Tumor necrosis factor alpha increases intestinal permeability in mice with fulminant hepatic failure

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Abstract

AIM: To determine the effect of tumor necrosis factor alpha (TNF-α) on intestinal permeability (IP) in mice with fulminant hepatic failure (FHF), and the expression of tight junction proteins.

METHODS: We selected D-lactate as an index of IP, induced FHF using D-galactosamine/lipopolysaccharide and D-galactosamine/TNF-α, assessed the results using an enzymatic-spectrophotometric method, transmission electron microscopy, immunohistochemistry, Western blotting and real-time quantitative polymerase chain reaction. The effect of the administration of anti-TNF-α immunoglobulin G (IgG) antibody, before the administration of D-galactosamine/lipopolysaccharide, on TNF-α was also assessed.

RESULTS: IP was significantly increased in the mouse model of FHF 6 h after injection (13.57 ± 1.70 mg/L, 13.02 ± 1.97 mg/L vs 3.76 ± 0.67 mg/L, P = 0.001). Electron microscopic analysis revealed tight junction (TJ) disruptions, epithelial cell swelling, and atrophy of intestinal villi. Expression of occludin and claudin-1 mRNA was significantly decreased in both FHF models (occludin: 0.57 ± 0.159 fold vs baseline, P = 0.000; claudin-1: 0.3067 ± 0.1291 fold vs baseline, P = 0.003), as were the distribution density of proteins in the intestinal mucosa and the levels of occludin and claudin-1 protein (occludin: 0.61 ± 0.0473 fold vs baseline, P = 0.000; claudin-1: 0.9467 ± 0.0186 fold vs baseline, P = 0.000). Prophylactic treatment with anti-TNF-α IgG antibody prevented changes in IP (4.50 ± 0.97 mg/L vs 3.76 ± 0.67 mg/L, P = 0.791), intestinal tissue ultrastructure, and the mRNA levels of occludin and claudin-1 expression (occludin: 0.8865 ± 0.0274 fold vs baseline, P = 0.505; claudin-1: 0.85 ± 0.143 fold vs baseline, P = 0.1), and in the protein levels (occludin: 0.9467 ± 0.0285 fold vs baseline, P > 0.05; claudin-1: 0.9533 ± 0.0186 fold vs baseline, P = 0.148).

CONCLUSION: Increased in IP stemmed from the downregulation of the TJ proteins occludin and claudin-1, and destruction of the TJ in the colon, which were induced by TNF-α in FHF mice.

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INTRODUCTION

Fulminant hepatic failure (FHF) is a devastating disease with high mortality. The intestinal mucosal barrier plays an important role in the body's protection against luminal pathogens and antigenic molecules. Increased intestinal permeability (IP) and disruption of the intestinal mucosal barrier have been observed in many hepatic diseases[1-4] that cause intestinal endotoxemia (IETM), also called gut-derived endotoxin. High levels and incidence of IETM have been reported in many hepatic diseases[5,6]. The IETM results in excessive inflammatory responses, with serious hepatic necrosis, further severe hepatitis, and even accelerated liver failure.

There are two potential pathways for passive permeation across the intestinal epithelia: the transcellular pathway (through the cells) and the paracellular pathway (between cells). The tight junction (TJ) is a semi permeable barrier that allows certain solutes (depending upon their size and charge) to pass through the paracellular pathway between the cells from one fluid component to the other, and even regulates the permeability of the paracellular spaces. It is thought that TJ may be functionally altered in model disease states, even when the intestinal epithelium remains confluent[7]. Structurally, TJs are composed of cytoplasmic proteins, including the zonula occludens (ZO) proteins, ZO-1, ZO-2, and ZO-3[8,9], as well as two distinct transmembrane proteins, occludin and claudins[8,10,11], especially claudin-1, the most widely distributed protein in the intestinal epithelium. Ocludin and claudins are the major transmembrane proteins that interact with intracellular plaque proteins such as ZO-1, ZO-2, and ZO-3, which in turn interact with the actin cytoskeleton to anchor occludin and other transmembrane proteins at the apical end of the lateral membrane[11]. It has been shown that TJ proteins and IP could be modulated by many factors, including shock[12], hyperthermia[13], alcoholic or nonalcoholic liver disease[14,15], and referred to the cytokines, tumor necrosis factor alpha (TNF-α), and interleukin (II)-1, and nitric oxide (NO)[16-17]. There are few studies on IP and its mechanism of modulation in FHF.

TNF-α is a key pro-inflammatory cytokine with a broad-spectrum of effects, and is primarily secreted by monocytes and macrophages. It can induce a hepatocyte necrosis in vivo or in vitro, and is elevated in FHF[16,19]. TNF-α can increase IP in vitro[14,17]; thus its role in the possible alteration of IP in FHF should be considered. D-lactate is produced by indigenous bacteria in the gastrointestinal tract[20]. Mammals do not have the enzyme systems to rapidly metabolize D-lactate. Evaluation of D-lactate levels indicates readily the change of IP[21]. We have tested TNF-α concentrations and intestinal ZO-1 expression in the FHF model in mice[22]. In this study, we utilized the same model and controls to investigate the changes in the permeability of the intestinal mucosa and attempted to elucidate the underlying molecular mechanisms by examining TJ ultrastructure and by measuring the expression of the TJ proteins, occludin, and claudin-1.

MATERIALS AND METHODS

Animals and treatment

Male, six-to eight-week-old BALB/c mice (China Medical University) were obtained from the China Medical University (Shenyang, China). They were housed and cared for in rooms maintained at a constant temperature and humidity. Food and water were allowed ad libitum. The mice were fasted overnight for the experiment. All animal experimental procedures were approved by the Ethics Committee of China Medical University before the commencement of the study. All mice were randomly divided into eight groups (n = 7 per group). One group of mice was given intraperitoneal injections of D-galactosamine (GalN; 800 mg/kg body weight; Sigma, Saint Louis, United States) and lipopolysaccharide (LPS; 10 μg/kg body weight; Sigma) to induce acute liver failure (ALF). A second ALF-induction group was also given intraperitoneal injections of GalN (800 mg/kg body weight) and TNF-α (10 μg/kg body weight; Sigma). First group was given anti-TNF-α immunoglobulin G (IgG) (100 μg per mouse; US Biological, United States) antibody treatment prior to ALF induction. The anti-TNF-α IgG antibody was injected via the vena caudalis 30 min before GalN/LPS administration. There were four control groups, which were injected intraperitoneally with GalN, LPS, TNF-α, or normal saline (NS). In summary, the eight groups were: (1) GalN/LPS; (2) GalN/TNF-α; (3) GalN control; (4) LPS control; (5) TNF-α control; (6) NS control; and (7) anti-TNF-α antibody and GalN/LPS (named anti-TNF-α IgG antibody pretreated group). Mice in first group were euthanized 2, 6, 9, 12 and 24 h after treatment. Other aforementioned groups were euthanized 6 h after administration of GalN/LPS.

D-lactate determination

The plasma from systemic blood samples was obtained and subjected to a deproteinization and neutralization process by acid/base precipitation using perchloric acid and potassium hydroxide. The protein-free plasma was then assayed for D-lactate concentration by enzymatic-spectrophotometric method[23].

Detection and observation of colonic mucosal ultrastructure

Ultrathin (70 nm) colon sections were examined using a transmission electron microscope (Hitachi H-600, Japan).

Immunohistochemistry of occludin, claudin-1

Frozen colon tissue sections (5 μm thick) were fixed on glass slides by incubating them in acetone for 10 min at 4 °C. The slides were incubated with 3% H2O: for 20 min at room temperature and indirectly immunolabeled using an ABC kit (Takara, Japan) according to the manufacturer’s instructions. Slides were then blocked in goat serum for 30 min at 37 °C and incubated with a rabbit anti-mouse polyclonal occludin, claudin-1 antibody (dilution, 1:50; Santa Cruz Biotechnology, United States) at
Intestinal permeability in fulminant hepatic failure

RESULTS

D-lactate

Compared to the normal control, plasma D-lactate levels increased after 2 h and reached significant levels 6 h after injection in GalN/LPS-treated mice (13.57 ± 1.70 mg/L, 3.76 ± 0.67 mg/L, 4.69 ± 1.46 mg/L, 4.50 ± 0.97 mg/L, and 12.48 ± 2.49 mg/L) were electrochemically anti-TNF-α-immunoglobulin G antibody-pretreated groups. 
P < 0.05, P < 0.01 vs baseline (NS group). Data are reported as the mean ± SD.

Western blotting analysis of occludin, claudin-1

Intestinal tissue samples were homogenized in lysis buffer [20 mmol Tris-HCl (pH 7.5), 1% Triton X 100, 0.2 mol NaCl, 2 mmol ethylene diamine tetraacetic acid, 2 mmol ethylene glycol tetraacetic acid, 1 mol dithiothreitol and 2 mol aprotinin]. Proteins (50 μg) were electrophoresed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (8%) and transferred to a nitrocellulose membrane. Membranes were blocked with nonfat dried milk in tris buffered saline containing 0.05% Tween-20 (TTBS) for 1 h at room temperature and incubated with a rabbit anti-mouse polyclonal occludin, claudin-1 antibody (diluted 1:400; Santa Cruz Biotechnology) at 4 °C overnight. After three washes in TTBS, the membranes were reacted with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology) at 4 °C overnight. The immunoreaction was visualized using α-diaminobenzidine and β-naphthyl acid phosphate (Sigma, United States).

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated from intestinal tissues using TRIzol Reagent (Invitrogen, United States). RNA was purified using DNase I and depurified using PI-PCI-EHCO. SYBR-green-based real-time polymerase chain reaction (PCR) [TaKaRa SYBR reverse transcription-PCR kit, Japan] was used to measure relative gene expression in each sample. PCR was performed using Taq DNA polymerase (Qiagen, Valencia, United States) and oligonucleotide primers for mouse were occludin (forward 5'-GCTTATCCTTGGGAGCCCTGGACA-3', reverse 5'-GTCTTTGCTTGCTGATATGATTG-3'), claudin-1 (forward 5'-AGACCTGATTTGACATCTTGGT-3', reverse 5'-TGCAACATAGGCAGGACAAGATTTA-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: forward 5'-TGTTGCTCGTGATCTGA-3', reverse 5'-TTGTCTTGAAGTGCGAG-3'). PCR conditions were as follows: one cycle at 95 ℃ for 30 min followed by 45 cycles of PCR amplification, each consisting of 95 ℃ for 5 s and 57 ℃ for 20 s. The concentration of mRNA was calculated according to the standard curve and then normalized to that of GAPDH.

Statistical analysis

SPSS version 13.0 Software was used to perform the statistical analyses. All data were analyzed using analysis of variance followed by a least-squares difference test. A P < 0.05 was used to indicate statistical significance.

Ultrastructural characteristics of the colonic mucosa

We observed obvious ultrastructural changes in the intes-
tinal mucosa 6 h after GalN/LPS administration. Some epithelial cell microvilli were disordered and distorted, and they were sparsely distributed. The epithelial cells were swollen or shrunken. Mitochondrial swelling was observed; cristae breakdown and disrupted tight junctions were also noted. The changes in the intestinal mucosa of mice treated with GalN/TNF-α were less severe than those of mice treated with GalN/LPS. There was no disruption of TJs in the control groups, including GalN, LPS and TNF-α groups; only less swelling of epithelial cells and distorted microvilli were observed. Pathological changes in the group prophylactically treated with antibody were less severe than those in the ALF groups, and even similar to the NS control group (Figure 2).

**Expression of occludin and claudin-1 proteins in the colon**

Immunohistochemical analysis revealed a high density of occludin and claudin-1 expression in the lateral membrane of the intestinal epithelium in the anti-TNF-α IgG antibody-pretreated group and the saline group. The traces of occludin and claudin-1 proteins weakened in the intestinal tissue 2 h after GalN/LPS treatment, and were most pale 9 h after the injections (Figure 3A and B). The traces slightly weakened even in the TNF-α control group.

The protein level of the NS group was set to 1 as the baseline. Western blotting analysis showed that occludin and claudin-1 expressions decreased significantly in the GalN/LPS-treated mice, particularly 6 h and 9 h after...
the GalN/LPS injections (occludin: 0.6733 ± 0.0433, 0.61 ± 0.0473 fold vs baseline, P < 0.01; claudin-1: 0.8600 ± 0.0208, 0.6633 ± 0.0328 fold vs baseline, P < 0.01) (Figure 4A and C). Expressions of these proteins were decreased significantly in the GalN/TNF-α and TNF-α groups 6 h after injection (occludin: 0.6133 ± 0.0145 fold vs baseline, P < 0.01, 0.8933 ± 0.01 fold vs baseline, P < 0.05; claudin-1: 0.6267 ± 0.0145 fold vs baseline, P < 0.01, 0.92 ± 0.0173 fold vs baseline, P < 0.05), which were less in the later group (Figure 4B and D). The protein expression in the antibody-treated group was close to the normal range (occludin: 0.9467 ± 0.0285 fold vs baseline, P > 0.05; claudin-1: 0.9533 ± 0.0186 fold vs baseline, P > 0.05) (Figure 4B and D).

Expression of occludin and claudin-1 mRNA in the colon

The mRNA level of the NS group was set to 1 as baseline. We obtained a reasonable amplification curve and a standard curve of occludin, claudin-1 and GAPDH RNAs, respectively. The correlation coefficients of all standard curves were 0.999. Real-time PCR quantitative analysis showed that there were marked decreases in occludin and claudin-1 expression in ALF mice 6 h and 9 h after GalN/LPS treatment (occludin: 0.57 ± 0.159 fold vs baseline, 0.345 ± 0.0247 fold vs baseline, P < 0.01; claudin-1: 0.3067 ± 0.1291 fold vs baseline, 0.2233 fold vs baseline, P < 0.01).

Figure 3  The tight junction proteins, occludin and claudin-1 determined by immunohistochemistry (400×). A: Ocludin staining in the saline group; B-D: Ocludin staining 2 h, 6 h, and 9 h after injection in the D-galactosamine (GalN)/lipopolysaccharide (LPS)-treated mice; E-H: Ocludin staining 6 h after injection in the anti-tumor necrosis factor alpha (TNF-α) immunoglobulin G (IgG) antibody pretreated group within the TNF-α-treated group; the LPS-treated group and the D-GalN-treated group, respectively; I: Claudin-1 staining in the saline group; J-L: Claudin-1 staining 2, 6, and 9 h after injection in the GalN/LPS-treated mice; M-P: Claudin-1 staining 6 h after injection in the anti-TNF-α IgG antibody pretreated group, the TNF-α-treated group, the LPS-treated group and the D-GalN-treated group, respectively. The mucosal tissue sections were double-labeled for proteins (brown color). Labeled sections were analyzed immunohisto-chemically. Decreased protein staining in the epithelial cells were observed at 2-9 h after injection in the GalN/LPS-treated mice and the TNF-α-treated group, and was absent in the other controls and the antibody pretreated group.
± 0.1155 fold vs baseline, \( P < 0.01 \) (Figure 5A and C), as well as 6 h after GalN/TNF-\( \alpha \) treatment and TNF-\( \alpha \) treatment (occludin: 0.2562 ± 0.0945 fold vs baseline, \( P < 0.01 \); 0.6421 ± 0.164 fold vs baseline, \( P < 0.05 \); claudin-1: 0.1366 ± 0.0661 fold vs baseline, \( P < 0.01 \); 0.5356 ± 0.1874 fold vs baseline, \( P < 0.05 \)) (Figure 5B and D). The mRNA expression in the antibody-treated group was close to the normal range (occludin: 0.8865 ± 0.0274 fold vs baseline, \( P > 0.05 \); claudin-1: 0.85 ± 0.1437 fold vs baseline, \( P > 0.05 \)) (Figure 5B and D).

**DISCUSSION**

The intestinal mucosa is the physical and metabolic barrier that separates cytotoxic components from gut lumen. Disruption of the gastrointestinal barrier function and the diffusion of luminal toxins and pathogens into the systemic circulation are central to the pathogenesis of a number of diseases. Increased IP has been reported in many hepatic diseases\(^2\)\(^-\)\(^4\)\(^-\)\(^6\). IETM shows an inclination correlation with the D-lactate levels in liver cirrhosis.
and severe injuries\cite{24,25}, and appears to be related to IP. The incidence of IETM reaches 80%-100% in severe hepatitis, and plays an important role in the secondary liver injury observed in FHF\cite{9}. How is IETM generated in FHF? When derived from overproduced bacteria in the lumen of the colon and terminal ileum\cite{26,27}, endotoxin (LPS) is translocated through the intestinal mucosa and into blood, causing IETM. Binding to LPS-binding protein, high density lipoprotein and low density lipoprotein\cite{28,29}, IETM is transported into the portal vein and achieves a higher level in the portal circulation than in peripheral veins\cite{30}. When beyond the detoxification capability of debilitated monocytes and Kupffer cells, IETM damages hepatocytes and accelerates liver failure, even causing systemic injury\cite{9,31}. To detect IP, we utilized D-lactate as an index. IP reached a peak 6 h post-injection of GalN/LPS. In GalN/LPS-treated mice, TNF-\alpha levels reached its first peak 2 h post-injection\cite{22}. When TNF-\alpha was blocked with anti-TNF-\alpha IgG antibody, there was a significant decrease in the IP of the mice. Considering that TNF-\alpha causes an increase in the epithelial barrier permeability in vivo and in vitro, it is concluded that TNF-\alpha is a crucial factor in the increased IP in FHF mice.

Borgstrom et al\cite{32} have showed that at least 85% of the passive flow across the mammalian small intestine takes the paracellular route. Since the cotransporters of the apical membrane are saturated at a low nutrient concentration (below 25 mmol for glucose), but after a meal, nutrient concentrations in the proximal intestine often exceed 200 mmol, it follows that the paracellular pathway may be the major route of nutrient uptake. Drewe et al\cite{33} has indicated that LPS is taken up by the intestinal mucosa predominantly by a transcellular pathway through enterocytes. Under ischemic conditions, the permeability of LPS is increased mainly by an enhanced paracellular translocation across the gut wall, whereas little apoptosis was observed in the intestinal epithelium. In several studies TNF-\alpha induced an increase in the TJ permeability of Caco-2 cells, and did not induce apoptosis in the cells\cite{14,34}, indicating that apoptosis was not responsible for the increase in epithelial TJ permeability. In our previous study, terminal dUTP nick end labeling-positive enterocytes were not seen in the intestine of

![Figure 5](image_url)
GalN/LPS and GalN/TNF-α-treated mice until 12 h after injection [35]. The increased IP observed 6 h after GalN/LPS and GalN/TNF-α treatment was not related significantly to the transcellular pathway. Because TJ regulates the paracellular route in the leakage of macromolecules, we examined TJ with TEM and tested the protein and mRNA levels of TJ proteins. Disrupted TJ structures, down-regulated occludin and claudin-1 protein and RNA levels, and a lower density of proteins distributed in the intestinal mucosa were observed in GalN/LPS- and GalN/TNF-α-treated mice 6 h after injection. These were not observed in normal controls and in the group treated prophylactically with anti-TNF-α IgG antibody. Similar results were obtained with another TJ protein, ZO-1, in our previous study [23]. These observations indicate that TNF-α plays a central role in the regulation of TJ proteins, resulting in the disruption of TJ structure. Considering that TNF-α disrupts TJ structure and increases transepithelial permeability in vitro and in vivo, we conclude that the altered IP is induced by TNF-α via the destruction of TJ. The destruction of TJ by TNF-α was the result of down-regulation of TJ proteins. In the TNF-α-treated group, because of the lower extent of down-regulation of the TJ proteins, ZO-1 [22], occludin and claudin-1 in the TNF-α-treated group, TJs were not visibly disrupted.

When IP was increased in GalN/LPS-treated mice 6 h after injection, the TJ structure was disrupted. In our previous study, ZO-1 showed an insignificant down-regulated expression [23], whereas occludin and claudin-1 were significantly down-regulated in GalN/LPS-treated mice 6 h after injection. This indicated a crucial role of occludin and claudin-1 in the composition of TJ. In vitro, TNF-α had a dose-dependent increasing effect on the permeability of the epithelium [35], and IETM had a severe effect on IP [36]. TNF-α could stimulate the expression of IL-1 and NO, which could disrupt TJ structure and increase epithelium permeability [14,18,37,38], and even synergize with interferon-α to induce intestinal epithelial barrier dysfunction [39]. In vitro on Caco-2 cell monolayers, TNF-α decreased the expression of phosphorylated occludin in detergent-insoluble fractions, but did not affect the expression of non-phosphorylated occludin protein. It also caused a decrease in Caco-2 transcellular resistance and an increase in transepithelial permeability to a paracellular marker, Lucifer yellow [19]. It seemed that the combined decreased effect on IP was maximized 6 h after injection of GalN/LPS in the mouse model of FHF. Few studies on the mechanism of TNF-α-mediated decrease in the induction of TJ proteins were found. This effect might be related to nuclear factor kappa B (NF-κB). NF-κB activation and nuclear translocation of NF-κB p65 has been observed when TNF-α induces an increase in the TJ permeability of Coca-2 cell monolayers [14]. In our previous experiments on Caco-2 cell monolayers, TNF-α induced an increase of TJ permeability to Lucifer yellow, which could be partially inhibited by preincubation with 2 μg/mL anti-TNF receptor I monoclonal antibody and anti-TNF receptor II monoclonal antibody (data not shown). This observation suggests that TNF receptor I and TNF receptor II mediate the TNF-α induced increase in tight junction permeability. Further studies are necessary to clarify the underlying signal transduction system of the TNF-α-induced decrease in the induction of TJ proteins and the increase in TJ permeability in vitro and in vivo.

In conclusion, we found that the increase in IP stems from the downregulation of TJ proteins, especially occludin and claudin-1, and the subsequent destruction of the TJ structure that were induced by TNF-α in the mouse model of FHF.

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