MAJOR ARTICLE

Berberine Ameliorates Intestinal Epithelial Tight-Junction Damage and Down-regulates Myosin Light Chain Kinase Pathways in a Mouse Model of Endotoxinemia

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Background. This study aimed to examine the protective effect of berberine in endotoxin-induced intestinal tight-junction injury in a mice model of endotoxinemia.

Methods. Endotoxinemia was induced by intraperitoneal injection of lipopolysaccharide (10 mg/kg). Mice were randomized to 5 groups: control mice, berberine-treated mice, lipopolysaccharide (LPS)–injected mice, mice pretreated with berberine, and mice administered berberine following LPS injection. Samples were collected 12 h after LPS treatment.

Results. Ileal mucosal permeability to fluorescein isothiocyanate dextran assay indicated that berberine reduced the permeability of the gut barrier in endotoxinemia. Transmission electron microscopy revealed that pretreatement with berberine partly prevented ultrastructural disruption of tight junctions by LPS. Immunofluorescence and Western blot analysis were performed, and the results demonstrated that pretreatement with berberine partially reversed the redistribution of tight-junction proteins in colon epithelium and in membrane microdomains. Our data also indicated that pretreatement with berberine could suppress translocation, from cytoplasm to the nucleus, of nuclear factor-κB and myosin light chain kinase activation in the intestinal epithelium.

Conclusions. Pretreatement with berberine attenuates disruption of tight junctions in intestinal epithelium in a mice model of endotoxinemia. This may possibly have been mediated through down-regulation of the nuclear factor-κB and myosin light chain kinase pathway.

Sepsis and septic multiple organ dysfunction and injury are causes of high mortality in critically ill patients worldwide. Sepsis can occur through infection with Gram-positive bacteria as well as with fungi and viruses. However, the sepsis syndrome mainly occurs in response to lipopolysaccharide (LPS) from Gram-negative bacteria, and LPS-induced endotoxinemia is frequently used in animal models. Previous studies have implicated the alteration of intestinal function as playing a critical role in the development of polymicrobial sepsis [1, 2], generating the theory that the gut represents the ''motor'' of the systemic inflammatory response [3]. Intestinal barrier function depends primarily on the integrity of the intestinal epithelium, which prevents bacteria constituting intestinal flora from translocating via both transcellular and paracellular pathways [4]. The tight junction (TJ) is most important for defining the characteristics of the gastrointestinal barrier. Enteric pathogens disrupt TJ by either altering the cellular cytoskeleton or by affecting specific TJ proteins, such as the integral membrane protein occludin [5], zonula occludens (ZO)–1 [6], and members of the claudin family [7]. Previous studies have indicated that TJ architecture and TJ protein redistribution in TJ membrane microdomains were altered during polymicrobial sepsis [8], and the opening of TJs is driven by myosin light chain (MLC) phosphorylation, which depends on

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activation of MLC kinase (MLCK) [9]. An in vivo model demonstrated that inhibition of MLCK could prevent deleterious effects of LPS-induced sepsis on the TJ barrier [10]. Moreover, the nuclear factor (NF)–κB signaling pathway has been demonstrated to mediate increases in MLCK expression in response to proinflammatory stimuli, thus resulting in TJ barrier breakdown [11].

Berberine (BBR) has been used for thousands of years in China as a traditional herbal medicine to treat gastrointestinal disorders and bacterial infection, and there have been no toxic effects reported to date in clinical studies [12, 13]. Since the beginning in the previous century, BBR has been extensively investigated and found to possess a wide variety of pharmacological and biological activities, such as anti-inflammatory [14], antioxidant [15], antitumor [16], and cholesterol-lowering [17] effects. In vitro studies indicated that the anti-inflammatory activities displayed by BBR may be mediated, in part, through the suppression of the NF-κB activation pathway [18]. BBR could also attenuate LPS-induced lung injury and reduce the mortality rate [19]. However, the effect of BBR on TJ barrier function and MLCK activation in endotoxinemia is still unknown.

In this study, we investigated the effects of BBR on MLCK activation and TJ barrier dysfunction in LPS-induced endotoxinemia to test the hypothesis that treatment with BBR attenuates endotoxinemia-induced TJ breakdown through MLCK and the NF-κB signaling pathway.

MATERIAL AND METHODS

Experimental Animals

The present study was performed in accordance with the guidelines of the China Laboratory Animal Management Committee. Male C57BL/6 mice, aged 6–8 weeks and weighing 20–25 g, were obtained from the Model Animal Research Center of the Nanjing University (Nanjing, China). All animals were housed in plastic cages with standard mice chow and water and allowed to acclimate to their environment for 1 week before the experiment. The mice were anesthetized with pentobarbital after a specified protocol.

Reagents

LPS from the *Escherichia coli* serotype 055:B7 and BBR chloride was procured from Sigma. Antibodies to occludin, ZO-1, claudin-1, claudin-4, NF-κB, and MLCK were purchased from Zymed Laboratories. Alexa 635 secondary antibodies, the Alexa 488-conjugated streptavidin and DAPI (4′,6′-diamidino-2-phenylindole) were purchased from Molecular Probes.

Experiment Protocols

Before experimentation, mice were allowed to adapt to the experimental environment for 1 week. The mice were randomly divided into 5 groups (= 8 mice pergroup): control, BBR alone, LPS, BBR +plus LPS, and LPS + plus BBR. Mice in the BBR plus+ LPS group mice received 200 mg/kg BBR intragastrically once daily for 7 days. This dosage was chosen on the basis of a review of previously published studies [20], and each dose was dissolved in normal saline and diluted to obtain a final concentration of 10 mg/mL. The control, LPS, and LPS +plus BBR groups were administered an equal volume of normal saline intragastrically. The LPS (10 mg/kg) was dissolved in normal saline or an equal volume of normal saline and was injected intraperitoneally 1 h after intragastrical treatment on day 7. The LPS +plus BBR group mice received 200 mg/kg BBR administered intragastrically 30 min after an injection of LPS. During experiments, mice had free access to water and food. Mice were killed 12 h after intraperitoneal injection of LPS or saline.

Clinical Score

At specified time points, animals were assessed for weight, food intake, and water consumption. In addition, clinical condition was assessed as a symptom score by grading the severity of conjunctivitis, diarrhea, ruffled fur, and lethargy on a 3-point scale [21]. As a result of obvious differences in appearance among treatment groups, grading was performed in a blinded fashion by 3 independent researchers. The means of 3 assessments were taken for grading. The scoring system is presented in Table 1.

Histopathological Evaluation

Segments of distal ileum and colon were excised, transferred into 10% phosphate-buffered saline (PBS) buffered formalin and embedded in paraffin. Sections measuring 5-μm in thickness were cut, placed on glass slides, and stained with hematoxylin and eosin (H&E). Images were obtained using a Zeiss Image A1 light microscope at × 20 magnification with AxioVision V4.5 software. 3 pathologists, blinded to the source of the slides, analyzed and reported on each slide. The degree of histopathologic changes was graded semiquantitatively by using the histologic injury scale described by Chiu et al [22]. (Table 2)

Transmission Electron Microscopy of TJ

2-mm sections of colon were washed and fixed with 4% glutaraldehyde for 2 h and then post-fixed with 1% osmium tetroxide. Tissues were embedded in Epon 812 and thinly sectioned. Thin sections were cut and stained with uranyl acetate and lead citrate and were examined with an H-600 (Hitachi, Japan) transmission electron microscope operated at 75kV.

Intestinal Permeability Assay

The assay of intestinal permeability was modified from a method described previously [23]. At 3, 6, and 12 h after injection of LPS, animals from each experimental group (3 per group) were subjected to general anesthesia with ketamine. A midline laparotomy was performed and the distal ileum exposed. A 5-cm segment of distal ileum was isolated between silk ties. A solution

Table 1. Clinical Scoring System

	Score		
Variable	0	1	2
Conjunctivitis	Eyes closed or bleared with serous discharge	Eyes opened with serous discharge	Normal, no conjunctivitis
Stool consistency	Diarrhea	Loose stool	Normal stool
Hair coat	Rough and dull fur, ungroomed	Reduced grooming, rough hair coat	Well groomed, shiny fur
Activity upon moderate stimulation	Lethargic, only lifting of the head after moderate stimulation	Inactive, less alert, <2 steps after moderate stimulation	Normal locomotion and reaction, >2 steps

containing 25 mg of 4kDa FITC-Dextran, diluted in 0.1 mL of PBS, was injected into the intestinal lumen. The bowel was then returned to the abdominal cavity and the skin was closed. The animal was kept under general anesthesia. A blood sample was drawn via cardiac puncture 30 min after the injection of FITC-Dextran. Blood was placed in ice-cold heparinized tubes and centrifuged at 10,000*g* for 10 min. The plasma was then analyzed to determine the concentration of FITC-Dextran by using a fluorescence spectrophotometer (F7000; Hitachi) at excitation and emission wavelengths of 495 nm and 520 nm, respectively. A standard curve was obtained by diluting serial concentrations of FITC-Dextran in mouse serum.

Immunofluorescence

Immunostaining was performed as described elsewhere [24]. After rinsing with ice-cold PBS, tissues were fixed in 3% paraformaldehyde and embedded in optimal cutting temperature compound (OCT; Sakura Finetech) and 5-μm frozen sections were transferred to coated slides, fixed in 1% paraformaldehyde, and washed 3 times with PBS. Thereafter, nonspecific binding was blocked with 5% normal goat serum in PBS. After incubation with monoclonal antibodies against occludin, ZO-1, claudin-1, claudin-4, and MLCK (1:200) in PBS with 1% goat serum overnight at 4°C, sections were washed and incubated with Alexa 635-conjugated or Alexa 488-conjugated secondary antibodies for 60 min. Images were obtained using a Leica TCS SP2 laser confocal scanning microscope (Leica Microsystems; Heidelberg).

Isolation of TJ Membrane Microdomains

Mucosal samples from colonic tissue were homogenized in an extraction buffer (Tris, 50 mmol/L; KCl, 25 mmol/L; $MgCl_2$, 5 mmol/L, EDTA, 2 mmol/L; NaF, 40 mmol/L; Na_3VO_4, 4 mmol/L; pH 7.4) containing 1% Triton X-100 and protease inhibitor mixture solution, and homogenized by using 50 strokes of a dounce homogenizer. TJ membrane microdomains were isolated and TJ proteins were analyzed by Western blot according to our previously reported method [25].

Tissue Nuclear Protein Extraction

Nuclear extracts were prepared by following a standard protocol [26]. Approximately 2 cm of proximal colon, collected 1 cm from the cecocolonic junction, was ground in a mortar with liquid nitrogen. The powder was suspended in a buffer: (NaCl, 150 mmol/L; HEPES, 10 mmol/L [pH 7.9]; EDTA, 1 mmol/L; PMSF, 0.5 mmol/L; and 0.6% NP-40) and briefly homogenized, and large debris were extracted by centrifugation. The supernatant was incubated on ice for 5 min and then centrifuged at 5000 g for 5min. The pelleted nuclei were lysed by resuspending them in 0.5 mL of buffer containing HEPES, 20 mmol/L (pH, 7.9); NaCl, 420 mmol/L ; MgCl, 21.2 mmol/L; EDTA, 0.2 mmol/L; DTT, 0.5 mmol/L; PMSF, 0.5 mmol/L; benzamidine, 2 mmol/L; 25% glycerol, and 5 μg/mL each of

Table 2. Intestinal Mucosal Damage Grading Score

Grade	Histological characteristic(s)
Grade 0	Normal mucosal villi
Grade 1	Subepithelial Gruenhagen's space (oedema), usually at the apex of the villus
Grade 2	Extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria
Grade 3	Massive epithelial lifting down the sides of villi; a few tips may be denuded
Grade 4	Denuded villi with lamina propria and dilated capillaries exposed
Grade 5	Digestion and disintegration of lamina propria; haemorrhage and ulceration

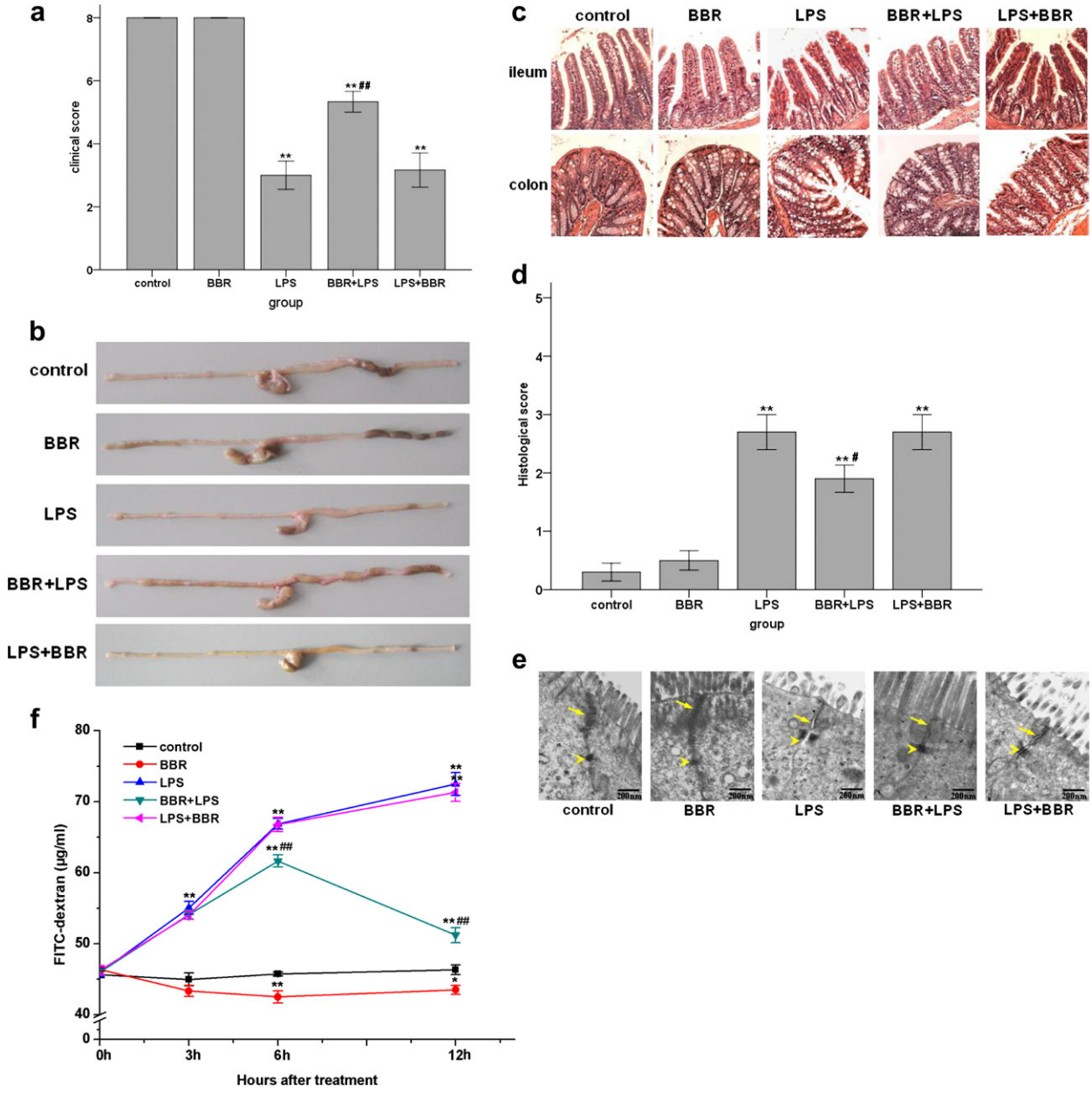

Figure 1. Clinical score, macroscopical features, histological appearance of distal ileum and colon, histological score of 5 group mice, ultrastructure micrographs of tight junctions (TJs), and intestinal Permeability. *A,* At indicated time points, animals were assessed for clinical score. *B,* photographs of the distal ileums and colons from 4 groups. Grossly, the stool pellets were smaller and the colon was thinner in sepsis group, and pretreatment berberine (BBR) prevented this change induced by lipopolysaccharide (LPS). *C,* Photomicrographs of sections of iliac and colonic samples. Sections of distal ileum were stained with hematoxylin and eosin (H&E) and viewed using a light microscope (original magnification, ×40). Histological injury is noted in endotoxemic mice, characterized by atrophy of the villi and discrete submucosal. In contrast, less injury was observed mice that received pretreatment BBR. *D,* Mucosal damage grading was assessed. The mean histological score of BBR-pretreated mice is significantly lower than that of endotoxemic mice, but subsequent treatment of BBR could not attenuate this damage. *E,* Ultrastructure of TJs in colon was observed by transmission electron microscope. In LPS-treated mice, the TJ and desmosomes were not intact, and the electron-dense materials were diminished. BBR before treatment attenuated this disruption of TJ morphology in endotoxemic mice. Arrows indicate the location of the TJs, and arrowheads indicate the desmosomes. (Scale bar = 200 nm). *F,* Effect of BBR on LPS-induced increase of intestinal permeability. The systemic concentration of 4 kDa FITC-Dextran was measured 3, 6, and 12 h after injection. Endotoxinemia resulted in a significant increase in intestinal permeability to 4 kDa FITC-Dextran; pretreatment of BBR but not subsequent treatment of BBR partially prevented the increased permeability; treated with BBR alone may partially reduce intestinal permeability compared with control group. Data are mean ± standard error of the mean (SEM), with 8 mice per experimental group. $^{*}P < .05$.** $P < .01$, compared with the control group. $^{\#}P < .05$, compared with the LPS group. $^{\#\#}P < .01$, compared with the LPS group.

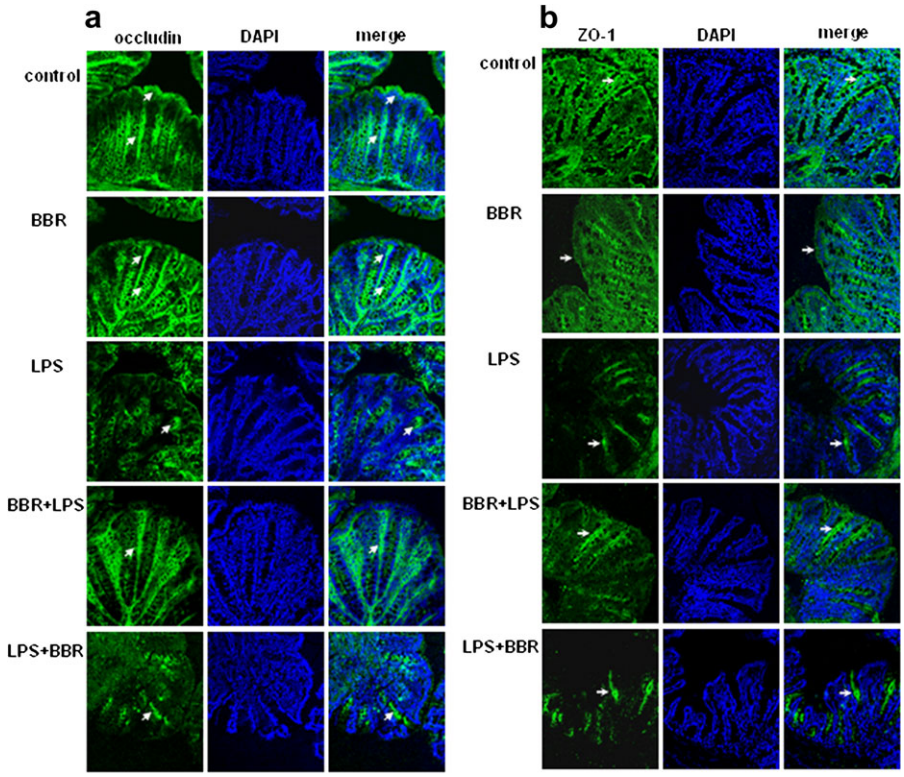

Figure 2. Distribution of occludin and ZO-1 in the colon. Immunolocalization of occludin *(A)* and ZO-1*(B)* at a 200× magnification is shown. In control and berberine (BBR) group mice, occludin and ZO-1 staining were continuous along the villous epithelium (green fluorescence). Nuclei were stained by DAPI (blue). Lipopolysaccharide (LPS) caused significant down-regulation of both proteins in the epithelium. In contrast, BBR pretreatment ameliorated the deleterious effect of LPS on the localization of both proteins, but subsequent treatment with BBR could not change this effect of LPS. Arrows show the staining of TJ proteins. Sections from at least 3 mice were examined for each condition.

pepstatin, leupeptin, and aprotinin. After centrifugation at 10,000*g* to remove nuclear debris, nuclear extracts were stored at −80°C. Protein concentration was determined by bicinchoninic acid assay (Pierce Biochemical).

Western Blot Analysis

Western blot analyses were performed for measurement of protein expression of NF-κB, MLCK, and TJ. For MLCK analysis, 2 cm of proximal colon were collected 1 cm from the cecocolonic junction and washed with saline, and the mucosa was obtained by scraping. Then proteins were extracted with the RIPA buffer for analysis. Protein concentration was determined by bicinchoninic acid assay (Pierce Biochemical).

Equal amounts of each extract were electrophoresed in 6% SDS–PAGE and then electrotransferred onto PVDF membranes (Bio-Rad). The membrane was blocked for 90 min at room temperature in 3% bovine serum albumin and 0.1% Tween-20 in Tris-buffered saline (TBS) followed by overnight incubation with primary antibodies diluted in 1% bovine serum albumin in TBS at 4°C. Membranes were washed 3 times in TBS (containing 0.1% Tween-20), and then incubated with the secondary antibodies for 90 min at room temperature. The membrane was developed by incubating for 5 min with SuperSignal Reagent (Pierce). Band intensities were quantified by densitometry and expressed as mean area density with use of Quantify One software (Bio-Rad), version 4.1.1. For total protein blots, mean area density was expressed relative to actin expression.

RESULTS

Clinical Score and Gross Morphological and Histopathologic Evaluation

Using a mouse model of endotoxemia, we observed that animals subjected to pretreatment with BBR prior to the intraperitoneal challenge with LPS had a significantly improved clinical score (Figure 1a). The amount of stool pellets in the colon from the BBR + plus LPS–treated group appeared to be greater, the cecum less edematous, and the colon thicker, compared with findings for endotoxemic mice (Figure 1b).

H&E staining of specimens from the ileums and colons of control and BBR group mice revealed integrated villi and compactly arrayed epithelium. The histological appearance of the intestine demonstrated marked atrophy and blunting of the villi; the brush border was discontinuous and the epithelium was disarrayed in endotoxemic mice. The group subjected to pretreatment with BBR exhibited minimal changes in the villi, and

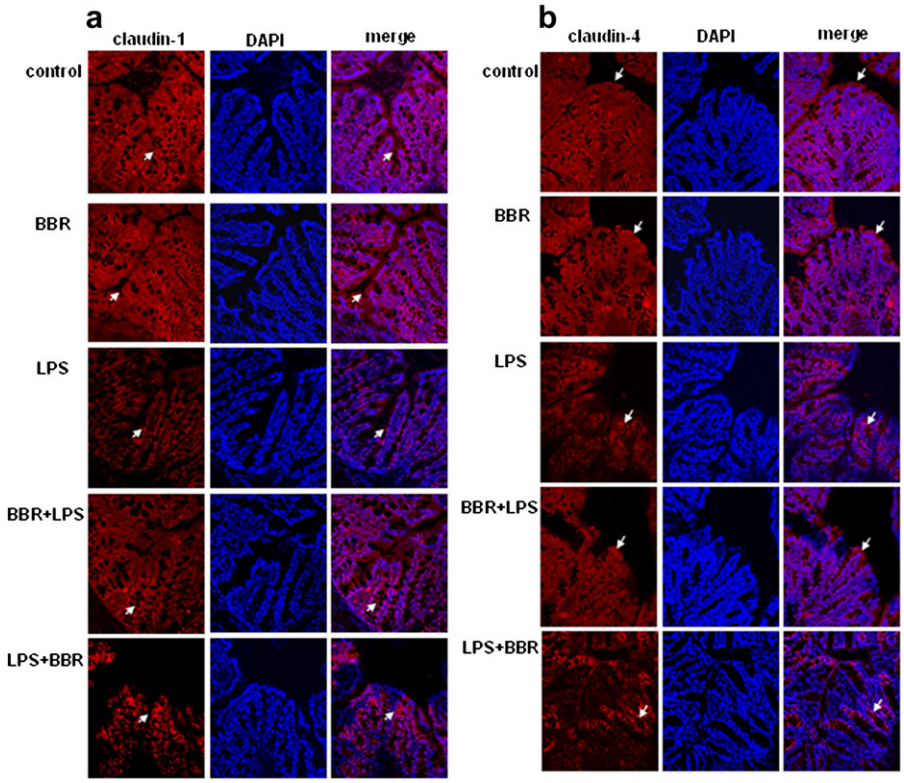

Figure 3. Distribution of claudin-1*(A)* and claudin-4 *(B)* in control mice, mice treated with berberine (BBR) alone, mice exposed to lipopolysaccharide (LPS) for 12 h, and endotoxemic mice that were pretreated and subsequently treated with BBR. Claudin staining *(red)* and DAPI staining *(blue),* as well as merged claudin and DAPI images, are presented. In animals in the BBR plus + LPS group, claudin-1 and claudin-4 distributions were regular, discrete, and similar to those in tissue-sections of control mice. However, in the LPS plus+ BBR group, claudin-1 and claudin-4 distributions were similar to those in the LPS group. Arrows indicate areas of staining. Images were shown at 200× the original magnification. Sections from at least 3 mice were examined for each condition.

the appearance resembled that of the sham group (Figure 1c). On the basis of an established mucosal damage score, pretreatment with BBR was found to prevent intestinal mucosal damage induced by LPS. However, subsequent treatment of BBR cannot reverse the effect of LPS (Figure 1d).

Treatment of BBR Prevented Ultrastructural Disruption of TJ in Sepsis Mice

In control and BBR groups, the TJ appeared to have an intact structure and desmosome. After 12 h of LPS treatment, we observed loss of TJ membrane fusions and decrease of electron-dense materials between the adjoining cells. Pretreatment with BBR alleviated the distortion of TJ ultrastructure. However, administration of BBR after LPS injection did not reverse the damage (Figure 1e).

Intestinal Permeability Assay

The amount of FITC-Dextran that permeabilized through the small intestinal epithelium into systemic circulation was significantly higher after LPS injection, compared with the control group. However, pretreatment with BBR decreased the amount of circulating FITC-Dextran (Figure 1f). This result indicates that pretreatment with BBR could alleviate increased intestinal permeability in endotoxemic mice. Our results also indicate that BBR alone may partially reduce intestinal permeability, compared with findings for the control group; this finding is consistent with our previous results obtained from in vitro research [27].

Immunofluorescence of TJ Protein

In our study, occludin and ZO-1 appeared as continuous bands along the epithelial sheet from the crypt to the villous tip both in control and BBR groups (Figures 2a and 2b). In the LPS group, occludin and ZO-1 appeared less distinctly, and the staining across the entire colonic epithelium was extensively decreased with certain residual staining in crypts. However, in the BBR + plus LPS group, the loss of occludin and ZO-1 organization was largely attenuated, and TJ distribution was similar to that in normal controls. Similarly, in control and BBR groups, claudin-1 and claudin-4 were predominantly distributed in the microvillus surface of epithelial cells and along the lateral membranes of cells. The distribution of claudin-1 and claudin-4 in colonocytes was completely disrupted in colonic epithelium of endotoxemic mice. Pretreatment of mice with BBR could partially

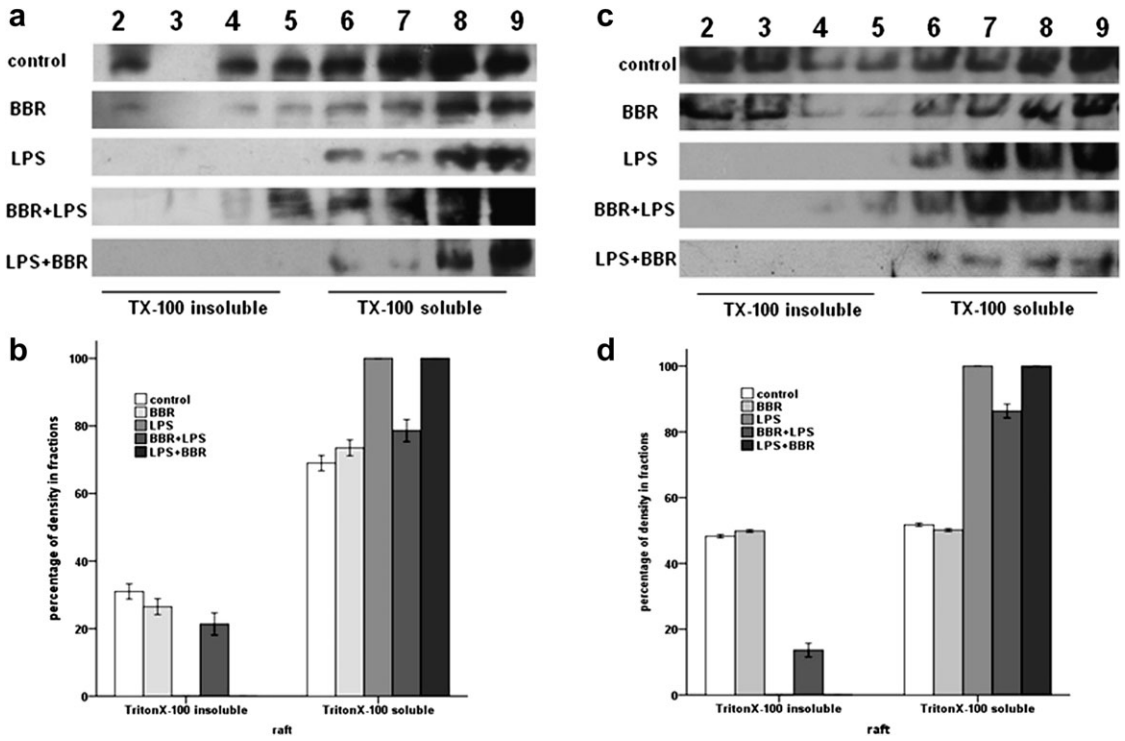

Figure 4. Localization of tight junction (TJ) protein occludin *(A* and *B)* and ZO-1 *(C* and *D)* in raft fractions. Homogenates were subjected to sucrose density gradient centrifugation. Equal amounts of protein from each fraction were analyzed by Western blotting. Blots were analyzed and quantified by densitometry with Quantity One analysis software. The blots shown are representative of 3 experiments. Data are shown as means ± standard errors.

attenuate LPS-induced redistribution of claudin-1 and claudin-4 (Figures 3a and 3b), but the intensity of staining was rather less than that observed in control mice. In contrast, the administration of BBR after LPS injection could not ameliorate the destruction.

Distribution of TJ Proteins in Membrane Microdomains

In this study, sucrose density gradients were used to examine the distribution of TJ proteins, including occludin, ZO-1, claudin-1, and claudin-4. Densitometry analysis was presented as a percentage of the total protein detected on the same immunoblot. Our results indicate that, in the control group, the amount, in membrane microdomains of TJs, was 51.72% ± .44% of occludin (Figures 4a and 4b), 31.01% ± 1.96% of ZO-1(Figures 4c and 4d), 42.77% ± .73% of claudin-1(Figures 5a and 5b), and 28.12% ± 1.76% of claudin-4 (Figures 5c and 5d). The distribution of TJ proteins in membrane microdomains was not altered in the BBR group; however, it was completely displaced from TJ membrane microdomains in endotoxemic mice. In the BBR +plus LPS group, the distribution of TJ proteins in raft fractions amounted to 13.64% ± 1.82% for occludin, 21.36% ± 2.83% for ZO-1, 22.39% ±1.92% for claudin-1, and 12.56% ± 1.32% for claudin-4. Our results indicate that the redistribution of TJ proteins from raft fractions was partially reversed by pretreatment with BBR.

Immunohistochemical Staining for MLCK

Increased MLCK activity results in alteration of colonic epithelial barrier function [28]. In our study, there was increased staining for MLCK in endotoxemic mice, compared with control and BBR groups; the images indicate the location of MLCK at the periphery of the intestinal epithelial cells. There was decreased staining in endotoxemic mice thatwere pretreated with BBR, but treatment with BBR after exposure could not alleviate the effect of LPS (Figure 6a).

Intestinal Nuclear NF-κB p65 and MLCK Protein

In the endotoxinemic state, the intestinal NF-κB p65 subunit is activated and translocates into the nucleus; the activated NF-κB then binds to the MLCK promoter region and increases the expression of MLCK [29, 30]. From Figures 6b and 6c, we observe that after 12 h of LPS stimulation, there was a marked increase in the nuclear expression of NF-κB, which was inhibited by pretreatment with BBR. The change in MLCK protein expression from colonic mucosa is depicted in Figures 6d and 6e. There was a significant increase of MLCK expression in endotoxinemia, and pretreatment with BBR also partially prevented an LPS-induced increase in MLCK expression, which is consistent with the observations for NF-κB.

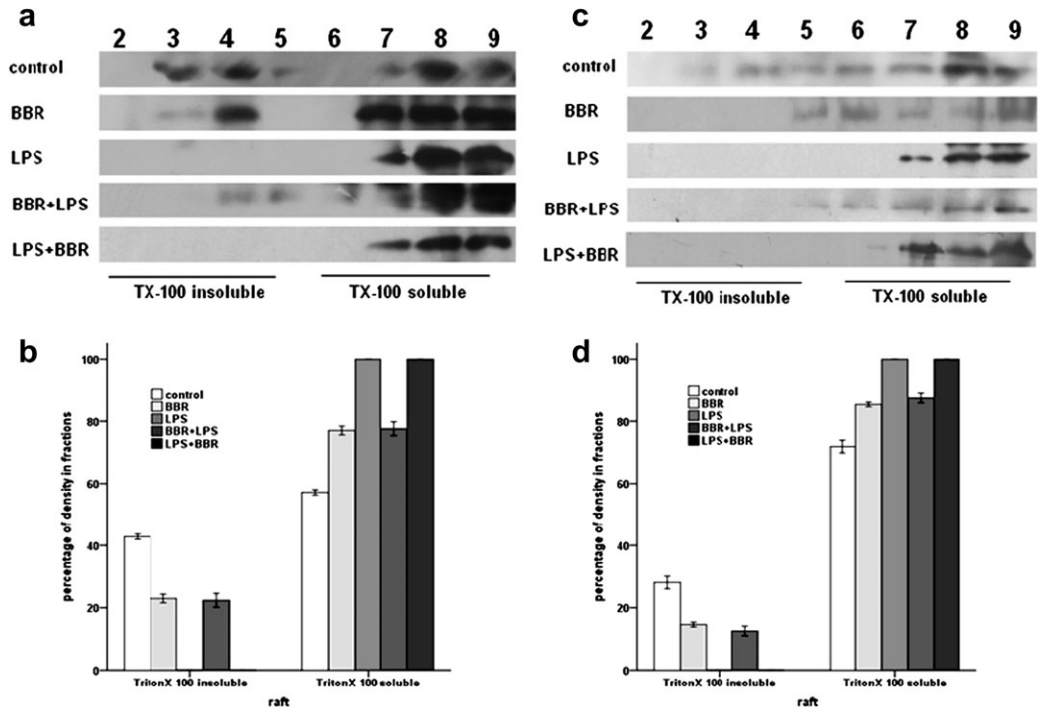

Figure 5. Localization of tight junction (TJ) protein claudin-1 *(A* and *B)* and claudin-4 *(C* and *D)* in raft fractions. Homogenates were subjected to sucrose density gradient centrifugation. Equal amounts of protein from each fraction were analyzed by Western-blotting. Blots were analyzed and quantified by densitometry with Quantity One analysis software. The blots shown are representative of 3 experiments. Data are shown as means ± standard errors.

DISCUSSION

In this study, we demonstrated that the administration of BBR could partly attenuate intestinal epithelial TJ barrier dysfunction in endotoxemic mice. Macroscopic observation and H&E-stained sections of distal ileum and colon indicated that BBR alleviates morphological changes induced by LPS. The FD4 assay revealed that BBR reduced the increased permeability of gut barrier in endotoxinemia. By use of immunofluorescence and immunoblotting, we demonstrated that BBR could partially prevent the decreased expression of several key TJ proteins in colonic mucosa during LPS-induced endotoxinemia and reverse the displacement of these TJ proteins from raft fractions. Finally, by using a combination of immunofluorescence and Western blot, we obtained evidence that this beneficial effect of BBR on TJ may partially be mediated through the inhibition on NF-κB and the MLCK pathway.

The intestinal epithelium restricts free passage of toxic and infectious molecules from the gut lumen while allowing selective paracellular absorption across the TJ. Therefore, an intact TJ barrier is critical to normal physiological function and prevention of disease. During critical illness, such as endotoxemic sepsis, there is a marked increase in intestinal permeability and a compromise of the TJ barrier integrity [8]. Regulation of the intestinal TJ—the key determinant of intestinal permeability—may constitute an important target for therapy.

The pathogenesis of intestinal TJ injury in endotoxemia remains unclear. However, several mechanisms have been explored to mediate the pathologic processes, such as NF-κB and MLCK signaling pathway. The NF-κB signaling cascade is a common pathway involved in the regulation of a variety of proinflammatory genes that are critical to the inflammatory response. The most abundant form of NF-κB in many cell types is a heterodimer consisting of p65 and p50. During endotoxemia, the inhibitory factor κB (I-κB) in intestinal mucosa is degraded, resulting in cytoplasmic-to-nuclear translocation of NF-κB p65 [29]. Thereafter, the activated NF-κB p65 binds to the MLCK promoter region and increases MLCK mRNA expression [30, 31]. MLCK is an important regulator of intestinal TJ. The MLCK-mediated MLC phosphorylation leads to contraction of actin-myosin filaments, resulting in alteration of TJ protein localization and expression and functional opening of TJ barrier [32, 33]. Therefore, drugs developed to inhibit the NF-κB and MLCK may have potential to repair the intestinal TJ barrier in endotoxemia.

BBR is widely used in the treatment of gastroenteritis and diarrhea in China. Previous studies have demonstrated that BBR exerts an anti-inflammatory effect partially through the NF-κB signaling pathway. The mechanism, in part, is ascribed to inhibition of I-κB kinase and results in the stabilization of I-κB and suppression of nuclear translocation of NF-κB p65 as well as, finally, the inhibition of NF-κB activity. BBR also suppresses

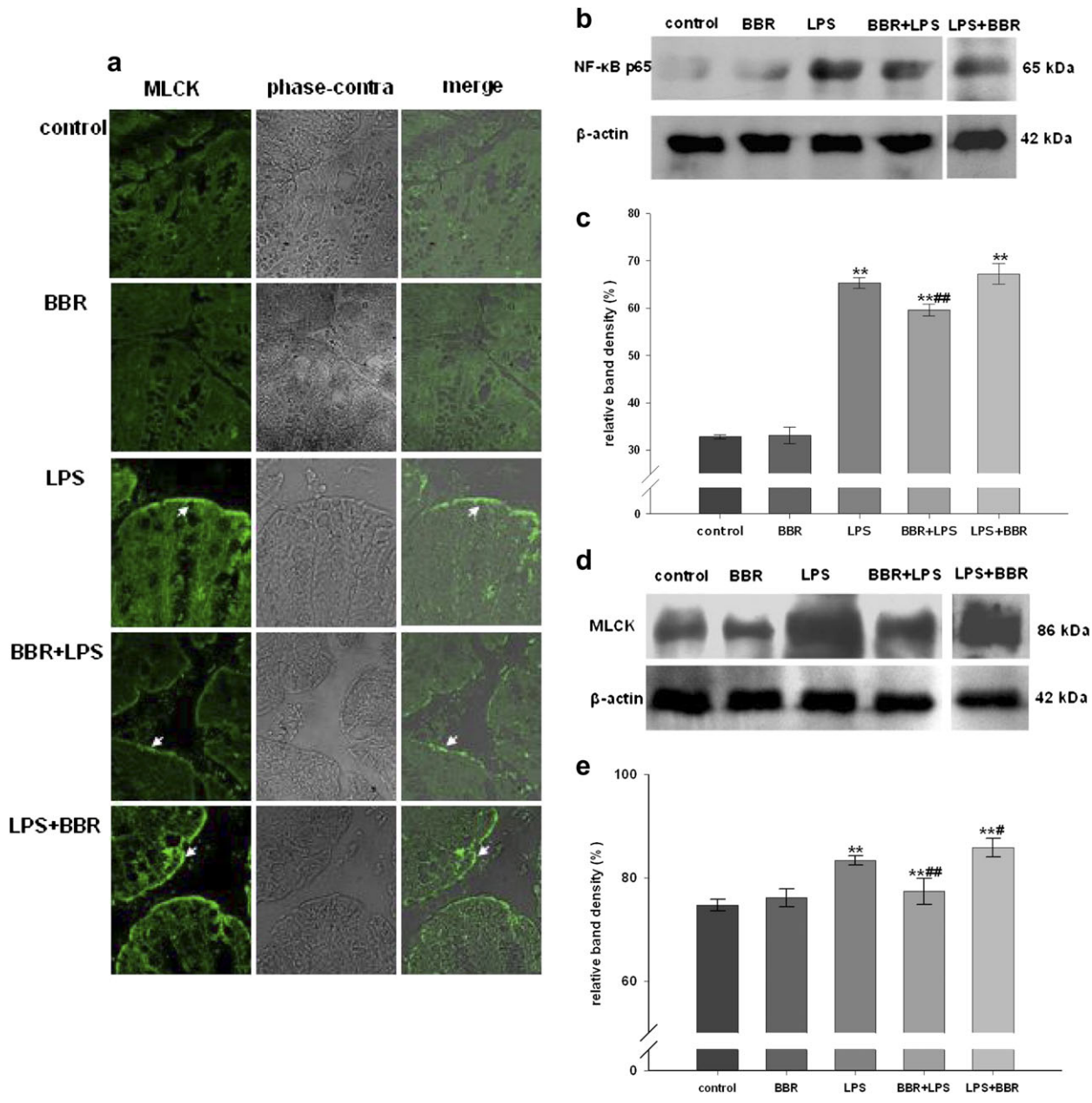

Figure 6. *A,* Immunohistochemical staining of intestinal sections for myosin light chain kinase (MLCK). An overlay of MLCK and phase contrast image was shown to demonstrate colocalization. Increased staining for MLCK (green) was seen in endotoxemic animals. In endotoxemic animals pretreated with berberine (BBR), there was a decrease in staining for MLCK. Subsequent treatment of BBR cannot reverse the effect of lipopolysaccharide (LPS). Arrows show the location of the staining. *B,* Intestinal nuclear factor (NF)-κB p65 expression in nuclear extracts analyzed by Western blotting. Equal loading of proteins is illustrated by β-actin bands. The blots shown are representative of 3 experiments. *C,* Blots analyzed and quantified by densitometry with Quantity One analysis software. Endotoxinemia resulted in increase in nuclear translocation of NF-κB, and pretreatment with BBR inhibited NF-κB p65 nuclear translocation induced by LPS. *D,* Representative Western blots for MLCK protein expression β-actin was used as a loading control. The blots shown are representative of 3 experiments. *E,* LPS-induced increase in MLCK expression, compared with the control group. Pretreatment with BBR attenuated the LPS-induced increase in MLCK expression. Data are shown as means ± standard errors. $^{*}P < .05$. $^{**}P < .05$, compared with the control group. $^{\#}P < .05$, compared with the LPS group. $^{\#\#}P < .01$, compared with the LPS group.

the expression of NF-κB-regulated gene products, including survivin, cyclin D1, and cyclooxygenase-2 [18]. At present, BBR has been demonstrated to ameliorate indomethacin- and radiation-induced intestinal injury [34, 35] and also appears to have beneficial effects against LPS-induced lung injury [19]. Our previous in vitro study also demonstrated, for the first time, that BBR could reduce intestinal epithelial TJ permeability [27]. However, to our knowledge, the effect of BBR on intestinal epithelial TJ, NF-κB p65 expression, and MLCK activation in endotoxemia has not been investigated.

The opening of tight junctions is primarily dependent on the composition and organization of the TJ proteins, especially components, such as occludin, ZO-1, and claudins, which are responsible for the barrier function. In the present study, these proteins exhibited varying degrees of displacement from the tips of villi to crypts in colonic epithelium. This result was consistent with the decline in permeability of TJ. BBR partially reversed this redistribution of TJ proteins and the increased permeability. This indicates that BBR may prevent TJ disruption in endotoxemia. Our results also implied that BBR could reduce the TJ permeability in normal mice; this finding is consistent with results in our previous in vitro study [27]. Lipid rafts are specialized structures on the plasma membrane, and the disruption of lipid rafts is associated with TJ opening [36]. Previous studies have revealed that the redistribution of TJ proteins in TJ membrane microdomains leads to TJ barrier dysfunction during sepsis [8]. In the present study, we examined the distribution of tight junction proteins within plasma membranes by sucrose density centrifugation using Triton X-100 as a detergent. BBR also reversed the distribution of TJ proteins from lipid raft fractions; this could explain the possible mechanism of protection for the TJ.

The gut is often considered the source of systemic infection because of increased mucosal permeability in systemic illness. MLCK-mediated regulation of TJ opening appears to be a common intermediate in the pathophysiological pathway of endotoxinemia. Thus, therapies targeting the inhibition of MLCK to specifically restore this barrier function may be attractive candidates for application in therapeutic intervention [37]. Therefore, we explored the effect of BBR on NF-κB and MLCK expression in endotoxinemia. Our data indicate that BBR can reduce the overexpression of nuclear NF-κB p65 and the MLCK in intestinal mucosa.

Furthermore, previous studies have indicated that mediators, such as nitric oxide and high-mobility group box-1, are upregulated upon LPS injection and that they contribute to gut-barrier dysfunction [38, 39]. Whether BBR acts directly on epithelial cells or on the expression of these mediators in endotoxemia and the possibility of a relationship between the protective effects of BBR on TJs are as yet unknown. These investigations will form the focus of our future work.

To replicate the clinical situation, we also administrated BBR following LPS injection. However, the effect of treatment with BBR subsequent to development of endotoxemia is not significant; we speculate that this result could be attributed to the pharmacokinetic features of BBR [40]. Therefore, we believe that BBR can be used in the late stage or during decubation of endotoxemia. Future clinical studies should identify conditions in which treatment can be commenced.

In conclusion, our data indicate—to our knowledge, for the first time—that BBR can ameliorate intestinal epithelium TJ disruption in a mice model of endotoxinemia, and the possible mechanism for this may be related to the inhibition of NF-κB and the MLCK signaling pathway. We speculate that BBR, individually or in combination with other therapy, may serve as a potential new therapeutic agent that restores the normal intestinal barrier function in endotoxinemia.

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