Lipopolysaccharide disrupts tight junctions in cholangiocyte monolayers by a c-Src-, TLR4-, and LBP-dependent mechanism

P. Sheth, N. Delos Santos, A. Seth, N. F. LaRusso, and R. K. Rao

1Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee; and 2Department of Medicine, Mayo Clinic, Rochester, Minnesota

Submitted 21 December 2006; accepted in final form 11 April 2007


Cholangiocytes, the epithelial cells that line the intra- and extrahepatic biliary tree form an important barrier to the diffusion of injurious factors from the bile duct lumen into the hepatic parenchyma. The tight junction (TJ), a multiprotein complex at the apical end of lateral membrane, provides for the barrier function to the biliary epithelium. Disruption of the TJ and regurgitation of bile acids and other injurious factors may play an important role in chronic cholestatic liver diseases like primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (7, 13, 15, 36, 41). However, very little is known about the structure and regulation of TJs in bile duct epithelium.

In the intestinal and renal tubular epithelia, the TJ is formed by the organization of a number of specific proteins including occludin, zona occludens (ZO-1, ZO-2, ZO-3), claudins and junctional adhesion molecule (1). The TJ proteins interact with the perijunctional actomyosin ring. Additionally, a variety of intracellular signaling molecules such as c-Src, phosphatidylinositol 3-kinase, ERK, PKCζ, and PP2A are localized at the TJ (1, 4, 33). A significant body of evidence indicates that the TJ and paracellular permeability are regulated by signaling molecules, such as intracellular calcium (38), cyclic AMP (5), GTPase switch protein (9, 29, 34, 46) and protein kinases (4, 32, 33, 39).

In the gastrointestinal and renal epithelia, inflammatory mediators such as reactive oxygen species, toxins, and cytokines are capable of disruption of TJs and increasing paracellular permeability to macromolecules (4, 30, 33, 40). Recent studies have demonstrated that oxidative stress and acetaldehyde affect TJs and increase paracellular permeability in Caco-2 cell monolayers by a mechanism dependent on phosphatidylinositol 3-kinase and tyrosine kinases such as c-Src (2, 4, 32, 33, 39). The expression of dominant negative c-Src in Caco-2 cells delayed the oxidative stress-induced disruption of TJ, while overexpression of wild-type c-Src potentiated the effect of oxidative stress (4).

Nearly 80% of patients with PSC show the symptoms of inflammatory bowel disease (IBD), particularly ulcerative colitis (14, 24, 42). An increase in TJ permeability to macromolecules is associated with IB and an increased permeability to endotoxins from the colonic lumen appears to play a crucial role in the pathogenesis of IBD. Endotoxemia may affect the integrity of bile duct epithelial TJs. Therefore, in the present study, the effect of lipopolysaccharide (LPS) on TJ integrity was evaluated in NRC-1 cell monolayers. Results show that 1) LPS disrupts the TJs and increases paracellular permeability in a dose-dependent manner, 2) LPS-induced disruption of the TJ was dependent on a nonreceptor tyrosine kinase, c-Src, 3) LPS induces an increase in occludin and ZO-1 tyrosine phosphorylation and a dramatic reduction in occludin threonine phosphorylation by a tyrosine kinase-dependent mechanism, and 4) LPS-induced disruption of TJs was mediated by Toll-like receptor 4 (TLR4), LPS binding protein (LBP), and myosin light chain kinase (MLCK).

MATERIALS AND METHODS

Materials

Cell culture supplies were obtained from Invitrogen (San Jose, CA) and Transwell inserts and other cell culture plastic ware were purchased from Costar (Cambridge, MA). Rat-tail collagen (type I), the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: R. K. Rao, Dept. of Physiology, Univ. of Tennessee Health Science Center, 894 Union Ave., Memphis, TN 38163 (e-mail: rkrao@physio1.utmem.edu).
FITC-inulin, polymixin B, ML-7 (an MLCK inhibitor), LPS (*Escherichia coli* 055:B5), propidium iodide, IFN-γ, and protein-A Sepharose were obtained from Sigma Chemical (St. Louis, MO). Fugene-6 was purchased from Roche (Indianapolis, IN) and SMART pool small interfering RNA (siRNA) specific for c-Src, LBP, and TLR4, and control RNA was obtained from Dharmacon (Lafayette, CO). Other fine chemicals and laboratory supplies were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical.

**Antibodies**

Mouse monoclonal anti-occludin antibody and rabbit polyclonal anti-occludin, anti-ZO-1, anti-claudin-1, anti-claudin-4, anti-claudin-5, and anti-phospho-threonine (p-Thr) antibodies were purchased from Zymed Laboratories (South San Francisco, CA). Biotin-conjugated anti-phospho-tyrosine (p-Tyr) antibody and anti-actin antibodies were purchased from BD Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-Src and anti-Abl antibodies were purchased from Upstate USA (Lake Placid, NY). AlexaFluor-488-conjugated anti-mouse IgG was obtained from Molecular Probes (Eugene, OR). Cy3-conjugated anti-rabbit IgG and mouse monoclonal anti-LBP and anti-TLR4 antibodies were purchased from Sigma Immunochemicals (St. Louis, MO). Anti-active caspase-3 antibody was purchased from Chemicon (Temecula, CA).

**Cell Culture**

NRC-1 cells (normal rat cholangiocytes) were cultured in DMEM-F12 supplemented with 10% fetal bovine serum, vitamin mix, chemically defined lipid mix, insulin-transferrin-selenium mix, nonessential amino acids, and antibiotics (penicillin and streptomycin) as described before (21, 39). Experiments were conducted at passages 32–38. Cells were cultured on plates or Transwells coated with rat-tail collagen, type I. The baseline transepithelial electrical resistance (TER) on day 5 or 6 varied from 500–800 Ω·cm², and all experiments were performed in confluent monolayers on day 5 or 6 post-seeding.

**Transfection of NRC-1 Cells With siRNA**

NRC-1 cells were seeded in six-well plates and allowed to attain 70–75% confluence. The cells were then incubated in serum-free and

---

**Fig. 1. LPS increases paracellular permeability in NRC-1 cell monolayer.**

A and B: NRC-1 cell monolayers were incubated in the absence (●) or presence (■) of LPS (500 ng/ml) for varying times. Transepithelial electrical resistance (TER; A) and unidirectional flux of inulin (B) were measured. Values are means ± SE (n = 6). *Significantly (P < 0.05) different from control values. C and D: NRC-1 cell monolayers were incubated with varying concentrations of LPS (100–500 ng/ml) for 90 min. TER (C) and inulin flux (D) were measured. Values are means ± SE (n = 6). *Significantly (P < 0.05) different from control values. E and F: NRC-1 cell monolayers were incubated with 10 or 30 μg/ml of polymixin B for 1 h before the administration of 250 or 500 ng/ml LPS. TER (E) was measured at varying times and inulin flux (F) was measured at 1 h after LPS administration. Values are means ± SE (n = 3). *Significantly (P < 0.05) different from corresponding control values. **Significantly (P < 0.05) different from corresponding values for LPS-treated cells.
antibiotic-free medium for 24 h and transfected with SMART pool siRNA for c-Src, TLR4, or LBP or control RNA (scrambled sequence). Control RNA and siRNAs were complexed with Oligo-lectamine Plus reagent and Fugene-6 and added to the cell monolayers and incubated for 6 h. Serum (FBS) level was restored to 10% and the cells were allowed to stabilize for 20 h. Cell monolayers were then trypsinized and seeded onto Transwell inserts for further experiments.

**LPS Treatment**

Cell monolayers were preincubated for 60 min in serum-free DMEM as a control or with appropriate concentrations of inhibitors for 1 h before LPS treatment. LPS (100–500 ng/ml) was administered to both the apical and basal chambers. The paracellular permeability was evaluated by measuring TER and unidirectional flux of FITC-inulin.

**Measurement of TER**

TER was measured as described before (17) by using a MillicellERS electrical resistance system (Millipore, Bedford, MA). The TER was recorded in empty Transwell inserts (usually 50–80 Ω·cm²) and was subtracted from all values.

**Unidirectional Flux of Inulin**

Inulin permeability was measured by incubating cell monolayers in the presence of 0.5 µg/ml FITC-inulin in the apical chamber. At varying times, 100-µl aliquots of basal medium were withdrawn and fluorescence was measured in a microplate fluorescence reader (FLx800, Bio TEK Instruments, Winooski, VT). Flux of FITC-inulin into the basal well was calculated as the percentage of total fluorescence administered into the apical well per hour per square centimeter of surface area.

**Immunofluorescence Microscopy**

Under various experimental conditions cell monolayers were fixed in acetone-methanol (1:1, vol/vol) at 0°C for 5 min. The fixed membranes were rehydrated in PBS and permeabilized with 0.2% Triton X-100 in PBS. Cell monolayers were blocked with 4% nonfat milk in Tris-buffered saline containing 0.05% Tween-20. Cells were then stained with a mixture of mouse monoclonal anti-occludin and rabbit polyclonal anti-ZO-1, anti-claudin-1, anti-claudin-4, or anti-claudin-5 antibodies. A mixture of AlexaFluor-488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG secondary antibodies was used. Cells were mounted and observed using a Zeiss LSM 5 PASCAL laser scanning confocal microscope with LSM 5 PASCAL software (Release 3.2). A series of images was collected from 1.0-µm XY sections. Images were stacked by use of the Image J software and processed by Adobe Photoshop (Adobe Systems, San Jose, CA).

Image J software was used to evaluate the amounts of occludin and ZO-1 present at the intercellular junctions by semiquantitatively measuring fluorescence density in the selected areas. Values are presented as pixels per square millimeter.

**Cell Viability and Apoptosis**

**DNA staining.** Cell monolayers under various experimental conditions were incubated with propidium iodide (1 µg/ml). The monolayers were analyzed for nuclear staining at 2 and 20 h of incubation with or without LPS or IFN-γ. As a positive control, cell monolayers were incubated with 0.1% Triton X-100 in F12 medium. Both phase-contrast and fluorescent images were collected and merged to obtain a composite picture using Metamorph imaging software.

**Caspase activation.** NRC 1 cells were grown in 100 mm culture dishes at 95% confluence. They were incubated with or without 250 or 500 ng/ml LPS for 3 h. Cell monolayers were rinsed with ice-cold PBS and proteins were extracted in lysis buffer-D (0.3% SDS, 10 mM Tris, 10 mM sodium fluoride, 1 mM sodium vanadate, and 1 µl/ml proteinase inhibitor cocktail (SIGMA) at pH 7.4). Proteins were immunoblotted for anti-active caspase-3 antibody.

**DNA fragmentation.** Quantitative analysis of DNA fragmentation was done by ELISA. NRC1 cells were grown in 24-well culture plates. At 3 days postseeding, the cells were treated with 250 ng/ml LPS or DMEM for 3 h. The cells were then washed two times with PBS, lysed, and centrifuged to remove the nuclei. An aliquot of the nuclei-free supernatant was placed in streptavidin-coated wells and incubated with biotin-conjugated anti-histone antibody and peroxidase-conjugated anti-DNA antibody for 2 h at room temperature. After incubation, the sample was removed, and the wells were washed three times with incubation buffer and mixed with 100 µl of the substrate (2,2′-azinodi-3-ethylbenzthiazolin-sulfonate). The absorbance was read at 405 nm by use of a plate reader.

**Preparation of Detergent-Insoluble Fractions**

Detergent insoluble fractions were prepared as described previously (16). Briefly, cell monolayers were lysed in Tris buffer containing 1.0% Triton X-100, 2 mM vanadate, 1 mM PMSF, protease inhibitors (2 µg/ml each of pepstatin, leupeptin, bestatin), and 10 mM NaF. Extracts were centrifuged at 15,600 g for 4 min at 4°C to sediment high-density actin cytoskeleton. The pellet was suspended in 200 µl of Tris buffer and sonicated to homogenize actin filaments. After withdrawal of aliquots for protein assay, detergent-insoluble fractions were mixed with Laemmlli’s sample buffer and heated at 100°C for 5 min.

Fig. 2. Polarity of LPS effect on paracellular permeability. NRC-1 cell monolayers were incubated with LPS (250 ng/ml) in apical, basolateral, or both surfaces. Control cell monolayers received no LPS. TER (A) and inulin flux (B) were measured at 2 h of LPS treatment. Values are means ± SE (n = 4). *Significantly (P < 0.05) different from control values.
**Immunoprecipitation and Immunoblotting**

Proteins in Triton-soluble and Triton-insoluble fractions were extracted under denaturing conditions using lysis buffer D (50 mM Tris buffer, pH 8.0, containing 0.3% SDS, 2 mM vanadate, 10 mM sodium fluoride, and protease inhibitors as described above) and heated at 100°C for 10 min. Biotin-conjugated anti-p-Tyr or rabbit polyclonal anti-p-Thr antibodies were used to immunoprecipitate p-Tyr and p-Thr. Immunocomplexes were precipitated with streptavidin-agarose or protein A-Sepharose and immunoblotted for occludin and ZO-1.

**Immunoblot Analysis**

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed for occludin, ZO-1, c-Src, c-Abl, LBP, or TLR4. Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies were used as secondary antibodies. The blots were developed by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL).

**Statistics**

Comparison between two groups was made by the Student’s t-test for grouped data. The significance in all tests was derived at 95% or greater confidence level.

---

**RESULTS**

**LPS Increases Paracellular Permeability in NRC-1 Monolayers**

Treatment with LPS results in loss of TJ integrity and an increase in paracellular permeability in corneal, retinal, and alveolar epithelial monolayers (11, 48, 49). In the present study we evaluated the effect of LPS on bile duct epithelial monolayers using NRC-1 cholangiocytes. Administration of LPS (100–500 ng/ml) resulted in a time-dependent (Fig. 1, A and B) and dose-dependent (Fig. 1, C and D) decrease in TER and increase in inulin permeability. Pretreatment of cell monolayers with polymixin B (an LPS inhibitor) attenuated the LPS-induced decrease in TER (Fig. 1E) and increase in inulin flux (Fig. 1F). Reduced TER and increased inulin permeability were observed when LPS was administered to either apical or basal chamber (Fig. 2). However, the effect was more pronounced when LPS was administered to the basal chamber. The effect was additive when LPS was administered to both apical and basal surfaces simultaneously. To rule out the possibility of apoptosis as a cause of LPS-induced transepithelial permeability, cell monolayers, incubated with or without LPS, were stained for apoptotic cells using propidium iodide. Incubation of cell monolayers with LPS for 2 or 20 h showed no sign of apoptosis (Fig. 3A). However, incubation...
of cell monolayers with IFN-γ for 20 h resulted in DNA staining in nearly 30% of cells (Fig. 3A). Almost all cells were stained by propidium iodide in cell monolayers treated with 0.1% Triton X-100 for 10 min. LPS (250–500 ng/ml) also failed to induce activation of caspase-3 (Fig. 3B) and DNA fragmentation (Fig. 3C).

**LPS Disrupts TJ**

To determine the effect of LPS on TJs, the cell monolayers were double labeled for occludin and ZO-1 by immunofluorescence method. Images were collected by using a confocal laser-scanning microscope. **B**: fluorescence at the junctions was measured by densitometric analysis using the software Image J. Values are means ± SE (n = 6).

*Significantly different from corresponding control (0 ng/ml) values.

**Fig. 4.** LPS induces redistribution of occludin and zonula occludens (ZO-1). A: NRC-1 cell monolayers were incubated in the absence (control) or presence of LPS (100 or 250 ng/ml) for 2 h. Cell monolayers were fixed in acetone-methanol and stained for occludin (green) and ZO-1 (red) by immunofluorescence method. Images were collected by using a confocal laser-scanning microscope. **B**: fluorescence at the junctions was measured by densitometric analysis using the software Image J. Values are means ± SE (n = 6).

Fig. 5. LPS induces redistribution of claudin-1 and claudin-4. NRC-1 cell monolayers were incubated with or without 500 ng/ml LPS for 1 h and fixed in paraformaldehyde. Cell monolayers were labeled for claudin-1, claudin-4, and occludin by immunofluorescence staining.
and ZO-1 are colocalized at the intercellular junctions in control cell monolayer. Treatment with LPS for 2 h reduced the distribution of occludin and ZO-1 at the intercellular junctions in a dose-dependent manner (Fig. 4). LPS at a low concentration (100 ng/ml) dramatically reduced the stain for ZO-1 at the intercellular junctions with only a slight effect on occludin distribution (Fig. 4A). However, at a higher dose (250 ng/ml), LPS reduced the junctional staining for occludin as well. Densitometric analysis of the fluorescence at the intercellular junctions confirms that junctional distribution of ZO-1 is reduced at a much more greater rate than that of occludin (Fig. 4B). The loss of staining for ZO-1 in both the intercellular junctions and the intracellular compartments suggests a possible degradation of ZO-1 after its release from the junctions. Although claudin-5 was undetectable in NRC-1 cell monolayers, significant portions of claudin-1 and claudin-4 were localized at the intercellular junctions (Fig. 5). LPS induced redistribution of both claudin-1 and claudin-4 from the intercellular junctions. Occludin predominantly localized at the intercellular junctions, and only a small portion of claudin-4 colocalized with occludin (Fig. 5).

LPS-Induced Disruption of Barrier Function Is Mediated by a Tyrosine Kinase-Dependent Mechanism

Previous studies have suggested that tyrosine kinase activity mediates the disruption of TJs caused by oxidative stress and acetaldehyde in an intestinal epithelial monolayer (4, 32, 33, 39). To determine the role of tyrosine kinase activity in LPS-induced disruption of TJs in NRC-1 cell monolayers we evaluated the effect of genistein (a general tyrosine kinase inhibitor) and PP2 (a selective inhibitor of Src kinases). LPS-induced decrease in TER (Fig. 6A) and increase in inulin permeability (Fig. 6B) were significantly reduced by the pretreatment of cell monolayers with genistein. Genistein by itself produced no significant effect on TER or inulin flux. PP2 by itself significantly reduced TER and increased inulin permeability; however, the effect of LPS on inulin permeability was not altered in the presence of PP2.

A previous study showed that oxidative stress-induced disruption of TJs in Caco-2 cell monolayers is mediated by the activation of c-Src (4). PP2 is known to inhibit all members of the Src kinase family. Therefore, to determine the specific role of c-Src in an LPS-induced disruption of TJ, NRC-1 cell monolayers were transfected with siRNA designed against the nucleotide sequence of the rat c-Src gene. The effect of LPS on TER and inulin flux was determined in cells transfected with c-Src siRNA or control RNA. Transfection with siRNA reduced the level of c-Src (Fig. 6, A and B), without altering the level of c-Abl. The level of c-Abl was slightly higher on day 4 in cells transfected with either control RNA or siRNA. LPS treatment significantly increased inulin permeability in cells transfected with control RNA, whereas there was no significant change in LPS-induced inulin permeability in cells transfected with c-Src siRNA (Fig. 7C). Confocal microscopy (Fig. 7D) and densitometric analysis (Fig. 7E) indicate that LPS significantly reduced the junctional distribution of ZO-1 in cells transfected with control RNA, whereas junctional distribution of ZO-1 was unaffected by LPS in c-Src siRNA-transfected cells.

![Fig. 6. Tyrosine kinase inhibitors prevent LPS-mediated disruption of tight junction (TJ) NRC 1 cell monolayers were pretreated with PP2 (3 μM) or genistein (150 μM) for 60 min. Cell monolayers were then incubated with (solid bars) or without (open bars) LPS (250 ng/ml) in both apical and basolateral compartments. TER (A) and unidirectional flux of inulin (B) were measured. Values are means ± SE (n = 4). *Significantly (P < 0.05) different from corresponding control values.](http://ajpgi.physiology.org/)

LPS Alters the Phosphorylation Status of Occludin and ZO-1

Occludin and ZO-1 are hyperphosphorylated on Ser and Thr residues in intact epithelium, while their Tyr phosphorylation is maintained at a low level. Previous studies demonstrated that disruption of TJs by oxidative stress is associated with an increase in phosphorylation of occludin on Tyr residues (4, 33). In the present study, we evaluated the effect of LPS on occludin and ZO-1 phosphorylation on Tyr and Thr residues. Significant levels of Tyr-phosphorylated occludin was detected in the control cell monolayer, but LPS further increased Tyr-phosphorylation in a time-dependent manner (Fig. 8A). Only a trace amount of Tyr-phosphorylated ZO-1 was detected in control cell monolayers, but LPS increased the Tyr-phosphorylation of ZO-1 in a time-dependent manner (Fig. 8A). There were only minor changes in the total amounts of occludin present in Triton-insoluble and Triton-soluble fractions. However, the ZO-1 level in Triton-insoluble fractions was gradually reduced in LPS-treated cell monolayers. The ZO-1 level in Triton-soluble fractions appeared unaerltered until 3 h. Pretreatment of cell monolayers with PP2 or genistein prevented both LPS-induced occludin and ZO-1 Tyr phosphorylation (Fig. 8B).

* Immunoprecipitation of p-Thr followed by immunoblot analysis for occludin and ZO-1 showed that occludin and ZO-1 are both phosphorylated on Thr residues in untreated NRC-1 cell monolayers (Fig. 9). LPS induced a rapid dephosphoryla-
tion of occludin on Thr residues, while Thr-phosphorylation of ZO-1 was unaffected by LPS. Pretreatment of cell monolayers with either genistein or PP2 prevented LPS-induced dephosphorylation of occludin (Fig. 9).

LPS-Induced Disruption of the TJ Is Mediated by TLR4 and LBP

Previous studies demonstrated that TLR4 and LBP are involved in LPS-mediated cellular responses (19, 43). To determine the role of TLR4 and LBP in LPS-induced disruption of TJs, NRC-1 cells were transfected with siRNA for rat TLR4, siRNA for rat LBP or nonspecific control RNA. TLR4 siRNA reduced the level of TLR4, without altering the level of LBP (Fig. 10, A and B). Transfection with siRNA to LBP reduced the level of LBP without significantly affecting the level of TLR4 (Fig. 10C). LPS significantly increased inulin permeability in the cells transfected with control RNA (Fig. 10D) but failed to increase inulin permeability in cells transfected with TLR4 siRNA or LBP siRNA. Confocal immunofluorescence microscopy showed that LPS treatment induced redistribution of ZO-1 from the intercellular junctions in cells transfected with control RNA (Fig. 10E), whereas junctional distribution of ZO-1 remained unaffected in LPS-treated cells transfected with siRNA for TLR4 or LBP (Fig. 10E).

LPS-Induced Paracellular Permeability Is Mediated by MLCK

Previous studies showed that activation of MLCK plays an important role in the regulation of intestinal epithelial TJs by TNF-α (47) and LPS-induced barrier disruption in colonic...

Fig. 7. Reduced expression of c-Src by small interfering RNA (siRNA) prevents LPS-induced TJ permeability. NRC-1 cells were transfected with siRNA targeting the rat c-Src sequence or with control RNA (scrambled sequence). Transfected cells were seeded on Transwell inserts. The effect of LPS (250 ng/ml) on TJ disruption was assessed on day 4 after transfection. A: effect of gene silencing on the protein levels was determined by immunoblot analysis. B: densitometric analysis of c-Src bands in A. All values on day 3 and 4 were normalized to corresponding value for day 2 (1.0). Values are means ± SE (n = 3). *Significantly (P < 0.05) different from control values. C: cell monolayers were incubated with or without LPS (250 ng/ml) and inulin permeability was measured after 2 h. Values are means ± SE (n = 4). *Significantly (P < 0.05) different from corresponding control values. D: cell monolayers after incubation for 2 h with (LPS) or without (Control) LPS were fixed and stained for ZO-1 by immunofluorescence method. Fluorescence images were collected using confocal laser scanning microscope. E: fluorescence at the intercellular junctions was measured by densitometric analysis. Values are means ± SE (n = 6). *Significantly different from corresponding control values.

Fig. 8. LPS increase Tyr-phosphorylation of ZO-1 and occludin by tyrosine kinase-dependent mechanism. A: NRC-1 cell monolayers were incubated with LPS (250 ng/ml) for varying times. Triton-soluble and Triton-insoluble fractions were prepared and p-Tyr was immunoprecipitated (IP). Both total protein extracts and immunocomplexes were immunoblotted (IB) for TJ proteins, occludin, and ZO-1. B: cell monolayers were preincubated with or without genistein or PP2 followed by incubation with LPS for varying times. P-Tyr in proteins extracted from Triton-insoluble fractions was immunoprecipitated and immunoblotted for occludin and ZO-1.
Therefore, we evaluated the effect of ML-7, a MLCK inhibitor, on LPS-induced permeability in NRC-1 cell monolayers. Pretreatment with ML-7 attenuated an LPS-induced decrease in TER (Fig. 11A) and increase in inulin flux (Fig. 11B), indicating that MLCK plays an important role in LPS-induced permeability in the cholangiocyte monolayer.

**DISCUSSION**

Elevated absorption of LPS due to disruption of the intestinal epithelial barrier function plays a crucial role in tissue injury in a variety of diseases such as IBD and alcoholic liver disease and in multiorgan failure in burn and trauma patients. An association of PSC patients with the symptoms of IBD suggests a potential role of endotoxemia in bile duct injury. The present study demonstrates that LPS rapidly disrupts barrier function in the NRC-1 cell monolayer, a rat bile duct epithelium. LPS disrupts the TJ and increases paracellular permeability to macromolecules by a c-Src-dependent mechanism that involves TLR4 and LBP. This observation may provide insight into the mechanism involved in bile duct injury in patients with PSC.

NRC-1 cells attained a TER of 500 – 800 Ω cm². These TER values are similar to the TER of Caco-2 cell monolayers, an intestinal epithelium, indicating that NRC-1 cell monolayer form well-developed TJs. On the other hand, the TER values for Mzh1 cell monolayers and mouse primary cholangiocyte monolayers were less than 100 Ω cm². Therefore, NRC-1 cells form an appropriate model system to study the TJ barrier function of the bile duct epithelium. Reduction of TER and increased inulin permeability indicate that the LPS dose dependently disrupts barrier function and increases paracellular permeability in NRC-1 cell monolayers. Under our experimental conditions LPS did not induce apoptosis as determined by the lack of DNA staining when cells were incubated with propidium iodide or assessed for caspase activation and DNA

---

**Fig. 9.** LPS decreases Thr-phosphorylation of occludin by a tyrosine kinase-dependent mechanism. Cell monolayers were preincubated with or without genistein or PP2 followed by incubation with LPS (250 ng/ml) for varying times. Phospho-threonine (p-Thr) in proteins extracted from Triton-insoluble fractions were immunoprecipitated and immunoblotted for occludin and ZO-1.

**Fig. 10.** LPS-induced TJ disruption is mediated by Toll-like receptor 4 (TLR4) and LBP. NRC-1 cells were transfected with a control RNA (scrambled sequence), rat TLR4-specific siRNA, or rat LBP-specific siRNA. The cells were seeded onto Transwell inserts and the effect of LPS on TJ permeability was evaluated on day 4. A and B: levels of TLR4 and LBP protein were determined by immunoblot analysis. C: densitometric analysis of bands for LBP and TLR4 on day 4 and 5. Values normalized to corresponding value for day 2 (1.0), and are means ± SE (n = 3). D: effect of LPS on TJ permeability was determined by inulin flux. Values are means ± SE (n = 4). *Significantly (P < 0.05) different from the corresponding control value. E: monolayers incubated for 2 h with (LPS) or without (Control) LPS were fixed and immunostained for ZO-1.
fragmentation. On the other hand, LPS induced a redistribution of occludin and ZO-1 from the intercellular junctions. Occludin and ZO-1 are two major TJ proteins in different epithelia (1), and the interaction between the COOH-terminal intracellular domain of occludin with ZO-1 is crucial for the organization and the stability of the TJ (39, 40). Immunofluorescence staining of NRC-1 cell monolayers for occludin and ZO-1 demonstrated a very sharp organization of these proteins at the intercellular junctions. This indicates the presence of well-developed TJs in NRC-1 cell monolayers. Treatment with LPS resulted in a reduced distribution of both occludin and ZO-1 at the intercellular junctions, suggesting that an LPS-induced increase in inulin permeability was caused by the disruption of TJs. At low dose (100 ng/ml), LPS dramatically induced a redistribution of ZO-1, whereas junctional staining of occludin remained relatively intact. However, at a higher concentration (250 ng/ml), LPS induced a redistribution of both occludin and ZO-1. This observation indicates that ZO-1 is first released of occludin from the junctions in response to LPS by a mechanism that involves a loss of interaction between occludin and ZO-1. The loss of interaction with ZO-1 then results in the redistribution of occludin from the TJ. Immunoblot analysis demonstrated that the ZO-1 level in the detergent-insoluble fraction was gradually reduced by LPS, whereas the ZO-1 level in the detergent-soluble fraction was not significantly altered until 3 h. This observation suggests that there is a gradual decrease in overall amount of ZO-1 in the LPS-treated cells. The loss was predominantly in the detergent-insoluble fraction of ZO-1, which represents the pool of ZO-1 that is associated with the TJ (33). The release of ZO-1 from the TJ may lead to its degradation by cytosolic proteolytic activity.

Several isoforms of claudins, including claudin-1 and claudin-4, are involved in the organization of TJs in different epithelia (1). The present study shows that claudin-1 and claudin-4 are expressed in NRC-1 cells, and a part of these proteins was organized at the intercellular junctions. Similar to occludin levels, the levels of claudin-1 and claudin-4 at the intercellular junctions were also reduced by LPS. Although occludin appears to be the major transmembrane protein localized at the TJs, claudin-1 and claudin-4 may also contribute to the assembly of TJs to some extent in NRC-1 cell monolayers.

The pathogenesis of biliary and sclerosing cholangitis is not well understood and there are no good animal models that mimic such cholangitis in vivo. However, abnormal accumulation of endotoxin in biliary epithelial cells has been demonstrated in PSC (37). LPS directly affects cholangiocyte proliferation by inducing the secretion of IL-6 (31) and induced the overexpression of MUC2 and MUC2AC in cultured cholangiocytes (50). LPS signaling enhances hepatic fibrogenesis in experimental cholestasis in mice (18) and increases mortality in bile duct-ligated rats (38). Our present in vitro study shows that LPS disrupts TJs of NRC-1 cell monolayers. Therefore, it is likely that the disruption of bile duct epithelial barrier function is one of the effects of LPS in the liver. It is not clear whether the LPS-induced disruption of TJs is mediated by the release of cytokines. LPS has been previously shown to release TNF-α in many cells (47) and IL-6 in cholangiocytes (31). A previous study showed that TNF-α disrupts the barrier function of cholangiocyte monolayers (26). Therefore, it is possible that LPS-induced disruption of TJs is mediated by TNF-α secretion.

Inhibition of LPS-induced inulin permeability by genistein indicates that LPS disrupts the TJs of NRC-1 cell monolayers by a tyrosine kinase-dependent mechanism. Previous studies have demonstrated that oxidative stress and acetaldehyde-induced disruption of TJs in an intestinal epithelial monolayer is mediated by tyrosine kinase activity (2, 4, 33, 39). Inhibition of LPS-induced inulin permeability in NRC-1 cell monolayers by PP2 (a Src kinase-selective inhibitor) indicates that Src kinase activity plays an important role in an LPS-induced disruption of TJs. The present study involving c-Src gene silencing by siRNA demonstrates that c-Src is involved in LPS-induced disruption of TJs. Reduced expression of c-Src by siRNA resulted in a loss of LPS-induced TJ permeability. LPS failed to change TER, inulin permeability, or redistribution of ZO-1 from the junctions in cells transfected with c-Src specific siRNA. Therefore, this study demonstrates that c-Src plays a crucial role in LPS-induced disruption of TJs. The role of c-Src in LPS-mediated activation of macrophages has been previously reported (8, 20), and c-Src has been shown to play an important role in the oxidative stress-induced disruption of TJs in an intestinal epithelium (4). In the present study, we also observed that the inhibition of Src kinases by PP2 significantly increased paracellular permeability by itself; however, reduced expression of c-Src by siRNA did not alter the basal permeability level. This observation suggests that increased permeability by PP2 may have been caused by the inhibition of some other isoform of Src kinase.
The structure and the regulation of TJs in bile duct epithelium are not very well understood. However, evidence suggests that the TJ of intestinal epithelium may be regulated by phosphorylation of various TJ proteins on Tyr, Ser, and Thr residues (4, 12, 19, 33, 35, 36, 40). Previous studies showed that the oxidative stress-induced disruption of TJs in intestinal epithelium is associated with the Tyr-phosphorylation of occludin and ZO-1 (4, 33). It was also demonstrated that Tyr phosphorylation of occludin resulted in a loss of its interaction with ZO-1 and ZO-3 (19). The results of this study show that LPS increases Tyr-phosphorylation of both occludin and ZO-1 in NRC-1 cell monolayers. Therefore, Tyr-phosphorylation of these proteins may reflect a loss of interaction between occludin and ZO-1 as a mechanism involved in the LPS-induced disruption of TJs in NRC-1 cell monolayers. Interestingly, both genistein and PP2 prevented LPS-induced Tyr-phosphorylation of occludin and ZO-1. C-Src may be either directly or indirectly involved in the phosphorylation of occludin and ZO-1.

The present study also shows that both occludin and ZO-1 are phosphorylated on Thr residues in untreated NRC-1 cell monolayers. Phosphorylation of occludin on Ser and Thr residues has previously been shown in intestinal and renal epithelia (12, 35). Occludin undergoes phosphorylation on Ser/Thr residues during the disruption of TJ by calcium depletion, phorbol ester, or bacterial infection (6, 12, 35, 40), suggesting that Ser/Thr phosphorylation of occludin may play a crucial role in the maintenance of the TJ integrity. The present study shows that LPS induces a rapid reduction in the Thr-phosphorylation of occludin in NRC-1 cell monolayers. Therefore, the dephosphorylation of occludin on Thr residues may be involved in the LPS-induced mechanism of TJ disruption. Additionally, LPS-induced dephosphorylation of occludin on Thr residues was prevented by genistein and PP2, suggesting that c-Src may indirectly mediate the LPS-induced Thr dephosphorylation of occludin. This observation indicates that there is a cross talk between Tyr phosphorylation and Thr-dephosphorylation of occludin in the mechanism involved in the regulation of TJ integrity in NRC-1 cell monolayers.

Previous studies have indicated that LPS-induced cell injury is predominantly mediated by the activation of TLR4 (10, 16). The present study shows that the LPS-induced disruption of TJs and increases in NRC-1 cell monolayer permeability are mediated by TLR4. Reduced expression of TLR4 by siRNA attenuated the LPS-induced inulin permeability and redistribution of ZO-1 from the intercellular junctions. Previous studies demonstrated that LBP is involved in LPS-induced monocyte activation (16, 21, 28). The present study also showed that reduced expression of LBP by siRNA abrogated the effect of LPS on inulin permeability and redistribution of ZO-1. LBP is therefore involved in LPS-induced TJ disruption and permeability increases. LBP is therefore involved in LPS-induced TJ disruption and increased permeability.

Previous studies showed that MLCK plays an important role in the regulation of intestinal epithelial TJs (47). TNF-α-induced disruption of TJs in intestinal epithelia monolayers was mediated by the increased expression and activation of MLCK. A recent study indicated that LPS-induced disruption of colonic epithelial barrier and bacterial translocation in rats is mediated by MLCK (27). Therefore in the present study, we evaluated the role of MLCK in LPS-induced permeability increases in NRC-1 cell monolayers. Attenuation of LPS-induced TER decrease and inulin permeability increases by MLCK inhibitor demonstrate that MLCK does play an important role in the LPS-induced disruption of TJ and barrier function. However, at the present time it is not clear how MLCK activation is related to c-Src and TLR4 activation.

Therefore, the present study demonstrates that LPS rapidly disrupts TJs and increases paracellular permeability in a bile duct epithelium by a c-Src-, TLR4-, and LBP-dependent mechanism. Such LPS-induced TJ disruption and increased paracellular permeability (to bile salts and other injurious factors) may play an important role in the pathogenesis of PSC and/or PBC.

GRANTS

This study was supported by a grant from Allen and Mussette Morgan Jr. Foundation for Study on Primary Sclerosing Cholangitis and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-55532.

REFERENCES


