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Dysregulation of the Barrier Function in T84 Intestinal Epithelial Cell Monolayers Through the Modulation of Tight Junction Localization by Lipopolysaccharide

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Abstract：**Background & Aims:** Bacterial lipopolysaccharide（LPS）has been reported to be an important factor involved in experimental enteropathy and IBD. Our aim was to investigate the functional, as well as the morphological, changes of the tight junction（TJ）in response to LPS in T84 intestinal epithelial monolayers. **Methods:** LPS（10, 100 or 300 μg/ml）was added to the cultured T84 cells. The barrier function of the TJ was evaluated by measuring transepithelial electrical resistance（TER）. The expression and localization of TJ-associated proteins（ZO-1 and occludin）were examined by Western blotting and confocal laser scanning microscopy, respectively. **Results:** LPS induced a significant decrease in TER in a dose-dependent manner in T84 cells. Western blotting demonstrated no significant changes in the expression of either ZO-1 or occludin. Confocal laser scanning microscopy and densitometric analyses showed significantly altered localization of ZO-1 and occludin at a higher concentration of LPS（300 μg/ml）（$P < 0.001$）. **Conclusions:** These results suggest that LPS induces a disruption of the TJ in T84 cells due to an altered localization of ZO-1 and occludin, thus leading to barrier dysfunction. The dysregulation of TJ by LPS may therefore play a crucial role in the pathogenesis of IBD.

Key words：**Tight junction, Lipopolysaccharide, T84 cell monolayer, ZO-1, Occludin**

Introduction

Intestinal epithelial cells provide a barrier function against proinflammatory luminal factors. Intestinal epithelial tight junctions（TJ）are located in the paracellular space between intestinal epithelial cells, and their major function is a barrier to block the paracellular permeation of proinflammatory luminal factors [1, 2]. In addition, the TJ functions as a gate to regulate the passage of ions, water, and various macromolecules through paracellular spaces. The TJ complex is composed of many different proteins including zonula occludens（ZO）-1, ZO-2, ZO-3, occludin, claudins-1/-2, and junctional adhesion molecules（JAM）[3-8]. ZO-1, ZO-2 and ZO-3 are cytoplasmic proteins, whereas occludin, claudins and JAM are transmembrane protein. The TJ is disrupted in experimental colitis, inflammatory bowel disease（IBD）, and several liver diseases [9-13]. In IBD tissues with active inflammation, the expression levels of TJ proteins（ZO-1, occludin）were decreased, whereas the expression levels of TJ proteins were almost unchanged in cases of inactive inflammation [9]. In IBD, the downregulation of the expression of junctional molecules is associated with inflammatory processes.

The human intestinal tract contains hundreds of different bacterial species. The intestinal epithelium is constantly exposed to lipopolysaccharide（LPS）derived from the intestinal bacterial flora. In an experimental enteropathy rat model, bacterial LPS plays a major role

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in indomethacin enteropathy [14]. Bacterial LPS induces an exacerbation of murine ileitis, and the LPS scavenger, polymixin B, ameliorates murine ileitis [15]. Alkaline phosphatase was able to detoxify LPS while significantly reducing inflammation in a dextran sodium-induced rat model [16]. Bacterial products, in particular LPS, have been reported to be important factors involved in the pathogenesis of IBD [17-19]. Therefore, LPS is considered to play an important role in experimental and human enteropathy.

The administration of LPS to the apical side of the cultured intestinal epithelial cells activate the MAPK family and transcription factor NF-κB [20]. LPS stimulates the release of proinflammatory cytokines such as TNF-α and IL-8 in intestinal epithelial cells [21, 22]. The disruption of the TJ is associated with pro-inflammatory cytokines such as TNF- α and interferon- γ (IFN- γ) [23-28]. LPS disrupts the barrier function and TJ in various epithelial and endothelial cells [29-32]. However, the functional responses of the TJ to stimulation by LPS in cultured intestinal epithelial cells have not been fully examined. In the present study, we investigated the functional as well as the morphological changes of the TJ in response to LPS in cultured intestinal epithelial cells.

Materials And Methods

Cell culture

T84 epithelial cells were grown in 1/1 DMEM and Ham's F-12 medium supplemented with 15 mM HEPES (pH 7.5), 14 mM $NaHCO_3$, antibiotics, and 10% newborn cow serum. The cells were seeded on collagen-coated, permeable polycarbonate filters (5 μm pore size) with a surface area of 0.33, 1.0, or 4.7 cm^2, as described previously (Coaster, Cambridge, MA) [33]. Bacterial LPS from *Escherichia coli* serotype 026; B6 (Sigma, St. Louis, MO) was added apically to the monolayers for incubation times, varying from 6 to 24 hr. Control monolayers were incubated with the cell culture medium only.

Assessment of Cell viability

Cell viability was determined by lactate dehydrogenase (LDH) release. Confluent T84 monolayers on 1.0-cm^2 filters were exposed to the medium with or without LPS (10, 100, 300, or 1,000 μg/ml), which was added to the apical side of the filters. The LDH concentration in the supernatant was measured, after 6 hr incubation with or without LPS. LDH content in the supernatant was determined by the JSCC transferable method using Pureauto LD (Sekisui Medical, Tokyo, Japan) [34].

Electrophysiological measurement of TER of cultured T84 cells

The transepithelial electrical resistance (TER) of confluent T84 monolayers on 1.0-cm^2 filters was measured before each experiment using an epithelial voltohmmeter (EVOM/EndOhm; World Precision Instruments, Sarasota, FL) [26]. The cells were used only when their stable TER was more than 1,000 Ω·cm^2. Next, the monolayers were exposed to the medium alone or LPS (10, 100, or 300 μg/ml), added to the apical side of the filters for 24 hr. TER was measured at 6, 12, and 24 hr after incubation with LPS.

Immunoblotting for TJ proteins in T84 epithelial cells

Confluent T84 monolayers on 4.7-cm^2 filters exposed to LPS (100 or 300 μg/ml for 6 hr)or the control were washed with ice-cold PBS and lysed in ice-cold NP-40 buffer (25 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% NP-40, 1mM Na_3VO_4, 1 mM APMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Cells were scraped into a 1.5-ml microcentrifuge tube and gently rotated for 30 min at 4℃. After centrifugation (1,000 g for 30 min), the supernatant was collected as the NP-40-soluble fraction. The pellet was resuspended in SDS buffer (25 mM Hepes, pH 7.5, 4 mM EDTA, 25 mM NaF, 1% SDS, 1 mM Na_3VO_4) using a homogenizer. The homogenate was combined with NP-40 buffer and passed 10 times through a 27 G needle, and then gently rotated again for 30 min at 4℃. After centrifugation (1,000 g for 30 min), the supernatant was used as the NP-40-insoluble fraction. The NP-40-insoluble material was then solubilized with SDS. The NP-40-insoluble fraction contained the cytoskeleton and associated proteins, while the NP-40-soluble fraction was not associated with the cytoskeleton [34, 35]. Equivalent protein concentrations in the NP-40-soluble fraction and the NP-40-insoluble fraction from the control and treated monolayers were subjected to SDS-PAGE and immunoblotting analysis for TJ proteins as described previously [35]. A quantitative densitometric analysis was done using the ImageGauge software program (Fujifilm, Tokyo, Japan) (n=4 in each group).

Immunofluorescence for ZO-1 and occludin in confluent T84 cells

Confluent T84 monolayers on 1.0-cm^2 filters exposed to LPS (100 or 300 μg/ml for 6 hr) or the medium only were fixed with 3.7% paraformaldehyde (10 min at room temperature) and permeabilized with 0.5% TX-100. Nonspecific background was blocked with 5% normal goat serum (1 hr at room temperature). The monolayers were incubated with primary antibodies to ZO-1 or occludin (1/400; Zymed Laboratories, San Francisco, CA) for 1 hr. The monolayers were washed and probed with Alexa-Fluor-488 (1/1,000, Molecular Probes) secondary antibodies. All monolayers were observed on a confocal laser scanning microscope (LSM 5 PAS-CAL, Zeiss, Jene, Germany) as previously described [36]. A series of images was collected from 0.06-μm XY sections. The images were stacked using the image J software program and then was processed by Adobe Photoshop (Adobe Systems, San Jose, CA). The Image J software program was used to evaluate the amounts of occludin and ZO-1 present at the intercellular junction by semiquantitatively measuring fluorescence density in the selected areas [32]. The values are presented as pixels per square millimeter.

Statistics

Results were expressed as the mean ± SD. Student's *t*-test was used to compare the results, and $P < 0.05$ was considered to indicate statistical significance.

Results

Assessment of cell viability

After incubation with LPS (10, 100, 300, or 1,000 μg/ml) or medium alone, cell viability was determined by LDH release. At concentrations of LPS (10, 100, or 300 μg/ml), LDH release was not increased after 6 hr in comparison to the control group (**Figure 1**). At a higher concentration of LPS (1,000 μg/ml), LDH release was significantly increased in comparison to the control group ($P < 0.01$). From these results, the monolayers were further exposed to the medium alone or LPS (10, 100, or 300 μg/ml).

The effect of LPS on the barrier function of TJ

After the cultured T84 cells were incubated with LPS (10, 100, or 300 μg/ml) or medium alone, the barrier function was determined by the measurement of TER. LPS induced a significant decrease in TER in a dose-dependent manner (**Figure 2**). At LPS concentrations of 100 and 300 μg/ml, the TER significantly decreased by 49.0% and 57.4% ($P < 0.01$), respectively, after 6 hr. After 24 hr, the TER partly recovered but significantly

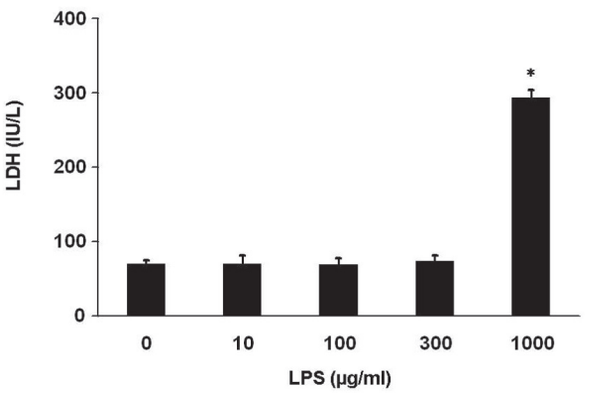

Fig. 1 Cell viability was determined by LDH release. After incubation with LPS (10, 100, 300, or 1,000 μg/ml) or medium alone for 6 hr, LDH contents were measured. At lower concentrations of LPS groups (10, 100, or 300 μg/ml) LDH release was not increased in comparison to the control groups. At a higher concentration of LPS (1,000 μg/ml), LDH release was significantly increased in comparison to the control group ($P < 0.01$) (*, $P < 0.01$ in comparison to the control group). Results represent the mean ± SD (n = 5 in each group).

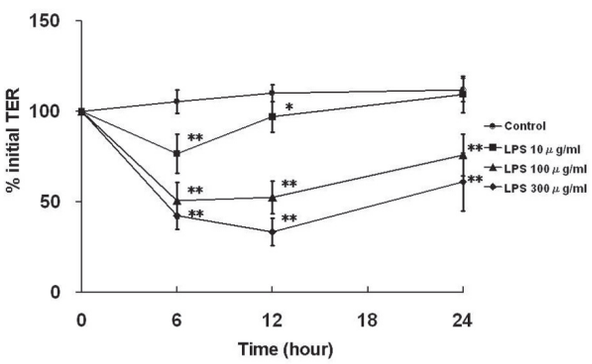

Fig. 2 Electrophysiological measurement of the TER of cultured T84 cells. Confluent T84 intestinal monolayers were incubated with LPS (10; squares, 100; triangles, or 300 μg/ml; diamonds) administered to the apical side of the monolayers or with medium alone (circles). The control monolayers maintained a TER of more than 1,000 Ω・cm2. LPS induced a significant dose-dependent decrease in TER in the T84 monolayer (*, $P < 0.05$; **, $P < 0.01$ in comparison to the control group). After 24 hr in the LPS (10 μg/ml) group, TER returned to the control level. The results represent the mean ± SD (n = 5 in each group).

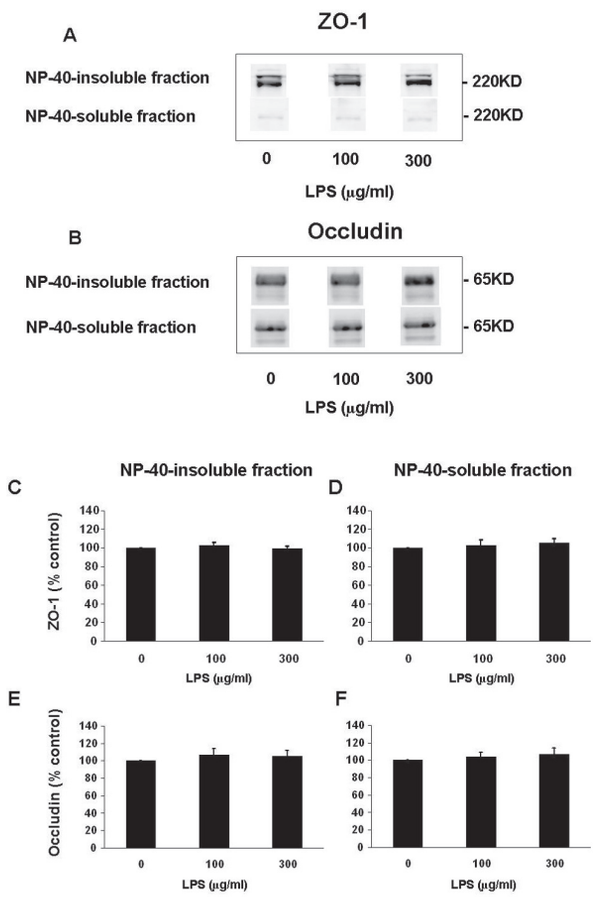

Fig. 3 Immunoblotting for TJ proteins. Confluent T84 intestinal monolayer cells were incubated for 6 hr with LPS (100 or 300 μg/ml) or with the cultured medium alone. The NP-40-soluble and the NP-40-insoluble fractions were immunoblotted with antibodies to ZO-1 and occludin. LPS treatment did not affect the expression level of the TJ-associated proteins, ZO-1 (A) and occludin (B). The expression of both ZO-1 and occludin showed no significant differences between the control and LPS (100 or 300 μg/ml) groups (C, D, E, and F). The results represent the mean ± SD (n = 4 in each group).

decreased ($P < 0.01$). A lower concentration of LPS (10 μg/ml) resulted in a significant decrease in TER by 23.2% after 6 hr ($P < 0.01$), but after 24 hr, the TER returned to the control level. These data indicated that LPS decreased TER in a dose-dependent manner, and that the TER tended to recover after 24 hr.

Expression of TJ-associated proteins, ZO-1 and occludin

After T84 cells were incubated with LPS (100 or 300 μg/ml) or medium alone, the expression of ZO-1 and occludin in the cells were determined by an immunoblotting analysis. In the control group, ZO-1 was mainly found in the NP-40-insoluble fraction (**Figure 3A**), but occludin was found in both the soluble and insoluble fractions (**Figure 3B**). LPS treatment did not alter the expression level of these proteins in either the NP-40-soluble or insoluble fractions. Furthermore, densitometric analyses of the insoluble and soluble fractions revealed that the expression of neither the ZO-1 (**Figures 3C** and **D**) or occludin (**Figures 3E** and **F**) showed no significant changes between the control and the LPS groups (100 and 300 μg/ml).

Localization of ZO-1 and occludin

After T84 cells were incubated with LPS (100 or 300 μg/ml) or medium alone, localization of ZO-1 and occludin was examined by immunofluorescence histochemistry. In the control monolayers, confocal laser scanning microscopy revealed that both ZO-1 and occludin were regularly localized at the cellular border in a chicken wire pattern (**Figures 4A** and **B**). Treatment with LPS (300 μg/ml) for 6 hr altered the distribution of ZO-1 and occludin at the cellular border (**Figures 4C** and D). LPS reduced the staining for ZO-1 and occludin. After the treatment with LPS for 6 hr, fluorescence at the cellular border was measured by a densitometric analysis using the Image J software program. LPS (300 μg/ml) induced the disruption of ZO-1 and occludin at the cellular border by densitometric analysis (**Figures**

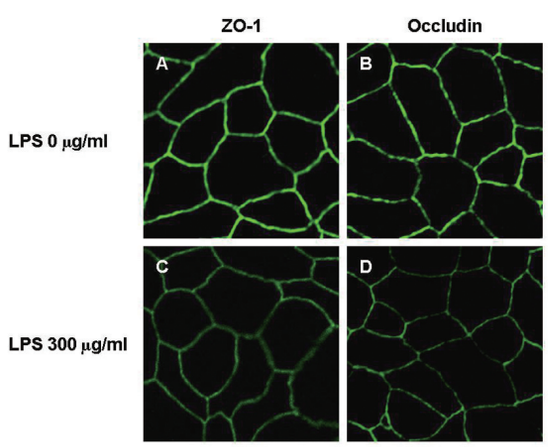

Fig. 4 Immunofluorescence for ZO-1 and occludin in confluent T84 cells. In the control group, confocal laser scanning microscopy showed that ZO-1 (A) and occludin (B) were linearly localized at the apical cellular border, while LPS (300 μg/ml) caused a disrupted appearance of ZO-1 (C) and occludin (D) (Original magnification, 400X). Treatment with LPS for 6 hr induced changes in the distribution of ZO-1 and occludin at the cellular border.

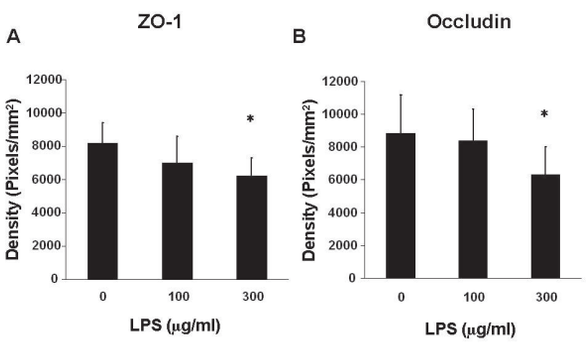

Fig. 5 Fluorescence density at the junctions was analyzed using the software Image J. Immunofluorescence densities for both ZO-1 (A) and occludin (B) were significantly decreased in the LPS concentration of 300 μg/ml, compared to the control group (*, $P < 0.05$ in comparison to the control group), however, no significant differences were observed between the LPS concentration of 100 μg/ml and the control group. The results represent the mean ± SD (n = 5 in each group).

5A and **B**). The density of immunofluorescence for ZO-1 and occludin were significantly decreased at the LPS concentration of 300 μg/ml, in comparison to the control group ($P < 0.05$). However, no significant differences between the LPS concentration of 100 μg/ml and the control group were noted. LPS, therefore, caused an altered localization of both ZO-1 and occludin.

Discussion

In the present study, LPS induced a decrease in TER in a dose-dependent manner, thus indicating that LPS disrupted the barrier function and the TJ. The disruption of the TJ is elicited by bacteria, cells of the immune system, and proinflammatory cytokines including TNF-α, INF-γ, IL-1, IL-4, and IL-13 [11, 24-26, 34, 37, 38]. These agents similarly induced a decrease in the TER and disrupted the barrier function [24, 26, 34, 37].

The functional responses of the TJ to LPS have not been fully examined. A number of previous studies, however, revealed that LPS stimulates the release of various proinflammatory cytokines such as TNF-α and IL-8 in intestinal epithelial cell lines via several pathways including Toll-like receptor (TLR) and NF-κB transcription factor [20-22]. TLRs, a group of pattern recognition receptors, have a role in pathogen recognition and host defense [39]. More than ten TLRs have been identified in mammalian species; these TLRs recognize distinct pathogen-associated molecular patterns. In particular, TLR4 serves as the main mediator of the innate immune response to LPS. In IBD patients, TLR4 expression is strongly upregulated [40]. TLR4 is constitutively expressed at the apical pole of T84 cells [41]. When LPS was administered to the apical side of T84 cells, the TLR4 and LPS complex activates an innate immune response, leading to the activation of transcription factor NF-κB, a key regulator of immune and inflammatory responses [42-44]. In addition, the complex stimulates the release of proinflammatory cytokines such as TNF-α and IL-8 in intestinal epithelial cell lines [21, 22]. Furthermore, these proinflammatory cytokines disrupt the TJ [24-26]. Therefore, such proinflammatory cytokines may be involved in a LPS-induced disruption of the barrier function. Further studies are required to elucidate the exact mechanism by which LPS disrupts the barrier function.

In the present study, LPS induced a decrease in the TER after 6 hr and TER showed a recovery after 24 hr, suggesting that a restoration mechanism may occur when stimulated with LPS. Several studies have investigated the mechanisms of tolerance to bacterial components such as LPS in intestinal epithelial cells [45-47]. Bacteria-derived LPS up-regulates peroxisome proliferator-activation receptor γ mediated via TLR4 and acts to suppress inflammation in colonic epithelial cells [45]. Otte *et al.* have shown that in intestinal epithelial cells prolonged incubation with LPS results in a state of hyporesponsiveness with no reactivity of cells to a second challenge, in which the phenomena were involved in a decrease in TLR surface expression and interleukin receptor associated kinase activity, and an increase in toll-interacting protein [47]. Cario *et al.* have shown TLR4 to be constitutively expressed at the apical pole of T84 cells [41]. After stimulation with LPS, TLR4 selectively passes to the cytoplasmic compartments near the basolateral membrane. Furthermore, LPS/TLR4-dependent cyclooxygenase-2 and prostaglandin E_2 production are important in mucosal homeostasis in intestinal epithelial cells, thereby protecting such cells against apoptosis [46]. Although the current study could not disclose the link between LPS and tolerance, these previous observations strongly indicate induction of LPS-induced tolerance in intestinal epithelial cells.

The functional responses of the apical side of TJ to the stimulation with LPS have not been fully examined. Kimura *et al.* and Xiao *et al.* showed that in rat intestinal crypt cell line IEC-6 cells the administration of LPS to

the basolateral side of the cultured cells decreased TER, but not when administered to the apical side [48, 49]. In contrast, Chin *et al*. showed that in the non-tumorigenic intestinal epithelial cell line, SCBN, the administration of LPS to the apical side of the cultured cells decreased the TER, but this did not occur when it was administered to the basolateral side [29]. The results of the present study also demonstrate that the administration of LPS to the apical side of T84 cells decreased the TER. Additionally, Rao *et al*. also showed that administration of hydrogen peroxide to cultured Caco-2 cells results in a greater decrease in the TER by apical administration compared with basolateral administration [50]. Jepson *et al*. have discussed the differences between apical and basolateral administration, and indicated that they may be related to the state of differentiation of the Caco-2 monolayers [51]. Taken together, the difference of these studies may be explained by the type of cultured cells and the cell differentiation.

The importance of actin in the maintenance and regulation of barrier function has been demonstrated in numerous studies [26, 37]. The TJ regulatory activity by actin has been reported to be localized to the apical portion of the cytoskeleton. Proinflammatory cytokines disrupt the TJ by decreasing the level of cytoskeleton-associated TJ proteins and by inducing cytoskeletal alterations. ZO-1 also interacts with the cytoskeleton by binding directly to actin [52]. ZO-1 may act as a direct or indirect link between the transmembrane component of the tight junction, occludin and the cytoskeleton. The interaction of TJ with the cytoskeleton is critical to the regulation of barrier function [26, 37]. The bacterial infection of epithelial cells also alters TJ-associated actin and subsequently disrupts the epithelial barrier function [38]. TJ proteins were investigated by separating the cytoskeleton-associated and non-associated proteins using NP-40 as described previously [34, 35]. In an analysis of the TJ proteins of the cytoskeleton-associated fractions, LPS did not decrease the protein level of cytoskeleton-associated ZO-1 and occludin. The results demonstrated that ZO-1 and occludin remains bound with cytoskeleton even after the treatment with LPS, and suggest that a decrease in the TER by LPS was associated with the altered localization of the ZO-1 and occludin.

In a morphological analysis, LPS altered the distribution of ZO-1 and occludin at the cellular border. LPS reduced the staining for ZO-1 and occludin. A densitometric analysis showed that LPS induced the disruption of ZO-1 and occludin at the cellular border. The TJ proteins have been shown to change their functions when the localization is altered without changes in TJ protein levels [25, 37, 53]. Therefore, LPS induced changes in localization but not in protein expression levels of the TJ proteins and these changes were responsible for a decrease in the TER. Blum *et al*. showed that the actin cytoskeleton plays a central role in endothelial TJ barrier regulation and that dynamic cytoskeletal alterations may primarily control vascular permeability [37]. The current results also suggest that altered localization of the ZO-1 and occludin may occur by cytoskeletal alterations.

In conclusion, these results suggest that LPS disrupts the barrier function in T84 cells through an altered localization of ZO-1 and occludin. As a result, TJ injury induced by LPS may therefore play an important role in the pathogenesis of IBD.

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