Published in final edited form as:

Exp Eye Res. 2009 September ; 89(3): 333–343. doi:10.1016/j.exer.2009.03.019.

Microtubule disassembly breaks down the barrier integrity of corneal endothelium

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Abstract

Increased contractility of the peri-junctional actomyosin ring (PAMR) breaks down the barrier integrity of corneal endothelium. This study has examined the effects of microtubule disassembly on Myosin Light Chain (MLC) phosphorylation, a biochemical marker of actomyosin contraction, and barrier integrity in monolayers of cultured bovine corneal endothelial cells (BCEC).

Exposure to nocodazole, which readily induced microtubule disassembly, led to disruption of the characteristically dense assembly of cortical actin cytoskeleton at the apical junctional complex (i.e., PAMR) and dispersion of ZO-1 from its normal locus. Nocodazole also led to an increase in phosphorylation of MLC. Concomitant with these changes, nocodazole caused an increase in permeability to HRP and FITC dextran (10 kDa) and a decrease in trans-endothelial electrical resistance (TER). Y-27632 (a Rho kinase inhibitor) and forskolin (known to inhibit activation of RhoA through direct elevation of cAMP) opposed the nocodazole-induced MLC phosphorylation, decrease in TER, and dispersion of ZO-1. Thrombin, which breaks down the barrier integrity of BCEC monolayers, also induced microtubule disassembly and MLC phosphorylation. Pretreatment with paclitaxel to stabilize microtubules opposed the thrombin effects. These results suggest that microtubule disassembly breaks down the barrier integrity of BCEC through activation of RhoA and subsequent disruption of the PAMR. The thrombin effect also highlights that signaling downstream of GPCRs can also influence the organization of microtubules.

Keywords

Microtubule disassembly; MLC phosphorylation; Corneal endothelium; Barrier integrity; Peri-junctional actomyosin ring

1. Introduction

Transparency of the cornea is dependent on deturgescence of its connective tissue stroma, which is enforced by an active fluid transport mechanism associated with the corneal endothelium (Bonanno 2003). Opposing this “fluid pump” function of the endothelium is the

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constant “fluid leak” from the anterior chamber into the stroma, which is induced by the hydrophilic glycosaminoglycans. However, the rate of fluid leak is determined by the barrier properties of the endothelium (Riley et al. 1998). Pro-inflammatory stimuli and iatrogenic events are known to cause stromal edema (Edelhauser 2006) but the mechanisms involved are not completely understood.

Our recent studies (Satpathy et al. 2004; Srinivas et al. 2004; Satpathy et al. 2005; Srinivas et al. 2006) have shown that the extent of phosphorylation of the regulatory light chain of Myosin II (also called myosin light chain, or MLC) is a major determinant of the barrier integrity of corneal endothelium, especially in response to pro-inflammatory agents. As in vascular smooth muscle cells, increased MLC phosphorylation induces actomyosin contraction in endothelial and epithelial cells (Somlyo and Somlyo 2003). Such a modulation of actomyosin contraction is known to influence the barrier integrity of the endothelial monolayers through disruption of the dense band of cortical actin associated with the apical junctional complex (referred to as the peri-junctional actomyosin ring, PAMR) (Turner 2006). Specifically, increased actomyosin contraction of the PAMR is believed to result in a breakdown of the barrier integrity, presumably by opposing interactions of the trans-membrane proteins at the apical junctional complex (Turner et al. 1997; Turner 2000; Dudek and Garcia 2001; Mehta and Malik 2006; Shen et al. 2006; Turner 2006).

In consistence with these reports, our recent studies have demonstrated that agents such as thrombin (Satpathy et al. 2004) and histamine (Srinivas et al. 2006), which induce MLC phosphorylation, lead to a breakdown of the barrier integrity of the corneal endothelium. In addition, adenosine (Srinivas et al. 2004) and ATP (Satpathy et al. 2005), which induce MLC dephosphorylation, opposed thrombin- and histamine-induced loss of the barrier integrity. In contrast to these agents, which affect MLC phosphorylation through signaling downstream of their GPCRs, recent reports have demonstrated that altered MLC phosphorylation could be produced in response to depolarization of the plasma membrane potential (Szasz et al. 2005) and microtubule disassembly (Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2005; Birukova et al. 2006). The latter, which forms the focus of this study, can occur in response to thrombin, (Birukova et al. 2004) TNF- α (Petrache et al. 2003), TGF- β 1 (Birukova et al. 2005), and oxidative stress (Banan et al. 2003). These molecules/stresses are also associated with corneal endothelial dysfunction (Sakamoto et al. 1995; Watsky et al. 1996; Chen et al. 1999; Kim et al. 2001; Funaki et al. 2003; Joyce 2003; Ayala et al. 2007).

The microtubules, as part of the cytoskeleton, are essential for structural integrity of cells and are involved in the trafficking of membrane proteins, migration, and polarity (Kelly 1990). Their disassembly is known to induce actomyosin contraction in different cell types (Kolodney and Elson 1995; Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2005; Birukova et al. 2005; Birukova et al. 2006) through upregulation of the small GTPase, RhoA (Birukova et al. 2004; Birukova et al. 2005). It has been suggested that RhoA-specific GEFs (guanine nucleotide exchange factors), essential for the activation of the RhoA and anchored to the microtubules (Ren et al. 1998; van Horck et al. 2001; Krendel et al. 2002; Birukova et al. 2006), are released in response to microtubule disassembly, leading to RhoA activation. The downstream effector of RhoA, Rho kinase, inactivates myosin light chain phosphatase (MLCP) by phosphorylation of its myosin-binding subunit (MYPT1; 110-120 kDa) at Thr-696 and Thr-853 (Somlyo and Somlyo 2003). This inactivation of MLCP enhances MLC phosphorylation (Somlyo and Somlyo 2003).

In the present study, we have investigated the effects of microtubule disassembly on the status of MLC phosphorylation and barrier integrity of monolayers of cultured bovine corneal endothelium. We have employed nocodazole to induce deliberate disassembly of microtubules

by its depolymerization (Eilers et al. 1989). Our results show that disassembly of microtubules results in the disruption of the PAMR, leading to a breakdown of the barrier integrity of corneal endothelial cells.

2. Materials and methods

2.1. Chemicals

Nocodazole, forskolin, rolipram, paclitaxel, Y-27632, and monoclonal α -tubulin antibody were obtained from Sigma (St Louis, MO). Bovine α -thrombin was purchased from Enzyme Research (South Bend, IN). Bovine calf serum was purchased from Hyclone (Logan, UT). Texas red conjugated phalloidin, goat anti-mouse Alexa 488, DAPI, and anti-fade agent were purchased from Molecular Probes (Eugene, OR). Phospho-Myosin Light Chain 2 (Thr18/Ser19) antibody was obtained from Cell Signaling Technology (Danvers, MA). ZO-1 antibody was obtained from Zymed (Long Island, NY). Electrodes for impedance sensing (8W10E+) were obtained from Applied Biophysics Inc (Troy, NY). The enhanced chemiluminescence kit was obtained from Amersham-Pharmacia Biotech (Piscataway, NJ).

2.2. Cell culture

Primary cultures of BCEC obtained from fresh bovine eyes were established as described previously (Satpathy et al. 2004; Srinivas et al. 2004; Satpathy et al. 2005; Srinivas et al. 2006). The growth medium contained Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum and an antibiotic-antimycotic mixture (penicillin: 100 U/mL, streptomycin: 100 μ g/mL and amphotericin-B: 0.25 μ g/mL). They were cultured at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Cells of the first/second passage were harvested and seeded on glass coverslips, Transwell™ filters (Corning Inc., Coburn, MA), Petri dishes, and electrodes and grown to confluence before use. Cell culture supplies were from Invitrogen (Long Island, NY).

2.3. MLC phosphorylation assay

MLC phosphorylation assays were carried out using confluent monolayers grown on Petri dishes as described previously (Satpathy et al. 2004; Srinivas et al. 2004; Satpathy et al. 2005; Srinivas et al. 2006). Cells were starved of serum for at least 12-16 hrs before use. Protein extracts obtained after drug treatment were dissolved in a urea sample buffer, equalized for protein content, and were then electrophoresed (25 μ g/lane) on polyacrylamide gels (containing acrylamide, bis-acrylamide, glycerol, Tris-base and glycine). The gels were blotted onto nitrocellulose paper and the phosphorylated and non-phosphorylated MLC were detected using a polyclonal anti-MLC antibody (1:3000). Blots were visualized using the enhanced chemiluminescence kit. The migration rate of phosphorylated and unphosphorylated MLC was in the following order: di-phosphorylated (PP) > mono-phosphorylated (P) > non-phosphorylated (NP). The band intensities were quantified by densitometry, using a custom-made software program.

2.4. Immunofluorescence for microtubule, F-actin, ppMLC and ZO-1

Cells grown on coverslips were washed with PBS after desired drug treatment, fixed with 3.7% paraformaldehyde and permeabilized using 0.2% Triton X-100 for 5 min. This was followed by blocking and incubation with monoclonal α -tubulin antibody (1:1000) for 12 hrs at 4°C. After another wash, cells were incubated with the secondary antibody (goat anti-mouse Alexa flour 488; 1:500) for 1 hr at room temperature. Finally, cells were treated with DAPI (1 μ g/mL) for 5 min to visualize the nuclei.

Using a similar protocol, cells were stained for actin by phalloidin conjugated to Texas Red (1:1000) for 45-60 min at room temperature. Cells were stained for ZO-1 after treatment with desired agents. The cells were fixed with PLP fixation buffer, followed by permeabilization with 0.01% saponin in PBS. Then the cells were blocked with a blocking buffer for 45 min and incubated with antibody for ZO-1 (1:25) overnight at 4°C. This was followed by washing and incubation with a secondary antibody (goat anti-mouse Alexa fluor 488; 1:1000) for 1 hr at room temperature. Identical protocol was employed to stain ppMLC. Rabbit corneal endothelium was isolated from eyes obtained from Pel-Freez Inc. (Rogers, AR). The isolated sheets were fixed on a pre-cleaned glass slide and subjected to staining protocol as used for cultured cells. Stained cells and the rabbit endothelium were mounted with an anti-fade agent and visualized using an epifluorescence microscope equipped with a 60x objective of 1.2 NA (Nikon, Tokyo, Japan). ppMLC and F-actin were imaged at the focal plane of ZO-1.

2.5. ECIS assay for Trans-endothelial electrical resistance (TER)

Electrical cell-substrate impedance was measured using ECIS™ (Applied Biophysics, Troy, NY). Through such cell-substrate impedance sensing, it is possible to extract TER (trans-endothelial electrical resistance), which is a measure of the barrier integrity of confluent cell layers (Tiruppathi et al. 1992). For measurement of cell-substrate impedance, cells were seeded at a density of 2×10^5 cells on gold electrodes (8W10E+, Applied Biophysics). Cells were grown to confluence and serum-starved for 1 hr before treatment with desired drugs/agents. Impedance across the cells was measured continuously at 4 kHz. The resistive portion of impedance was normalized to its initial value at time zero, a measure of relative changes in TER, when presented.

2.7. HRP flux measurements

HRP flux across cells grown on Transwell™ filters was quantified as a measure of barrier integrity, as described previously (Satpathy et al. 2004; Satpathy et al. 2005; Srinivas et al. 2006). The apical bath containing HRP (44 kDa, 440 µg/mL) was exposed to different drugs/reagents. Samples from the basolateral chamber were analyzed for peroxidase activity after 30 min using a colorimetric assay. Absorbance at 470 nm was proportional to HRP level in the basolateral chamber.

2.8. Trans-endothelial permeability assay

Cells were grown on 0.2 µm pore-size collagen IV (1 mg/ml)-coated tissue culture inserts (Nunc, Fisher Scientific, Pittsburgh, PA) until confluent. Monolayers were then serum starved for 1 hr and either left untreated or exposed to nocodazole in triplicate with desired agents. Following treatment, FITC-dextran (10 kDa) dissolved in the medium was placed in the apical compartment at a concentration of 0.4 µg/mL and allowed to equilibrate for 2 hrs. Samples were then taken from the basolateral chamber for fluorescence measurements. Cells were treated with forskolin and rolipram for 1 hr before exposure to nocodazole. Fluorescence was measured by excitation at 492 nm and the emission collected at 520 nm.

2.9. Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical differences between treatments were determined by analysis of variance (ANOVA) with Bonferroni's post hoc comparisons using Graph Pad (Software Inc., San Diego, CA, USA) statistical analysis software.

3. Results

3.1. Microtubule disassembly disrupts PAMR

Nocodazole induces depolymerization of microtubules (Eilers et al. 1989). Several recent studies (Verin et al. 2001; Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2005; Birukova et al. 2006) have employed nocodazole to examine the influence of microtubule disassembly on actin cytoskeleton. In BCEC, exposure to 10 μ M nocodazole for 30 min led to the disappearance of the fibrillary extensions of microtubules from around the nucleus to the cell periphery (Fig. 1B; n = 10). Similar treatment also led to disruption of the dense band of cortical actin localized at the plane of the apical junctional complex (referred to as the perijunctional actomyosin ring, or PAMR; Fig. 1D; n = 10) (Satpathy et al. 2004; Srinivas et al. 2004; Satpathy et al. 2005; Srinivas et al. 2006). For comparison, the organization of microtubules and PAMR without nocodazole treatment is shown in Figs. 1A and 1C.

3.2. Microtubule disassembly induces MLC phosphorylation

To determine the mechanism underlying the disruption of PAMR in Fig. 1D, we examined the effect of microtubule disassembly on MLC phosphorylation. Exposure to 2 μ M nocodazole for 30 min led to increased MLC phosphorylation (~ 150% compared to control). Typical Western blot after urea-glycerol gel electrophoresis in Fig. 2A shows an increased intensity of di-phosphorylated MLC in cells treated with nocodazole compared to untreated (n = 10). This enhancement in MLC phosphorylation was suppressed when cells were treated with nocodazole in the presence of 10 μ M Y-27632, a selective Rho kinase inhibitor (Fig. 2A). Densitometric analysis of similar experiments (n = 5) is shown in Fig. 2B. The effect of nocodazole on MLC phosphorylation was also confirmed by performing immunolocalization of ppMLC. In untreated cells, the ppMLC along the cell periphery was moderate indicative of basal level of MLC phosphorylation (Fig. 3A). On treatment with Y-27632 alone, the ppMLC was reduced compared to untreated cells, indicative of MLC dephosphorylation (Fig. 3B). Microtubule disassembly increased the intensity of ppMLC staining (Fig. 3C), which is indicative of enhanced MLC phosphorylation. This response was opposed on co-treatment with 10 μ M Y-27632 (Fig. 3D). We also note that the localization of the increased ppMLC staining is along the locus of PAMR. Similar to the response in BCEC, nocodazole-induced microtubule disassembly also led to a disruption of the PAMR in the isolated rabbit endothelium (data not shown; n = 4). Furthermore, the disruption was opposed by co-treatment with Y-27632 (data not shown; n = 4).

3.3. Microtubule disassembly breaks down the barrier Integrity

As noted earlier, organization of PAMR is a principal determinant of barrier integrity of endothelial monolayers (Turner 2000). Since the PAMR was significantly affected by nocodazole, we examined the effect of microtubule disassembly on the barrier integrity of BCEC. The correlation between PAMR disruption and loss of barrier integrity was ascertained by using cytochalasin D, an actin-severing agent (Bereiter-Hahn et al. 2008). As expected, cytochalasin D led to a precipitous decline in TER in a dose dependent manner (Fig. 4). Exposure to 2 μ M nocodazole also decreased TER with a maximum decline within 10 min (Fig. 5A). The influence of nocodazole on the barrier integrity was further assessed by determining the permeability to HRP across monolayers (see Methods). Exposure to 2 μ M nocodazole for 30 min led to an increase in permeability to HRP by > 6-fold compared to control (n = 5) This decline persisted for more than 2 hrs, but a significant recovery was achieved when cells were pretreated with 2 μ M Y-27632 for 1 hr followed by addition of nocodazole, as shown in Fig. 5B. Histogram analysis of the % reduction in TER from similar experiments (n = 8) shown in Figs. 5A and 5B are summarized in Fig. 5C.

3.4. Microtubule disassembly causes dislocation of ZO-1

ZO-1 is a marker of TJ assembly, as it is bound to many associated trans-membrane molecules (Tsukita et al. 2001; Ikenouchi et al. 2007). In untreated cells, ZO-1 is distributed continuously and uniformly at the cell periphery (Fig. 6A), corresponding to intact tight junction assembly. Upon treatment with Y-27632 alone, the pattern of ZO-1 localization was similar to untreated cells (Fig. 6B). Exposure to 2 μ M nocodazole for 30 min led to discontinuities in the localization of ZO-1, along with its dispersion at the periphery (Fig. 6C). This nocodazole response was suppressed by pretreatment with 2 μ M Y-27632 for 1 hr (Fig. 6D). These responses were further confirmed in the isolated rabbit corneal endothelium (data not shown; n = 4).

3.5. Elevated cAMP attenuates the nocodazole-induced loss of barrier integrity

The effects of forskolin on the nocodazole-induced MLC phosphorylation and the breakdown of barrier integrity were examined to confirm the involvement of the RhoA-Rho kinase axis. As shown in Fig. 7A, when cells were co-treated with 10 μ M forskolin and 2 μ M nocodazole for 30 min, MLC phosphorylation was reduced compared to nocodazole treatment alone. A histogram of densitometric analysis of similar data from representative experiments (n = 4) shown in Fig. 7A is summarized in Fig. 7B. Co-treatment with forskolin significantly reduced the %pMLC when compared to nocodazole. Similar results were obtained in response to adenosine, which is known to elevate cAMP through activation of A2B receptors (Srinivas et al. 2004) (data not shown). In consistence with these responses, nocodazole-induced increase in ppMLC at the cell periphery (Fig. 8C) was also suppressed by co-treatment with forskolin and rolipram (a selective inhibitor of PDE4, a cAMP-dependent phosphodiesterase known to be active in corneal endothelial cells; (Srinivas et al. 2004)) (Fig. 8D). On treatment with forskolin and rolipram, the ppMLC was reduced when compared to untreated cells suggestive of MLC dephosphorylation (Fig. 8B).

Consistent with the suppression of MLC phosphorylation and consequent inhibition of the disruption of PAMR, pre-exposure to forskolin prevented the nocodazole-induced decline in TER (Fig. 9A). Forskolin by itself resulted in an increase in TER. Histogram analysis in terms of % reduction in TER of similar experiments (n = 3) in Fig. 9A is shown in Fig. 9B. Co-treatment with forskolin significantly attenuates the % reduction in TER induced by nocodazole. The influence of nocodazole on the barrier integrity was further assessed by determining the permeability to HRP across monolayers (see Methods). Exposure to 2 μ M nocodazole for 30 min led to an increase in permeability to HRP (Fig. 10A) by > 6-fold compared to control (n = 5). The influence of nocodazole on permeability was also determined by quantifying the flux of FITC-dextran (10 kDa) across BCEC monolayers (see Methods). Exposure to 5 μ M nocodazole increased permeability to FITC dextran in comparison to untreated control (Fig. 10B). Pretreatment with forskolin for 1 hr attenuated the nocodazole response.

3.6. Thrombin induces microtubule disassembly

Thrombin, a serine protease, induces loss of barrier integrity in BCEC through activation of PAR-1 receptors (Satpathy et al. 2004; Satpathy et al. 2005). This has been associated with an increase in MLC phosphorylation and a breakdown of the organization of the PAMR. In lung vascular endothelial cells (Birukova et al. 2004), thrombin brings about a similar loss of barrier integrity, partially through microtubule disassembly. To highlight the significance of our observations with nocodazole, we examined if the thrombin effect also involves a similar mechanism. As shown in Fig. 11C, exposure to thrombin 2 U/mL for 2 min induced disassembly of the microtubules similar to nocodazole (shown in Fig. 1B). This disassembly was prevented by pretreatment with 5 μ M paclitaxel for 1 hr (Fig. 11D), which is known to stabilize microtubules (Fig. 11B). Furthermore, pretreatment with paclitaxel also opposed the thrombin-induced MLC phosphorylation by ~ 37% (n = 6; Fig. 12).

4. Discussion

Despite being leaky, the corneal endothelium forms a significant barrier against facile entry of fluid from anterior chamber into the stroma. Accordingly, a breakdown in the barrier function of the endothelium is implicated in stromal edema in response to inflammatory mediators and iatrogenic effects (Edelhauser 2006). In continuation of our previous studies, which have established a role for the PAMR (Satpathy et al. 2004; Srinivas et al. 2004; Satpathy et al. 2005; Srinivas et al. 2006), this study has focused on the role of microtubules in the maintenance of the barrier integrity. The major finding of this study is that a disassembly of microtubules disrupts the PAMR, which in turn leads to a breakdown of the barrier integrity of corneal endothelium.

Our first observation, highlighted in Fig. 1D, is that the actin cytoskeleton at the apical junction (PAMR) is disrupted following microtubule disassembly. Similar observations have been reported (Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2005; Birukova et al. 2005; Birukova et al. 2006) with vascular endothelial cells. The efficacy of nocodazole in producing microtubule disassembly is indicated by the disappearance of microtubule extensions to the periphery and also by its condensation around the nucleus (Fig. 1B). Concomitant with these effects on microtubules, we have found destruction of the organization of the PAMR in Fig. 1D. This disorganization appears to be secondary to increased actomyosin contraction, as demonstrated by increased MLC phosphorylation by Western blot analysis (Fig. 2).

Furthermore, the increase in MLC phosphorylation is located along the locus of the PAMR, as shown by increased number of high intensity spots of ppMLC along the cell border in response to nocodazole (Fig. 3C) and its opposition by Y-27632 (Fig. 3D). It is noteworthy that a similar disorganization of the PAMR and spotty appearance of ppMLC have been demonstrated in our earlier studies with BCEC in response to thrombin (Satpathy et al. 2004) and histamine (Srinivas et al. 2006).

As noted earlier, MLC phosphorylation could be enhanced by activation of MLCK and/or inhibition of MLCP (Satpathy et al. 2004; Srinivas et al. 2006). As in the smooth muscle cells, MLCK is activated by Ca^{2+} -calmodulin complex (Kamm and Stull 2001; Somlyo and Somlyo 2003) and is not known to be regulated by microtubules directly or indirectly. However, as noted earlier, the catalytic subunit of MLCP (PPC1 δ ; 38 KDa) (Somlyo and Somlyo 2003) is inhibited by Rho kinase through phosphorylation of the myosin binding subunit, MYPT1 of MLC phosphatase (MLCP) (Kamm and Stull 2001; Somlyo and Somlyo 2003). Upstream of Rho kinase is the small GTPase RhoA, which is activated by microtubule disassembly. The latter is shown to result in a release of RhoA-associated GEFs (i.e., guanine nucleotide exchange factors such as p190RhoGEF and GEF-H1), which are bound to the microtubules (van Horck et al. 2001; Krendel et al. 2002; Zenke et al. 2004). Our findings in Figs. 2 and 3, which show enhanced MLC phosphorylation in a manner dependent on Y-27632, are indicative of the activation of RhoA upon microtubule disassembly.

Given our focus on the corneal endothelial barrier integrity in the context of stromal hydration control, we next determined if the loss in the organization of the PAMR secondary to microtubule disassembly can produce a breakdown in the barrier integrity. For this approach, we followed a well-established technique involving electrical cell-substrate sensing (Verin et al. 2001; Mehta et al. 2002; Birukova et al. 2004; Birukova et al. 2004; Birukova et al. 2004; Yin and Watsky 2005; Birukova et al. 2006; Mehta and Malik 2006). This technique has recently been applied to corneal endothelial cells by Yin and Watsky (Yin and Watsky 2005). To confirm our protocol, we first conducted control experiments in which we assessed changes in the measured TER in response to an actin severing agent, cytochalasin D (Fig. 4). Similarly,

we have observed that nocodazole induces a sustained reduction in TER in less than 10 min. This TER reduction is consistent with disorganization at the apical junctional complex, as noted in terms of disruption of the PAMR and dislocation of ZO-1 (Figs. 1, and 6). Interestingly, we failed to note a complete block in the initial reduction in TER by pre-treatment with Y-27632 (Fig. 5B). However, TER showed a significant recovery to values before treatment with nocodazole (Fig. 5C). The TER response suggests that the response to microtubule disassembly induces loss in barrier integrity through a disruption of actin cytoskeleton.

In previous studies (Srinivas et al. 2004; Satpathy et al. 2005), we have demonstrated that agents known to elevate cAMP in BCEC reduce MLC phosphorylation by inhibiting RhoA-Rho kinase pathway (Essler et al. 2000; Ellerbroek et al. 2003; Qiao et al. 2003; Goeckeler and Wysolmerski 2005). Furthermore, the consequent reduction in MLC phosphorylation has been shown to overcome thrombin- and histamine-induced disruption of actin cytoskeleton and concomitant loss in barrier integrity (Satpathy et al. 2004; Srinivas et al. 2006). Pre-exposure to forskolin, which attenuated nocodazole-induced MLC phosphorylation (Figs. 7 and 8), readily blocked decrease in TER (Fig. 9) as well as increase in permeability to FITC dextran (Fig. 10B). These results provide further evidence to the claim that microtubule disassembly induces activation of RhoA-Rho kinase axis while producing a breakdown in the barrier integrity. Another pathway that is known to impact the integrity of cell-cell junctions involves the newly discovered GEFs, the EPACs (Birukova et al. 2007; Birukova et al. 2008). These are implicated in the activation of small GTPase Rap1, which has been shown to enhance adherens junctions involving E cadherins. However, we failed to observe any significant changes in TER in response to selective and specific activators of EPAC1 and 2 (data not shown).

In order to emphasize the importance of microtubule disassembly *vis-à-vis* maintenance of barrier integrity, we also looked into the effect of thrombin. In this study, we have found that thrombin induces microtubule disassembly similar to nocodazole (Fig. 11C).

Furthermore, stabilization of microtubules by pre-treatment with paclitaxel opposed thrombin-induced MLC phosphorylation (Fig. 12). This clearly suggests that the thrombin-induced loss in barrier integrity is partly through microtubule disassembly.

In summary, this study implicates the integrity of microtubules in regulation of barrier integrity of the corneal endothelium. Specifically, we have demonstrated that the microtubule disassembly also leads to disruption of the characteristic assembly of cortical actin at the apical junctional complex, which results in a breakdown of the barrier integrity.

ACKNOWLEDGEMENTS

Supported by NIH grant R21-EY019119 (SPS), HL81453 (DM), and Faculty Research Grant, VP of Research, IU Bloomington, IN (SPS).

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A: Untreated **B: NDZ** **C: Untreated** **D: NDZ****Fig. 1.**

Effect of nocodazole on the organization of the cytoskeleton. Immunostaining of microtubule using antibody against α -tubulin in untreated cells (A) or cells treated with 10 μ M nocodazole (NDZ) for 30 min (B). Note the disassembly of microtubules, as evident by the loss of the fibrillary extensions to the periphery and the apparent condensation of microtubules around the nucleus (indicated by arrows). Staining for F-actin in untreated cells (C). The characteristic PAMR is indicated by the arrow. Exposure to nocodazole (D) induces disruption of the PAMR as shown by the arrow. The responses shown are typical of more than ten independent trials.

Fig. 2.

Effect of nocodazole on MLC phosphorylation. MLC phosphorylation was assayed using urea glycerol gel electrophoresis, followed by immunoblotting. Typical blot depicting the phosphorylation status of MLC (A) (NP: Non-phosphorylated MLC, P: Mono-phosphorylated MLC; PP: Di-phosphorylated MLC). Treatment with 2 μ M Nocodazole (NDZ) for 30 min enhances MLC phosphorylation, as indicated by the intense diphosphorylated (PP) MLC bands, when compared to control (n = 10). Co-treatment with 10 μ M Y-27632 (Y) opposes nocodazole-induced response. Summary of densitometric analysis of the representative experiments (n = 5) expressed as %pMLC (B) shown in panel (A). * indicates statistical significance at $P < 0.05$.

Fig. 3.

Localization of phosphorylated MLC (ppMLC). In untreated cells (A), ppMLC staining is diffuse and moderate in the cortical region, consistent with a low basal level of MLC phosphorylation. Treatment with 10 μ M Y-27632 for 30 min reduces the ppMLC when compared to untreated cells (B). Treatment with 2 μ M nocodazole (NDZ) for 30 min (C) increases the of ppMLC consistent with its ability to increase MLC phosphorylation. Co-treatment with nocodazole and Y-27632 opposes nocodazole response (D).

Fig. 4. Effect of cytochalasin D on TER. The influence of cytochalasin D on TER was monitored by ECIS. Exposure to cytochalasin D induces an immediate, dose-dependent decline in TER with a maximum decline observed at 2 µg/mL. The responses shown are representative of three independent trials.

Fig. 5.

Effect of nocodazole on TER. The changes in TER in response to nocodazole (NDZ) were measured by ECIS (A). Treatment with 2 μ M nocodazole induces a persistent reduction in TER with a peak decline occurring within 10 min ($n = 8$). Pretreatment with 2 μ M Y-27632 for 1 hr attenuates the decline in TER in response to nocodazole (B). The decline is not persistent, unlike with nocodazole (Fig. 5A), and is characterized by recovery to baseline by around 3 hr. Histogram analysis of representative experiments ($n = 8$) showing the recovery of TER in response to Y-27632 pretreatment (C). The % reduction in TER over 2 hr period with nocodazole alone was greater compared to when cells were pretreated with Y-27632. * indicates statistical significance at $P < 0.05$.

A: Untreated **B: Y-27632** **C: NDZ** **D: Y-27632 + NDZ****Fig. 6.**

Effect of nocodazole on localization of ZO-1. Immunostaining of ZO-1 in untreated cells (A). Note the continuous ZO-1 localization throughout the cell periphery (shown by arrow).

Exposure to 10 μ M Y-27632 for 30 min (B) does not alter the localization of ZO-1 and was similar to control. Exposure to 2 μ M nocodazole (NDZ) for 30 min (C) induces discontinuities in ZO-1 localization (shown by arrow), indicating barrier instability. Pretreatment with 2 μ M Y-27632 for 1 hr (D) opposes the nocodazole-induced response. The responses shown are typical of three independent trials.

Fig. 7.

Effects of cAMP on nocodazole-induced MLC phosphorylation. Co-treatment with 10 μ M forskolin (FSK) significantly inhibits the nocodazole-induced MLC phosphorylation (A), as evident by the loss of intense di-phosphorylated (PP) MLC bands compared to nocodazole (NDZ). Summary of densitometric analysis (B) expressed as %pMLC of the representative experiments ($n = 4$) shown in panel (A). * indicates statistical significance at $P < 0.05$.

Fig. 8.

Influence of cAMP on phosphorylated MLC (ppMLC). In untreated cells (A), the intensity of ppMLC along cell periphery is moderate, indicating basal level of MLC phosphorylation. Treatment with 10 μ M forskolin (FSK) and 50 μ M rolipram (B) reduced the intensity of ppMLC when compared to untreated cells indicating MLC dephosphorylation. Exposure to 2 μ M nocodazole for 30 min (C) increases ppMLC intensity in the cortical region, consistent with the observed increase in MLC phosphorylation. Co-treatment with 10 μ M forskolin and 50 μ M rolipram (D) opposes nocodazole response. The responses shown are typical of five independent trials.

Fig. 9.

Effects of cAMP on nocodazole-induced breakdown in the barrier integrity. Exposure to 2 μ M nocodazole led to a precipitous decrease in TER (A). Treatment with 10 μ M forskolin alone enhances TER compared to control. Pretreatment with 10 μ M forskolin for 30 min opposes the decrease in TER in response to nocodazole. (B) Histogram analysis of the representative experiments ($n = 3$) shown in (A). The % reduction in TER in response to nocodazole was significantly attenuated by pretreatment with forskolin 15 min onwards up to 1 hr. * indicates statistical significance at $P < 0.001$.

Fig. 10.

Influence of nocodazole on *in vitro* permeability. The changes in *in vitro* permeability in response to nocodazole was ascertained by quantifying the flux of HRP across cells grown on culture inserts (A). Treatment with 2 μ M nocodazole for 30 min significantly increases HRP flux compared to control (n = 5). Histamine (H) at the concentration of 100 μ M for 10 min was used as a positive control. * indicates statistical significance at $P < 0.001$. The ability of elevated cAMP to oppose nocodazole-induced increase in paracellular permeability was quantified by the flux of FITC dextran across cells grown on porous culture inserts (B). Treatment with 5 μ M nocodazole significantly increases permeability to FITC dextran in comparison to control. Pretreatment with 10 μ M forskolin (FSK) in combination with 50 μ M rolipram (ROL) for 1 hr significantly attenuates nocodazole-induced increase in permeability (n = 6). * indicates statistical significance at $P < 0.01$.

Fig. 11.

Effect of thrombin on microtubule organization. Immunostaining for microtubules in untreated cells (A). The characteristic uncondensed fibrillary extensions are evident. Treatment with 5 μ M paclitaxel (PTX) alone for 1 hr (B) stabilizes microtubule assembly. Exposure to 2 U/ml thrombin for 2 min (C) induces microtubule condensation (shown by arrows). Pretreatment with 5 μ M paclitaxel for 1 hr (D) opposes the thrombin response. The responses shown are typical of three independent trials.

Fig. 12.

Effect of microtubule stabilization on thrombin-induced MLC phosphorylation. Treatment with 2 U/ml thrombin for 2 min induces increased MLC phosphorylation when compared to untreated cells. Pretreatment with 5 μ M paclitaxel (PTX) for 1 hr (A) attenuates the thrombin (Th)-induced MLC phosphorylation. Summary of densitometric analysis (B) expressed as % pMLC of the representative experiments ($n = 6$) similar to that shown in (A). * indicates statistical significance at $P < 0.001$. ** indicates statistical significance at $P < 0.01$.

Fig. 13.

Cell signaling involved in the loss of barrier integrity secondary to microtubule disruption. RhoA-specific guanine nucleotide exchange factors (GEFs) on the microtubules are released upon disruption of microtubules, leading to activation of RhoA. Subsequently, Rho kinase inhibits MLC phosphatase (MLCP) by phosphorylating its MYPT1 subunit. The resulting increase in MLC phosphorylation induces actomyosin contraction, leading to disruption of the PAMR, which in turn causes breakdown in the barrier integrity. Activation of PKA, which prevents activation of RhoA, indirectly inhibits MLCP.