated by microtubule disruption and non-muscle contraction are uncertain but likely involve increased MLC phosphorylation and activation of the small GTP-binding protein Rho (40, 45). Our laboratory (21, 58) and others (29, 41, 48, 63, 64, 66) have previously shown a direct link between the level of MLC phosphorylation, EC retraction, and agonist-induced increases in EC permeability, indicating the importance of actomyosin-driven contraction in EC barrier dysfunction. Our present data demonstrate for the first time a correlation between microtubule disruption, activation of the contractile machinery (MLC phosphorylation, stress fiber formation), and a decrease in TER (barrier dysfunction). An increase in MLC phosphorylation after microtubule disruption in ECs (present study), previously noted in fibroblasts (40), suggests activation of biochemical cascades that lead to contraction and EC barrier failure. Importantly, fibroblasts precontracted with serum and having increased levels of MLC phosphorylation showed a decreased contractile response to microtubule disruption (40). This observation contradicts the "tensegrity" model, which suggests that microtubules function as compressive elements that oppose cellular contraction (10, 11, 32), and supports the active involvement of MLC phosphorylation and Rho activation in the initiation of contraction after microtubule disruption (16, 40).

Our laboratory (21, 57, 58) has previously shown that thrombin-mediated EC cytoskeletal rearrangement and barrier dysfunction are strongly correlated with increased Ca\(^{2+}\)/CaM-dependent MLCK activity, myosin PPase inhibition, and a net increase in MLC phosphorylation. In contrast to thrombin, microtubule inhibitors did not increase MLCK activity, and specific inhibition of MLCK (with ML-7) did not affect nocodazole-induced MLC phosphorylation, indicating that MLCK is not involved in the activation of the EC contractile machinery that is induced by microtubule inhibition. Increases in MLC phosphorylation and the initiation of contraction in smooth muscle and non-muscle cells also follow the activation of the small GTPase Rho (14, 53) and its target Rho kinase, which directly phosphorylates MLC in vitro and in vivo (4, 42, 44). In addition, Rho kinase phosphorylates the regulatory subunit of myosin-associated PPase, which leads to inhibition of myosin-associated PPase activity and an increase in MLC phosphorylation (37, 39, 49). Our laboratory (26) and others (12, 20, 27) have recently shown the involvement of Rho in agonist-induced EC barrier dysfunction, increases in MLC phosphorylation, and myosin PPase inhibition. Our present data indicate that specific Rho or Rho kinase inhibitors completely abolish increases in MLC phosphorylation induced by microtubule inhibitors, strongly implicating the involvement of the Rho pathway in the EC barrier dysfunction induced by microtubule disruption. Consistent with these data, Rho kinase inhibition significantly attenuated nocodazole-induced decreases in TER. Although microtubule depolymerization in fibroblasts also induces stress fiber formation via Rho activation (45), the precise mechanisms of Rho activation induced by microtubule disruption are not currently known. Our laboratory (Verin and Garcia, unpublished observations) demonstrated that Rho inhibition did not directly affect EC microtubule structure, indicating that Rho activation is a downstream event of microtubule disruption. To evaluate biochemical events leading to Rho-dependent increases in MLC phosphorylation and EC cytoskeletal rearrangement, we measured PPase activity in the myosin-enriched fraction, which includes myosin, actin, and regulatory and catalytic subunits of endothelial myosin PPase (57, 59, 60), and most likely represents myosin-specific PPase activity in endothelium. We found that the microtubule inhibitor vinblastine significantly decreased myosin-spe-
cific, but not total, PPase activity in bovine ECs, supporting the involvement of myosin PPase in the increased MLC phosphorylation, cytoskeletal rearrangement, and barrier dysfunction induced by microtubule disruption.

In summary, we have characterized the biochemical and physiological pathways leading to EC barrier dysfunction induced by microtubule disruption. Microtubule disassembly leads to increased MLC phosphorylation, stress fiber formation, and EC contraction via Rho GTPase-dependent, but not MLCK-dependent, mechanisms that are likely mediated via myosin PPase inhibition. Although the precise mechanism that links microtubules, Rho activation, and EC barrier dysfunction remains to be determined, our data demonstrate a significant role of microtubule dynamics in EC barrier regulation.

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