Experimental endotoxemia.

Document that bile HMGB1 mediates gut barrier dysfunction in mucosal permeability and bacterial translocation. The increase in endotoxemic rat bile to normal mice significantly increased both LPS-induced gut barrier dysfunction in rats. In addition, feeding time points. Duodenal injection with anti-HMGB1 antibody reversed and bile HMGB1 levels were significantly increased at the 8- to 12-h concentration in the bile was markedly elevated at the 3- to 4-h time points, significantly decreased at the 4- to 12-h time points, TNF-α release TNF-α into the bile. This study aims to test the hypothesis that in response to LPS stimulation, hepatic Kupffer cells and extrahepatic macrophages release a large amount of the inflammatory cytokine high-mobility group box 1 (HMGB1) into the bile and that bile containing HMGB1 contributes to gut barrier dysfunction in experimental endotoxemia. To test this, rat common bile ducts were catheterized and bile flow rate was monitored before and during the LPS administration. Eight hours after LPS challenge, anti-HMGB1 neutralizing antibody or nonimmune (sham) IgG was injected into the duodenal lumen of endotoxemic rats; normal mice were also gavaged with normal or endotoxemic rat bile (bile collected from LPS-treated rats). We found that after LPS challenge, the bile flow rate in rats was significantly decreased at the 4- to 12-h time points, TNF-α concentration in the bile was markedly elevated at the 3- to 4-h time points, and bile HMGB1 levels were significantly increased at the 8- to 12-h time points. Duodenal injection with anti-HMGB1 antibody reversed LPS-induced gut barrier dysfunction in rats. In addition, feeding endotoxemic rat bile to normal mice significantly increased both mucosal permeability and bacterial translocation. The increase in permeability and bacterial translocation was reversible following removal of HMGB1 from the endotoxemic rat bile. These findings document that bile HMGB1 mediates gut barrier dysfunction in experimental endotoxemia.

endotoxemia; gut barrier function

Sepsis. A lethal syndrome that develops in response to infection, occurs in 750,000 patients per year in the United States and is fatal for 20–40% of those cases (1, 15). Most of the deaths are related to multiple organ dysfunction syndrome (MODS). Currently, leaky gut is thought to be the “motor” that drives the development of MODS (7). The intestine is the biggest reservoir of bacteria in the body and leakage of bacteria or microbial products, notably LPS, from the lumen of the gut into the systemic compartment, leads to initiation or amplification of a deleterious inflammatory response and MODS.

LPS is an important factor in sepsis (24, 25). When LPS is administered by intraperitoneal injection in rodents, impaired gut barrier function (intestinal hyperpermeability and bacterial translocation) is observed (11). However, when administered orally, LPS fails to induce gut barrier dysfunction in rodents (23), indicating that gut mucosal epithelial cells do not respond to direct LPS exposure. The pathogenesis of gut barrier dysfunction in endotoxemic rodents is mediated, at least in part, by the following proinflammatory mediators: inducible nitric oxide synthase (iNOS), TNF-α, and high-mobility group box 1 (HMGB1) (11, 20, 21). To date, the underlying mechanism of gut barrier dysfunction in sepsis still remains unclear.

HMGB1 is a potent late proinflammatory mediator in sepsis (26). Exogenous recombinant HMGB1 given by intraperitoneal injection (50,000 ng/in 1 ml sterile PBS) is capable of inducing animal intestinal hyperpermeability and bacterial translocation at the 12-h time point (21), and treatment with anti-HMGB1 neutralizing antibody ameliorates intestinal hyperpermeability and bacterial translocation following hemorrhagic shock (30). These data support the notion that HMGB1 is capable of inducing gut barrier dysfunction in sepsis and hemorrhagic shock; however, it is still hard to make a conclusion that circulating HMGB1 is responsible for gut barrier dysfunction in sepsis from an animal experiment using a high dose of exogenous HMGB1.

It has been shown that bile acids, which circulate between the liver and the gut, are able to modulate intestinal barrier function (3, 4, 9, 18, 29), but the underlying mechanism is still not clear. Currently, almost all bile studies focus on bile acids and bilirubin, while research on bile cytokines is very minimal. The liver is a unique organ because Kupffer cells are the largest population of fixed tissue macrophages in the whole body, and macrophages are an important source of the inflammatory cytokines TNF-α, IL-6, and HMGB1 (24, 25). There is evidence showing that LPS stimulation increases the HMGB1 mRNA expression in both cultured primary hepatocyte and Kupffer cells; however, only Kupffer cells release HMGB1 protein into the culture media, whereas LPS-stimulated primary hepatocytes do not release HMGB1 protein into the culture media (32). This indicates that Kupffer cells, instead of hepatocytes, play an important role in triggering inflammation. LPS challenge not only decreases the rate of bile flow (2, 10), which is important to maintain gut homeostasis, but also stimulates macrophages to release TNF-α into the bile (12). It has been shown that TNF-α concentration in the bile of LPS-challenged animals is 30 times higher than that in the serum (12). However, this investigation did not test the role of bile TNF-α; instead, a high dose of exogenous TNF-α dissolved in saline was infused into duodenal lumen to cause intestinal mucosal injury (12). Since macrophages are the main
source of circulating HMGB1 (24, 25), it is possible that LPS could also stimulate hepatic Kupffer cells and extrahepatic macrophages to release a large amount of HMGB1 into the bile and that bile cytokines might play an important role in modulating gut barrier function in endotoxemia. To date, little is known about bile cytokines; no published data regarding bile HMGB1, and the role of bile cytokines is unknown. On the basis of this information, we hypothesize that after LPS stimulation, hepatic Kupffer cells and extrahepatic macrophages release large amounts of TNF-α and HMGB1 into the bile, and the high level of bile HMGB1 plays an important role in inducing gut barrier dysfunction in experimental endotoxemia. This investigation showed that after LPS challenge, bile TNF-α and HMGB1 concentrations were significantly increased; endotoxemic rat bile with high levels of TNF-α and HMGB1 was capable of inducing intestinal hyperpermeability and bacterial translocation in normal mice, and removal of HMGB1 from LPS-challenged rat bile could reverse endotoxicem in rat bile-induced gut barrier dysfunction in normal mice. In addition, neutralization of endotoxicemic rat bile HMGB1 could reverse LPS-induced gut barrier dysfunction in endotoxicemic rats. Collectively, our data support the notion that bile HMGB1 mediates gut barrier dysfunction in experimental endotoxemia.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Polyclonal antibodies against highly purified human HMGB1 (no detectable bacterial DNA/lipids) were raised in rabbits (Cocalico Biologicals, Reamstown, PA), and titers were determined by immunoblotting. Anti-HMGB1 antibodies were affinity purified by using cyanogen bromide-activated Sepharose beads following standard procedures. The neutralizing activity of anti-HMGB1 was confirmed in HMGB1-stimulated macrophage cultures by assay of TNF release. In the presence of anti-HMGB1 antibody, neutralizing antibody was defined as inhibiting > 80% of HMGB1-induced TNF release. Nonimmune (sham) rabbit IgG (cat. no. I5006) was purchased from Sigma-Aldrich. Anti-rat TNF-α neutralizing antibody (cat. no. AF-510-NA) was purchased from R&D Systems (Minneapolis, MN).

Animals. This research protocol complied with the regulations regarding the care and use of experimental animals published by the National Institutes of Health and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh Medical School. Male C57Bl/6 mice weighing 20 to 25 g and male Sprague-Dawley rats weighing 250 to 300 g (Charles River, Wilmington, MA) were used in this study. The animals were maintained at the University of Pittsburgh Animal Research Center with a 12:12-h light-dark cycle and free access to standard laboratory feed and water. Animals were not fasted before the experiments but were acclimatized for 7 days before being studied. Catheter, syringes, and tubing were flushed with heparin sodium (1,000 units/ml) before all procedures. Animals were not fasted for this experiment. 12 h prior to bile collection, six rats were anesthetized by intraperitoneal injection. A laparotomy was performed, and a polyethylene catheter (internal diameter = 0.58 mm, outside diameter = 0.965 mm; Becton Dickinson, Sparks, MD) was inserted into the common bile duct and secured with I-0 surgical silk (Azwell, Osaka, Japan). This procedure permitted bile to be collected externally. The catheter was used for continuous bile flow monitoring and collection. After 1-h normal control bile collection, each rat was given a single dose of LPS (cat no. L2630, 0111: B4; Sigma; 3 mg/kg in 1-ml sterile PBS, LD₅₀ = 37 mg/kg) by intraperitoneal injection. The bile flow rate was continuously monitored and collected for 12 h during which time a volume of Ringer’s lactate solution equal to the volume of collected bile was intraperitoneally injected every 2 h to prevent animals from being dehydrated. Simultaneously, another five normal control rats were treated in the same manner as described above (except that LPS was not given) to collect and monitor the normal bile flow rate for 12 h. Multiple small doses (30 mg/kg) of pentobarbital sodium were given as needed to maintain animals anesthetized during the whole bile collection procedure. After the completion of bile collection, all animals were immediately killed by cardiac puncture.

Experimental design. In the first experiment, we tested the role of bile HMGB1 in inducing gut barrier dysfunction in endotoxemic rats. Eighteen male Sprague-Dawley rats (250–300 g) were randomly divided into three groups (n = 6 for each group): the control group, the LPS+nonimmune (sham) IgG group, and the LPS+anti-HMGB1 antibody group. Each rat from the LPS+nonimmune IgG group and the LPS+anti-HMGB1 group was intraperitoneally injected with a single dose of LPS (3 mg/kg in 1-ml sterile PBS, LD₅₀ = 37 mg/kg). Control animals were given 1 ml PBS by intraperitoneal injection. Eight hours after LPS injection, LPS-challenged animals were anesthetized with 90 mg/kg pentobarbital sodium by intraperitoneal injection, after which the abdomens were opened. Then 300 μg anti-HMGB1 neutralizing antibody in 1-ml sterile PBS was slowly injected (27-gauge needle) into the duodenal lumen of the rats in the LPS+anti-HMGB1 group. The abdomens were then closed. An equivalent amount of nonimmune IgG was given in the same manner to rats in the LPS+sham IgG group. Twelve hours after LPS administration, all animals from each group were anesthetized with pentobarbital sodium, and the following procedures were performed: 1) blood was aspirated from the heart to measure serum HMGB1 concentration; 2) a segment of ileum was harvested for determination of mucosal permeability; 3) the mesenteric lymph nodes (MLN) complex were harvested to measure bacterial translocation.

Since a high concentration of TNF-α in the endotoxicemic rat bile lasted for 2 h and a high level of HMGB1 in the endotoxemic rat bile lasted for 4 h, we hypothesized that a combination of 2 h of gavage treatment with endotoxicemic rat bile collected at the 3-h time point (containing high levels of TNF-α) plus 4 h of gavage treatment with endotoxicemic rat bile collected at the 10-h time point (containing high concentrations of HMGB1) might be able to synergistically induce intestinal mucosal hyperpermeability and bacterial translocation in normal animals.

To test our hypothesis and avoid more rats from being subjected to external biliary drainage procedure, mice were used instead of rats for the bile gavage test, because the same number of mice would require a much smaller volume of rat bile. Mice were gavaged with bile hourly to avoid hourly duodenal injection-related gut barrier dysfunction, because the surgical procedure of abdominal opening, repeated duodenal injections, and gut manipulations would impair gut motility and influence gut barrier function. An experiment was performed to show that bile flow rate in normal mice (n = 3) is about 90 μl/h, considering that the stomach retains some amount of bile and stomach acids and enzymes might damage or attenuate activity of bile HMGB1 and bile TNF-α. Therefore, each mouse was gavaged with 100 μl rat bile per hour in the following experiment to prove HMGB1 in the endotoxemic rat bile is important in inducing gut barrier dysfunction in normal mice.

To test the uptake hypothesis that TNF-α and HMGB1 in the endotoxemic rat bile are able to synergistically induce gut barrier dysfunction in normal mice, another experiment was carried out to show that neither 2 h of gavage treatment with pooled endotoxemic rat bile collected at the 3-h time point [containing high levels of TNF-α (bile TNF-α concentration = 1,838.6 ± 103.1 pg/ml)] data were shown as means ± SE, n = 6] alone nor 4 h of gavage treatment with pooled endotoxemic rat bile collected at the 10-h time point [containing high levels of HMGB1 (bile HMGB1 concentration = 33.3 ± 5.0 ng/ml)] data were shown as mean ± SE, n = 6] alone could induce significant
intestinal mucosal hyperpermeability and bacterial translocation in normal mice compared with the normal control mice fed with an equivalent amount of normal control rat bile at equivalent time points (n = 3–4 for each group). However, a combination of 2 h of gavage treatment with endotoxemic rat bile collected at the 3-h time point (containing high levels of TNF-α) plus 4 h of gavage treatment with endotoxemic rat bile collected at the 10-h time point (containing high concentrations of HMGB1) was able to synergistically induce intestinal mucosal hyperpermeability and bacterial translocation in normal mice compared with normal mice gavaged hourly with an equivalent amount of normal control rat bile for a total of 6 h.

In the second experiment, an additional 24 normal mice were randomized into four groups (n = 6 for each group). Mice in the control group were gavaged with 100 μl pooled normal control rat bile hourly for 6 h; mice in the sham IgG group were gavaged with 100 μl pooled TNF-α enriched (TNF-α concentration = 1,838.6 ± 103.1 pg/ml, data were shown as means ± SE, n = 6) endotoxemic rat bile hourly for the first 2 h and with 100 μl pooled HMGB1-enriched (HMGB1 concentration = 33.0 ± 5.0 ng/ml, data were shown as means ± SE, n = 6) endotoxemic rat bile (pretreated with 80 ng nonimmune IgG/ml bile) hourly for additional 4 h; mice in the anti-HMGB1 group were treated the same as sham IgG group except HMGB1-enriched endotoxemic rat bile was pretreated with anti-HMGB1 neutralizing antibody (80 ng/ml bile) and mice in the anti-TNF-α group were treated the same as sham IgG group except TNF-α-enriched endotoxemic rat bile was pretreated with anti-TNF-α neutralizing antibody (3 ng/ml bile). After the gavage procedure, the animals were returned to their cages and allowed free access to food and water. Six hours after the first bile feeding, the animals were anesthetized with pentobarbital sodium (90 mg/kg im), and several procedures were performed. Blood was aspirated from the heart to measure serum HMGB1, a segment of distal ileum was harvested for determination of mucosal permeability, scraped mucosa (containing high levels of TNF-α) was able to synergistically induce intestinal mucosal hyperpermeability and bacterial translocation in normal mice and performed as described (11, 29) using following antibodies: rabbit anti-claudin-1, anti-occludin, anti-ZO-1, anti-β-actin (all from Zymed Laboratory).

Statistics. Data are presented as means ± SE. Bacterial translocation data colony-forming units between groups were analyzed using Mann-Whitney U-test. Significance was accepted at the 5% level.

RESULTS

LPS challenge reduces bile flow rate. All animals subjected to bile diversion procedure survived for 12 h and were not dehydrated. Their blood hematocrit (Hct) was comparable to that of the control and endotoxemia group rats. Compared with control time point (0 h time point), bile flow rate was significantly decreased by 30–40% from 4 to 12 h after LPS injection (n = 6 for each time point, P < 0.05) (Fig. 1). The bile flow rate in the control group subjected to bile diversion procedure (without LPS challenge) was not significantly reduced (P > 0.05).

LPS challenge increases bile TNF-α and HMGB1 concentrations. Compared with the 0 h time point control bile, bile TNF-α concentration was significantly increased at 3–4 h (P < 0.05) with the peak occurring 3 h after LPS challenge (Fig. 2A). Bile IL-6 levels were not statistically elevated (Fig. 2B), but bile HMGB1 concentration began to rise significantly from 8 to 12 h; the peak of HMGB1 secretion occurred 10 h after LPS injection (Fig. 2C). Bile IFN-γ and IL-1 were not detectable in each group at each time point. This result confirms the view that TNF-α is an early inflammatory cytokine and that HMGB1 is a late inflammatory cytokine. The concentrations of these bile cytokines were not increased at any time point in the normal control rats (without LPS treatment), and their concentrations were comparable to that of the 0 h basal level.

LPS challenge does not increase serum HMGB1 concentration at 12-h time point. Twelve hours after LPS injection, compared with the control group, the mean serum HMGB1 concentration (Fig. 3) was not significantly elevated in either LPS+sham IgG or LPS+anti-HMGB1 group, and serum HMGB1 concentration in the LPS+anti-HMGB1 group was not statistically higher than that in the LPS+ sham IgG group (P > 0.05).

Fig. 1. Effect of LPS challenge on bile flow rate in rats. After 1-h control bile (0 h) collection, 3 mg/kg ip LPS was injected into the rats (n = 6), the bile flow rate was continuously monitored, and bile samples were collected hourly from 1–2, 3–4, 8–12 h multiple time points. Four hours after LPS injection, bile secretion volume was significantly decreased compared with the value measured from the 0-h time point and control (*P < 0.01 vs. control).
Neutralization of gut luminal bile HMGB1 reverses LPS-induced intestinal barrier dysfunction in rats. Eight hours after LPS administration, bile HMGB1 concentration began to increase. Anti-HMGB1 antibody was injected into the duodenal lumen to neutralize endotoxemic rat bile-derived HMGB1, since this is where the endotoxemic rat bile enters the intestinal lumen. Compared with the control group, ileal mucosal permeability to the fluorescent macromolecule FD4 was significantly increased in the LPS challenged rats treated with sham IgG, and neutralization of intestinal luminal endotoxemic rat bile-derived HMGB1 reversed LPS-induced mucosal hyperpermeability (Fig. 4A). Bacterial translocation to MLN was minimal in the control group but was extensive in the LPS+sham IgG group, while anti-HMGB1 therapy significantly reduced LPS-induced bacterial translocation (Fig. 4B) ($P < 0.05$ vs. the control; $P < 0.05$ vs. LPS+sham IgG group).

Gavage treatment with endotoxemic rat bile induces intestinal barrier dysfunction in normal mice. Relative to values obtained by studying control bile-fed mice ($n = 6$ for each group), ileal mucosal permeability was significantly increased in the sham-IgG group (Fig. 5A, $P < 0.05$).

Similarly, bacterial translocation to MLN was minimal in the control group, but was extensive in the sham IgG group (Fig. 5B, $P < 0.05$). Yet, the removal of HMGB1 from endotoxemic rat bile by using anti-HMGB1 neutralizing antibody significantly abrogated endotoxemic rat bile-induced mucosal hyperpermeability and bacterial translocation ($P < 0.05$ vs. sham IgG treatment). The removal of TNF-α from endo-

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Fig. 2. Effect of LPS challenge on bile TNF-α, IL-6, and high-mobility group box 1 (HMGB1). TNF-α, IL-6, and HMGB1 were measured in bile samples collected at the following multiple time points: 0 h, 1 h, 2 h, 3 h, 4 h, 8 h (10 h only for HMGB1), and 12 h after LPS (3 mg/kg ip) injection ($n = 6$ for each time point). Results are means ± SE, *$P < 0.05$ vs. control.

Fig. 3. Effect of LPS challenge on rat serum HMGB1. Serum HMGB1 was assessed 12 h after LPS (3 mg/kg) challenge. Control rats were given a saline intraperitoneal injection. Eight hours after LPS challenge, rats in the LPS+sham IgG group were treated with a single dose of 300 µg nonimmune IgG administered by duodenal injection, and rats in the LPS+anti-HMGB1 (LPS+a-H) group were given a single dose of 300 µg anti-HMGB1 neutralizing antibody administered by duodenal injection ($n = 6$ for each group). Results are means ± SE.

Fig. 4. Effect of LPS on rat gut mucosal permeability (A) and bacterial translocation to mesenteric lymph nodes (MLN; B). Ileal mucosal permeability and bacterial translocation were assessed 12 h after LPS injection. Control rats were given a saline intraperitoneal injection. Eight hours after LPS challenge, rats in the LPS+sham IgG group were treated with a single dose of 300 µg nonimmune IgG administered by duodenal injection, and rats in the LPS+anti-HMGB1 group were given a single dose of 300 µg anti-HMGB1 neutralizing antibody administered by duodenal injection ($n = 6$ for each group). FD4, fluorescent tracer FITC-dextran with a molecular mass of 4,000 kDa. Results are means ± SE. (*$P < 0.05$ vs. the control; †$P < 0.05$ vs. LPS+sham IgG group).
Tight junction proteins was preserved when endotoxemic rat bile was pretreated with anti-HMGB1 antibody. There was no significant difference in ZO-1 expression among these three groups (*P > 0.05) (Fig. 6).

**DISCUSSION**

LPS is an important factor in gram-negative sepsis. It is well known that LPS given by intravenous or intraperitoneal injection induces intestinal hyperpermeability and bacterial translocation in rodents; however, the underlying mechanism is not completely understood. The effects of LPS on gut barrier function are dependent upon the method of delivery. Orally administered LPS does not induce intestinal hyperpermeability or bacterial translocation in rats (23). In addition, when inoculated with gut epithelial cell Caco-2 or IEC-6 cell, LPS has no capability of directly inducing epithelial cell monolayer hyperpermeability. Currently, it is postulated that LPS induces macrophages to release a large amount of proinflammatory cytokines (such as IL-1, TNF-α, HMGB1) into the bloodstream and that these high concentrations of circulating cytokines contribute to mucosal hyperpermeability. This view is supported by the evidence showing that cytokinom, a cytokine mixture of IFN-γ, IL-1, and TNF-α has the capability to induce intestinal epithelial cell Caco-2 cell monolayer to leak (20). Among these cytokines, TNF-α has been extensively studied because it is a key early inflammatory mediator in sepsis. It has been shown that after LPS injection, murine serum TNF-α concentration is increased at 0.5–2.5 h and that this peak lasts for 2 h and then decreases to the normal control level at the 3-h time point (24). Gut permeability in LPS-treated mice does not increase at the 6-h time point; however, the permeability begins to increase at the 12-h time point and remains high at the 18-h time point (11). This time course is consistent with the changes in bile HMGB-1. Although systemic treatment with anti-TNF-α neutralizing antibody failed to improve gut barrier dysfunction in LPS-challenged mice (R. Yang, unpublished observation), we still cannot exclude the possibility that circulating TNF-α contributes to gut barrier dysfunction in sepsis. In addition, our study showed that serum HMGB1 was not significantly increased in LPS-treated rats, suggesting that circulating HMGB1 is not likely to be responsible for sepsis-related gut barrier dysfunction. On the contrary, our investigation demonstrated that TNF-α and HMGB1 in the endotoxemic rat bile were capable of synergistically inducing gut barrier dysfunction in normal mice. Removal of HMGB1 from the endotoxemic rat bile could reverse endotoxemic rat bile-induced gut hyperpermeability and bacterial translocation in normal mice. Additionally, neutralization of the endotoxemic rat bile-derived HMGB1 by anti-HMGB1 neutralizing antibody administered by duodenal injection reversed LPS-induced gut barrier dysfunction in rats. Taken together, our data indicate that bile is changed qualitatively and quantitatively in experimental endotoxemia; except for the circulating proinflammatory mediators’ pathway, hepatic bile cytokines are able to modulate gut barrier function in experimental endotoxemia. The liver modulates gut barrier function at least partly through bile HMGB1 in endotoxemia, making bile HMGB1 a potential target for treating sepsis. However, the importance of bile cytokines has not been recognized.

Our data suggest that bile TNF-α and HMGB1 play an important role in inducing gut barrier dysfunction in sepsis.

Endotoxemic rat bile HMGB1 contributes to intestinal mucosal tight junction protein loss in normal mice. The regulation and maintenance of normal intestinal mucosal barrier function depends on the proper assembly and functioning of the tight junctions between adjacent epithelial cells. Formation of tight junctions requires the assembly of several proteins, including the transmembrane protein, occludin, and claudin-1. Therefore, we used Western blot analysis of whole cell extracts prepared from mucosal scrapings to assess changes in the level of these proteins. Compared with the results obtained by studying ileal mucosal scrapings from mice gavaged with control rat bile, the expression of occludin and claudin-1 was substantially decreased in mice gavaged with endotoxemic rat bile pretreated with nonimmune IgG (*P < 0.05 vs. the control; †P < 0.05 vs. LPS+anti-HMGB1 group). However, the expression of these tight junction proteins was preserved when endotoxemic rat bile was pretreated with anti-HMGB1 neutralizing antibody. The concentration is significantly abrogated endotoxemic rat bile-induced mucosal hyperpermeability (*P < 0.05 vs. sham IgG treatment) but did not statistically reduce bacterial translocation. Anti-TNF-α group mice had higher bacterial translocation than the anti-HMGB1 group (*P < 0.05 vs. anti-HMGB1 treatment).

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**DISCUSSION**

LPS is an important factor in gram-negative sepsis. It is well known that LPS given by intravenous or intraperitoneal injection induces intestinal hyperpermeability and bacterial translocation in rodents; however, the underlying mechanism is not completely understood. The effects of LPS on gut barrier function are dependent upon the method of delivery. Orally administered LPS does not induce intestinal hyperpermeability or bacterial translocation in rats (23). In addition, when inoculated with gut epithelial cell Caco-2 or IEC-6 cell, LPS has no capability of directly inducing epithelial cell monolayer hyperpermeability. Currently, it is postulated that LPS induces macrophages to release a large amount of proinflammatory cytokines (such as IL-1, TNF-α, HMGB1) into the bloodstream and that these high concentrations of circulating cytokines contribute to mucosal hyperpermeability. This view is supported by the evidence showing that cytokinom, a cytokine mixture of IFN-γ, IL-1, and TNF-α has the capability to induce intestinal epithelial cell Caco-2 cell monolayer to leak (20). Among these cytokines, TNF-α has been extensively studied because it is a key early inflammatory mediator in sepsis. It has been shown that after LPS injection, murine serum TNF-α concentration is increased at 0.5–2.5 h and that this peak lasts for 2 h and then decreases to the normal control level at the 3-h time point (24). Gut permeability in LPS-treated mice does not increase at the 6-h time point; however, the permeability begins to increase at the 12-h time point and remains high at the 18-h time point (11). This time course is consistent with the changes in bile HMGB-1. Although systemic treatment with anti-TNF-α neutralizing antibody failed to improve gut barrier dysfunction in LPS-challenged mice (R. Yang, unpublished observation), we still cannot exclude the possibility that circulating TNF-α contributes to gut barrier dysfunction in sepsis. In addition, our study showed that serum HMGB1 was not significantly increased in LPS-treated rats, suggesting that circulating HMGB1 is not likely to be responsible for sepsis-related gut barrier dysfunction. On the contrary, our investigation demonstrated that TNF-α and HMGB1 in the endotoxemic rat bile were capable of synergistically inducing gut barrier dysfunction in normal mice. Removal of HMGB1 from the endotoxemic rat bile could reverse endotoxemic rat bile-induced gut hyperpermeability and bacterial translocation in normal mice. Additionally, neutralization of the endotoxemic rat bile-derived HMGB1 by anti-HMGB1 neutralizing antibody administered by duodenal injection reversed LPS-induced gut barrier dysfunction in rats. Taken together, our data indicate that bile is changed qualitatively and quantitatively in experimental endotoxemia; except for the circulating proinflammatory mediators’ pathway, hepatic bile cytokines are able to modulate gut barrier function in experimental endotoxemia. The liver modulates gut barrier function at least partly through bile HMGB1 in endotoxemia, making bile HMGB1 a potential target for treating sepsis. However, the importance of bile cytokines has not been recognized.

Our data suggest that bile TNF-α and HMGB1 play an important role in inducing gut barrier dysfunction in sepsis.
Thus, in addition to the traditional circulating cytokines’ pathway, we propose another mechanism of LPS-mediated gut barrier dysfunction in sepsis: LPS stimulates hepatic Kupffer cells and extrahepatic macrophages to release large amounts of TNF-α and HMGB1 into the bile, and these high levels of TNF-α and HMGB1 in the endotoxemic rat bile contribute to gut barrier dysfunction. Increased bacterial translocation and/or increased mucosal permeability to LPS worsen the liver injury (6, 16, 28), and the inflamed liver/extrahepatic macrophages might release more HMGB1 and TNF-α into the bile to further enhance the gut barrier dysfunction. In addition, the increased liver reduces bile salt secretion and bile flow rate (2, 10, 17), and the decreased bile flow rate and bile acids contribute to mucosal barrier dysfunction as well, because adequate bile is required to maintain the intestinal homeostasis (17, 18).

HMGB1 exposure contributes to the loss of intestinal mucosal tight junction proteins (occludin and claudin-1), and the loss of tight junction proteins may facilitate gut bacterial translocation. As a result, HMGB1 exposure contributes to increased gut mucosal permeability and bacterial translocation in sepsis. In this study, the specific HMGB1 antibody results in significant but not complete restoration of the gut barrier dysfunction. This suggests that in addition to HMGB1, other factors might also be involved. Hydrophilic bile salts and phospholipids could have some effects on preventing gut barrier functions from impairment induced by bacteria and their toxins; further investigation is needed to study the effect of hydrophilic bile salts and phospholipids on gut barrier function in sepsis.

Our data showed that HMGB1-enriched bile alone did not induce intestinal hyperpermeability in normal mice. There are three possibilities for this, the first being that the effect of bile TNF-α on mucosal tight junction is neglected because TNF-α and HMGB1 in the endotoxemic rat bile were capable of synergistically inducing gut barrier dysfunction in normal mice. Second, it is possible that the exposure time of bile HMGB1 is not long enough (only 4 h) to induce mucosal hyperpermeability. The third possibility is that the bile HMGB1 is highly pure, and highly pure HMGB1 has a weak proinflammatory activity by itself but needs other factors (DNA/lipids) bound to it to cause a stronger proinflammatory reaction (19). The actual mechanism warrants further investigation.

In this study, TNF-α and HMGB1 in the endotoxemic rat bile were observed to be able to synergistically induce gut barrier dysfunction in endotoxemic rodents. Therefore, it is important to remove high concentrations of proinflammatory cytokines from bile to treat sepsis. However, these cytokines cannot be removed by external bile drainage, because external...
intraperitoneal injection can induce release of HMGB1, TNF-α, and other inflammatory cytokines from hepatic Kupffer cells and extrahepatic macrophages. However, it is unclear how much percentages of these cytokines are produced by hepatic Kupffer cells and extrahepatic macrophages, respectively. Under sepsis conditions, because liver functions are injured, many plasma components including HMGB1, TNF-α, and other inflammatory cytokines could enter bile from blood so that these components in bile could be a mixture produced by hepatic Kupffer cells and extrahepatic macrophages. Our work begins to provide evidence to support developing strategies targeting bile cytokines in an attempt to improve the situation of septic patients.

GRANTS

This study was supported by funding from the Department of Critical Care Medicine, University of Pittsburgh Medical Center. Pittsburgh, PA.

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