Lipopolysaccharide-binding protein modulates hepatic damage and the inflammatory response after hemorrhagic shock and resuscitation

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Lehnert, Mark, Tetsuya Uehara, Blair U. Bradford, Henrik Lind, Zhi Zhong, David A. Brenner, Ingo Marzi, and John J. Lemasters. Lipopolysaccharide-binding protein modulates hepatic damage and the inflammatory response after hemorrhagic shock and resuscitation. Am J Physiol Gastrointest Liver Physiol 291: G456–G463, 2006. First published May 18, 2006; doi:10.1152/ajpgi.00480.2005.—Hemorrhagic shock and resuscitation cause endotoxemia and hepatocellular damage. Because lipopolysaccharide-binding protein (LBP) enhances cellular responses to endotoxin, our aim was to determine whether LBP contributes to hemorrhage/resuscitation-induced injury by comparing LBP knockout and wild-type mice. Under pentobarbital anesthesia, wild-type and LBP-deficient mice were hemorrhaged to 30 mmHg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer solution. Serum alanine aminotransferase (ALT) necrosis, neutrophil infiltration, and 4-hydroxynonenal by histology/cytochemistry and stress kinase activation by immunoblot analysis were then determined. ALT in wild-type mice was 2,461 ± 383 and 1,418 ± 194 IU/l (means ± SE), respectively, at 2 and 6 h after resuscitation versus sham ALT of 102 ± 6 IU/l. In LBP-deficient mice, ALT was blunted at both time points to 1,108 ± 340 and 619 ± 171 IU/l (P < 0.05). Liver necrosis after 6 h was also attenuated from 3.5 ± 0.8% in wild-type mice to 1.3 ± 0.5% in LBP-deficient mice (P < 0.05). After hemorrhage/resuscitation, neutrophil infiltration increased 71% more in wild-type than LBP knockout mice. Similarly, hepatic 4-hydroxynonenal staining, indicative of lipid peroxidation, decreased from 33.8 ± 4.5% in wild-type mice to 11.6 ± 1.9% in knockout mice (P < 0.05). After hemorrhage/resuscitation, activation of MAPKs, JNK and ERK, occurred in wild-type mice, which was largely blocked in LBP-deficient mice. However, endotoxin in portal blood after resuscitation was not significantly different between wild-type and knockout mice. In conclusion, hemorrhagic shock and resuscitation to mice cause severe, LBP-mediated hepatocellular damage. An absence of LBP blunts hepatocellular injury with decreased neutrophil infiltration, oxidative stress, and c-Jun and ERK activation.

knockout mouse; hemorrhage/resuscitation; liver injury; stress kinases

PATIENTS THAT INITIALLY SURVIVE hemorrhage and resuscitation may develop a systemic inflammatory response that leads to injury and dysfunction of vital organs (8). Hemorrhagic shock and subsequent resuscitation are frequently associated with endotoxemia in animals and humans (41). The liver, with its crucial involvement in metabolism and homeostasis, is among the most frequently affected organs after hemorrhage-induced hypotension in humans (17). The liver also acts to clear and detoxify toxins and bacterial products entering via the portal vein from the gut. Previously, experimental strategies to decrease endotoxin [lipopolysaccharide (LPS)], an essential component of the outer membrane of gram-negative bacteria, improved survival and decreased the production of inflammatory cytokines after hemorrhage and resuscitation (7, 50).

LPS-binding protein (LBP) markedly enhances cellular responses to LPS (33). LBP catalytically transforms LPS from aggregates to monomers and facilitates the interaction of LPS with membrane CD14 receptors present on monocytes and macrophages. The binding of LBP-LPS complexes to CD14 receptors mediates intracellular signal transduction via Toll-like receptor 4 (TLR4). In the presence of LBP, less LPS is required to stimulate TNF-α production from inflammatory cells than in the absence of LBP (42, 44). LBP may also facilitate the transfer of LPS to lipoproteins and thereby decrease the ability of LPS to promote cytokine formation (48). In vitro, pro- or antiinflammatory effects of LPS can depend on the LBP concentration (16). Thus LBP can both enhance and neutralize the biological activity of LPS after hemorrhage/resuscitation, and the importance of LBP for tissue damage after hemorrhage/resuscitation remains to be elucidated.

Transgenic mouse models with defined genetic defects provide an important tool to determine the role of specific proteins involved in the acute stress response syndrome during hemorrhage/reperfusion-induced injury in vivo. Compared with pharmacological approaches, transgenic animal models eliminate the potentially confounding problems of drug side effects and nonspecificity. Here, using LBP-deficient mice (49), we present evidence that LBP plays a role in augmenting hepatocellular injury after hemorrhagic shock and resuscitation.

MATERIALS AND METHODS

Animals. Inbred C57BL/6J (wild type) mice and LBP-deficient (LBP knockout, C57BL/6-Lbp^null^) mice on a C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME). Knockout mice from a strain originally described in Ref. 49 were backcrossed 10 or more times into Jackson Laboratory’s C57BL/6 mice, as in previous studies (14, 46) comparing LBP-deficient mice with LBP-expressing mice. All mice used were male and 6–8 wk of age and 20–25 g in weight. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina (Chapel Hill, NC).

Experimental protocols. After an overnight fast, each mouse was anesthetized with pentobarbital sodium (80 mg/kg body wt). Under spontaneous breathing, both femoral arteries were exposed and cannulated with polyethylene-10 catheters (SIMS Portex). The catheters were flushed with normal saline containing heparin (100 IU/l) before...
Table 1. Arterial pressure during sham operation and hemorrhage/resuscitation

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type Sham</th>
<th>Knockout Sham</th>
<th>Wild-Type Shock</th>
<th>Knockout Shock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before shock</td>
<td>80±0.6</td>
<td>83±3.6</td>
<td>82±2.6</td>
<td>80±0.3</td>
</tr>
<tr>
<td>Shock</td>
<td>79±1.8</td>
<td>78±1.4</td>
<td>30±10</td>
<td>29±0.4</td>
</tr>
<tr>
<td>End of shock</td>
<td>85±0.4</td>
<td>83±1.4</td>
<td>29±4.0</td>
<td>30±1.1</td>
</tr>
<tr>
<td>Reperfusion 15 min</td>
<td>81±3.4</td>
<td>84±2.0</td>
<td>74±2.5</td>
<td>75±4.5</td>
</tr>
<tr>
<td>End of Reperfusion</td>
<td>82±3.6</td>
<td>86±1.4</td>
<td>80±1.1</td>
<td>85±3.6</td>
</tr>
</tbody>
</table>

Values are means ± SE (in mmHg). Mean arterial blood pressure was measured during sham operation and at different times in the hemorrhage/resuscitation protocol.

insertion. One catheter was connected via a transducer to a pressure analyzer (Micro-Med; Louisville, KY), and blood was withdrawn via the second catheter into a heparinized syringe (10 units) over 5 min to a mean arterial pressure of 30 mmHg. This pressure was maintained for 3 h by the withdrawal or reinfusion of shed blood (18). Body temperature was monitored and maintained at 37°C. After 3 h, mice were resuscitated with the shed blood plus a volume of lactated Ringer solution corresponding to 50% of the shed blood volume, which was infused with a syringe pump over 30 min (3, 4). Adequacy of resuscitation was determined by the restoration of blood pressure. Hypotension for 3 h was required, because shorter periods of hemorrhagic shock (1 and 2 h) produced much milder and more variable injury (18, 31). The catheters were then removed, the vessels were ligated, and the groin incisions were closed. Sham-operated animals underwent the same surgical procedures, but hemorrhage was not carried out. At 6 h after resuscitation, all mice were ambulatory with no obvious differences between the wild-type and LBP-deficient mice. No mortality in any group occurred over the course of the experiments.

Some wild-type and LBP knockout mice were anesthetized and killed by exsanguination immediately at the end of resuscitation. For the determination of hemorrhage/resuscitation-dependent liver damage, mice were anesthetized and killed by exsanguination 6 h after the end of resuscitation in a second set of experiments. For each mouse, the two right dorsal liver lobes were snap frozen in liquid nitrogen. The remaining liver was flushed with normal saline, infused and fixed with 10% buffered formalin through the portal vein, embedded in paraffin sections, and stained with hematoxylin-eosin.

Endotoxin assay. Portal vein blood was collected under sterile conditions in pyrogen-free heparinized syringes at the end of resuscitation. After centrifugation at 1,200 rpm for 10 min, the plasma was stored at −20°C in pyrogen-free glass tubes until the measurement of endotoxin with a Limulus Amebocyte Lysate test kit (Kinetic QCL, BioWhittaker; Walkersville, MD) (39).

Western blot analysis for intracellular signaling. Liver tissue was homogenized in lysis buffer [10 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5% Nonidet P-40, and 25% glycerol] with protease and phosphatase inhibitors (50 mM PMSF, 2.5 mg/ml aprotonin, 2.5 mg/ml pepstatin, and 5 mg/ml leupeptin) at 4°C, followed by centrifugation for 30 min at 4°C at 15,000 rpm. Supernatants were stored at −80°C for later analysis. Lysates (50 μg protein) were separated by electrophoresis on 10% polyacrylamide-SDS gels and transferred to nitrocellulose membranes (Schleicher and Schuell; Keene, NH). Equal loading was confirmed by Ponceau S staining. Phospho (p)-c-Jun was detected using rabbit anti-p-c-Jun antibody (Santa Cruz Biotechnology; Santa Cruz, CA). p-ERK was detected using rabbit anti-p-ERK antibody (Cell Signaling; Lexington, KY). Mouse anti-α-actin antibody (ICN Biomedicals; Irvine, CA) served as a loading control. Blots were blocked [5% nonfat dry milk in 20 mM Tris (pH 7.5), 125 mM NaCl, and 0.1% Tween 20] for 1 h, incubated 1 h at room temperature in primary antibody (1:1,000 in blocking buffer), and then incubated 1 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; 1:1,000 in blocking buffer). Proteins were detected with ECL detection reagents (Amersham; Arlington Heights, IL). Films were scanned, and the integrated density of individual bands was determined in TIF files using Adobe Photoshop CS (Adobe; Mountain View, CA).

Examination of liver injury. Sera were stored at −80°C for later analysis of alanine aminotransferase (ALT) using a kit (Sigma Chemical, St. Louis, MO). Focal liver necrosis was quantitated microscopically at a magnification of ×100 in 10 random fields for each liver in a blinded fashion, as described previously (31, 34). Necrotic areas were expressed as the percentage of the total area of the liver sections examined.

Detection of myeloperoxidase-positive leukocytes. Liver sections were deparaffinized and blocked with 2% hydrogen peroxide in PBS (pH 7.4) containing 1% Tween 20 for 5 min, followed by 1 h of 1% BSA in PBS with 0.4% Tween 20 and an additional 15 min in 2% BSA in PBS (pH 7.4) containing 1% Tween 20. Rabbit anti-myeloperoxidase (MPO) antibody (Sigma Chemical) was applied at a 1:200 dilution for 30 min at room temperature. Incubation with a secondary peroxidase-linked anti-rabbit IgG antibody (Peroxidase Envision Kit, DAKO; Carpinteria, CA) was performed for 15 min. Labeled proteins were visualized with diaminobenzidine (Peroxidase Envision Kit, DAKO), followed by counterstaining with hematoxylin. MPO-positive leukocytes were counted from a total of 50 high-power (×400) fields/mouse.

Detection of 4-hydroxynonenal. Paraffin-embedded sections of the liver were deparaffinized, rehydrated, and incubated with polyclonal antibodies against 4-hydroxynonenal (Alpha Diagnostics; San Antonio, TX) in PBS (pH 7.4) containing 1% Tween 20 and 1% BSA. Peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit, DAKO) were used to detect specific binding. A Universal Imaging Metamorph image acquisition and analysis system was used to analyze the density of 4-hydroxynonenal staining.
(Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss; Thornwood, NY) was used to capture and analyze the immunostained tissue sections at ×40 magnification. The extent of labeling was determined in randomly selected fields as the percentage of area within a preset color range determined by the software. Data from each tissue section (10 fields/section) were pooled to determine means, as described previously (31).

**Statistical analysis.** Differences between groups were determined by one-way ANOVA using a multiple-comparison procedure (Tukey test) and by Kruskal-Wallis ANOVA on ranks using a multiple-comparison procedure (Dunn’s method). A P value of <0.05 was considered significant. P values between 0.05 and 0.1 were also noted as nonsignificant trends. Data are given as means ± SE.

**RESULTS**

**Hemodynamic characteristics of hemorrhage and resuscitation.** Because endotoxemia affects blood pressure (19, 35), we first determined whether the blood pressures of wild-type and knockout mice were comparable before, during, and after hemorrhage/resuscitation. Before the imposition of hemorrhagic shock, the blood pressures of wild-type mice and knockout mice were nearly identical (Table 1). Blood pressures during the hypotensive period were equal for both knockout and wild-type mice. The increases of blood pressures after resuscitation were also not different between groups (Table 1). The amount of blood removed did not differ between groups (32 ± 1.3 and 29.7 ± 1.1 ml/kg body wt in wild-type and knockout mice, respectively). Wild-type and LBP mice undergoing the sham procedure maintained blood pressures throughout the experiment (Table 1). These results indicated that gene deficiency of LBP did not affect blood pressure either before or after shock or change the amount of blood loss required to produce hypotension of 30 mmHg. Moreover, the resuscitation protocol restored blood pressures to prehemorrhage levels.

![Fig. 2. Decreased liver necrosis after hemorrhage/resuscitation in LBP-deficient mice.](image)

WT and LBP-deficient mice were subjected to hemorrhage/resuscitation or sham operation, as described in Fig. 1, and livers were fixed for histology at 6 h after resuscitation. Top: sections of sham-operated WT (left) and LBP-deficient (right) mice. Middle: sections of WT (left) and LBP-deficient (right) mice after hemorrhage and resuscitation (shock). The arrows depict the borders of necrotic areas. Sections are stained with hematoxylin and eosin. Bar = 100 μm. Bottom: percentages of liver necrosis for the four groups, determined as described in MATERIALS AND METHODS. Group size was 6 mice/group. *P < 0.05 vs. WT.
Endotoxemia after hemorrhage/resuscitation. Endotoxin in portal blood at the end of resuscitation increased in both wild-type (291 ± 116 pg/ml) and knockout mice (376 ± 223 pg/ml) compared with mice that underwent the sham operation (below detection by amebocyte lysate assay). Differences of endotoxin between wild-type and LBP-deficient mice were not statistically significant. The increase of endotoxin after hemorrhage/resuscitation was transient and decreased by 50% and 80% at 30 min and 6 h, respectively, after resuscitation (data not shown).

Transaminase release after hemorrhage/resuscitation. In wild-type mice, ALT as a serum marker of liver damage (31) increased to 2,461 ± 383 IU/l at 2 h after resuscitation compared with 102 ± 6 IU/l in sham-operated wild-type mice (P < 0.05). ALT remained elevated at 6 h after resuscitation (1,418 ± 194 IU/l at 6 h after resuscitation vs. 86 ± 5 IU/l after sham operation, P < 0.05). These findings show that liver damage occurred early in our hemorrhage/resuscitation model (Fig. 1).

In LBP-deficient mice, ALT decreased by 55% (1,108 ± 340 IU/l) at 2 h after resuscitation and 57% (619 ± 171 IU/l) at 6 h after resuscitation compared with wild-type mice (P < 0.05; Fig. 1). ALT in sham-operated knockout mice was comparable to wild-type mice (104 ± 6 and 81 ± 6 IU/l at 2 and 6 h after resuscitation; Fig. 1). These results indicated that liver injury after hemorrhage/resuscitation was substantially decreased in LBP-deficient mice.

Liver necrosis after hemorrhage/resuscitation. Wild-type mice showed large, confluent areas of coagulative necrosis at 6 h after the end of resuscitation. The injury was most pronounced in midzonal and pericentral regions of liver lobules (Fig. 2). Morphometry revealed that 3.5 ± 0.8% of the area of liver sections became necrotic (Fig. 2). In LBP knockout mice, coagulative necrosis in tissue sections was substantially decreased (Fig. 2), and morphometry revealed that the extent of necrosis was three times smaller (P < 0.05; Fig. 2). Taken together, the results showed that liver injury after hemorrhage/resuscitation was substantially decreased in LBP-deficient mice.

Accumulation of MPO-positive leukocytes in the liver after hemorrhage/resuscitation. At 6 h after the end of resuscitation, liver neutrophils increased 10-fold in wild-type mice compared with sham-operated wild-type mice (P < 0.05; Fig. 3). In LBP knockout mice, the hemorrhage/resuscitation-induced increase of neutrophil infiltration was blunted by 71% (P < 0.05; Fig. 3). These findings indicate that hepatic neutrophil infiltration after hemorrhage/resuscitation was markedly reduced in LBP knockout mice.

Lipid peroxidation after hemorrhage/resuscitation. Tissues undergoing hemorrhage/resuscitation experience oxidative stress. Hepatic oxidative stress in vivo can be revealed by immunohistochemical detection of 4-hydroxynonenal, a marker of lipid peroxidation. After hemorrhage/resuscitation of wild-type mice, 4-hydroxynonenal staining in liver sections increased compared with sham-operated wild-type mice (Fig. 4). Parenchyma surrounding central venules and midzonal areas showed the most intense staining (Fig. 4). Compared with wild-type mice, hepatic 4-hydroxynonenal staining was decreased in LBP-deficient mice after hemorrhage/resuscitation. Morphometry revealed that 4-hydroxynonenal staining was three times greater in wild-type mice compared with LBP knockout mice (P < 0.05; Fig. 4). Taken together, these findings showed that considerable lipid peroxidation occurred in the liver after hemorrhage/resuscitation, which was attenuated in LBP-deficient knockout mice.

Activation of proinflammatory stress kinases after hemorrhage and resuscitation. To examine the inflammatory stress response to hemorrhagic shock and resuscitation, we assessed the phosphorylation of stress kinases. Six hours after hemorrhage/resuscitation, Western blot analysis was performed with antibodies specific for the phosphorylated forms of the MAPKs c-Jun, ERK, and p38 MAPK from whole liver extracts (Fig. 5). Phosphorylation of c-Jun increased 91% after hemorrhage/resuscitation in wild-type mice compared with sham-operated mice (P < 0.05). ERK phosphorylation also increased 66% after hemorrhage/resuscitation in wild-type mice, a trend that did not quite reach statistical significance (P = 0.07). In LBP knockout mice after hemorrhage/resuscitation, both c-Jun and ERK phosphorylation were significantly decreased compared with wild-type mice (Fig. 5). In contrast, phosphorylation of p38 was markedly decreased after hemorrhage/resuscitation compared with both sham-operated wild-type mice and sham-operated LBP knockout mice (P = 0.002), and no differences were observed between the two mouse strains. These results indicated that activation of JNK and ERK increased after hemorrhage/resuscitation, which was blunted in LBP-deficient mice. In an opposite fashion, hemorrhage/resuscitation decreased the phosphorylation of p38, an effect that was not influenced by deficiency of LBP.

DISCUSSION

Endotoxin release after hemorrhage/resuscitation in wild-type and LBP knockout mice. After hemorrhage/resuscitation, bacteria and bacterial cell-wall products such as endotoxin...
translocate the damaged gut mucosa into the portal blood to reach the liver (12, 27). Our results in mice confirm that portal blood endotoxin increases after hemorrhage/resuscitation. This increase of endotoxin after hemorrhage/resuscitation was transient and decreased by 50% at 30 min after resuscitation and by 80% after 6 h (data not shown), in agreement with a previous study in animals and humans (41). After hemorrhage/resuscitation, hepatic injury, neutrophil infiltration, and stress kinase activation were substantially greater in wild-type than LBP-deficient mice (Figs. 1, 3, and 5). However, endotoxin influx into the liver after resuscitation was not different. This implies that LBP is not an important mediator of endotoxin translocation by gut mucosa. Moreover, changes of the amount of endotoxin entering the liver cannot account for the differences observed between LBP-deficient and wild-type mice. Because LBP-deficient mice were used that had been backcrossed 10 or more times to C57BL/6J wild-type mice, the possibility that the differences observed were due to linked alleles from the SV129 founder mouse strain was minimized.

Some clinical studies have questioned the importance of mucosal translocation of endotoxin and/or bacterial translocation in promoting proinflammatory changes and adverse clinical outcome (36), whereas other studies have shown a possible connection of endotoxemia with adverse outcomes after trauma/hemorrhage (10, 13, 40, 41, 52). Lack of concordance of these clinical studies may reflect the methodological problems and confounding variables of measuring endotoxin, especially in patient populations. Our findings of decreased liver injury in LPB-deficient mice after resuscitated hemorrhage support an etiological role of endotoxin in the pathophysiology of organ damage after hemorrhagic shock and add evidence to the notion that endotoxemia may be clinically relevant in hemor-

Fig. 4. Decreased hepatic 4-hydroxynonenal (4-HNE) staining in LBP-deficient mice after hemorrhage/resuscitation. WT and LBP-deficient mice were subjected to hemorrhage/resuscitation or sham operation, as described in Fig. 1. 4-HNE protein adducts were visualized by immunocytochemistry, as described in MATERIALS AND METHODS. Top: sections of sham-operated WT (left) and LBP-deficient (right) mice. Middle: sections of WT (left) and LBP-deficient (right) mice after hemorrhage and resuscitation. Bar = 100 μm. Bottom: percentages of 4-HNE staining, determined as described in MATERIALS AND METHODS. Group size was 6 mice/group. *P < 0.05 vs. WT.
rhave and trauma. Our findings of decreased liver injury in LBP-deficient mice after resuscitated hemorrhage support an etiological role of endotoxin in the pathophysiology of organ damage after hemorrhagic shock.

Dependence of hepatic oxidative stress after hemorrhage/resuscitation on LBP. Reactive oxygen species mediate, at least in part, liver injury after hemorrhage/resuscitation, ischemia-reperfusion, and cold storage/reperfusion (23, 31, 32, 51). After no-flow ischemia, LPS amplifies radical generation and other proinflammatory responses by Kupffer cells, which are events that contribute to hepatic injury. Numerous other reports (6, 20, 21, 26) have also shown a role of Kupffer cells in inflammatory and prooxidant responses after hemorrhage, trauma, and sepsis. Our findings here show that LBP is part of this oxidative stress response, because hemorrhage/resuscitation-dependent hepatic lipid peroxidation was markedly decreased in LBP-deficient mice (Fig. 4).

The interdependence between endotoxemia and oxidative stress is well established (28). Various stresses such as sepsis and hemorrhage/resuscitation upregulate the acute-phase protein LBP in various tissues, including the liver (26, 47). LBP expression may enhance antimicrobial defenses by promoting the generation of reactive oxygen and proinflammatory cytokines (26). In the absence of LBP, oxygen radical production by human monocytes and TNF-α, IL-6, and nitric oxide production by Kupffer cells after LPS are blunted (15, 28). NADPH oxidase plays a major role in the pathogenesis of hepatic injury after hemorrhage/resuscitation, and we (31) have recently shown that hepatic necrosis, enzyme release, hepatic neutrophil infiltration, and the generation of reactive oxygen and nitrogen species are substantially decreased in NADPH oxidase-deficient mice after hemorrhage/resuscitation. c-Jun is a component of activator protein (AP)-1, and, in other recent work (1), diphenylelenedioneum, an NADPH oxidase inhibitor, was shown to decrease hepatic AP-1 activation after hemorrhage/resuscitation. On the basis of our observations here, that both oxidative stress and activation of hepatic ERK and c-Jun were blunted in LBP-deficient mice (Figs. 4 and 5), it is possible that a link exists between NAPDH oxidase-dependent oxidative stress and MAPK activation, especially because reactive oxygen species can activate ERK and JNK (11, 37). Future experiments will be needed to address this hypothesis.

We also attempted to measure TNF-α and IL-6 mRNA and protein levels after hemorrhage/resuscitation by ELISA and RNase protection assay. Although nonsignificant trends were observed that were consistent with LBP-dependent cytokine formation after hemorrhage/resuscitation, overall levels were low (data not shown). These results may be due to the fact that tissue was harvested at 6 h, and the formation of TNF-α after LPS is typically maximal at 1–2 h and then rapidly returns to baseline. In contrast, ERK activation and c-Jun phosphorylation (signifying JNK activation) are longer-lasting responses of macrophages to LPS stimulation that persisted at 6 h after resuscitation.

Importance of LBP for liver damage and the hemorrhage/resuscitation-induced intracellular inflammatory response. Serum ALT (Fig. 1), hepatic necrosis (Fig. 2), neutrophil infiltration (Fig. 3), and hepatic lipid peroxidation (Fig. 4) were up to three times greater in wild-type than LBP-deficient mice after hemorrhage/resuscitation. Similarly, the phosphorylation of stress kinases was greater in wild-type mice (Fig. 5). These findings support the conclusion that LBP amplifies hemorrhage/resuscitation-dependent liver injury by augmenting the LPS-dependent inflammatory response. The effects of LBP can be dose dependent, because low concentrations of LBP enhance LPS-induced activation of isolated human mononuclear cells, whereas higher levels of LBP neutralize activation (16). The results of the present study suggest that LBP at physiological levels in vivo promotes rather than suppresses injury after hemorrhage/resuscitation. After bacterial challenge, LBP-deficient animals are unable to contain infection at a local level, as shown in a pneumonia model with Klebsiella and after an intraperitoneal infection with Salmonella (14, 22). In contrast, monoclonal antibody against either LBP or CD14 dramatically decreases mortality from injected endotoxin (29). These results indicate that LBP contributes to toxicity in endotoxemia but confers protection against invasion by live bacteria. After alcohol treatment, liver injury is decreased in

Fig. 5. Decreased phosphorylation of Jun and ERK in LBP-deficient mice after hemorrhage/resuscitation. WT and LBP-deficient mice were subjected to hemorrhage/resuscitation or sham operation, as described in Fig. 1. Six hours after the end of resuscitation, livers were processed for Western blot analysis with antibodies against α-actin and the phosphorylated (p) forms of c-Jun, ERK, and p38, as described in MATERIALS AND METHODS. In A, lanes 1–4 are WT mice after sham operation (lane 1) or hemorrhage/resuscitation (shock, lanes 2–4). Lanes 5–8 are LBP-deficient mice after sham operation (lane 5) or hemorrhage/resuscitation (shock, lanes 6–8). Lane 9 contained internal standards (control [Ctrl]). In B, densitometric measurements normalized to α-actin staining are plotted. Group sizes were 2–3 mice/group. *P < 0.05 vs. sham; †P = 0.07 vs. sham; #P < 0.05 vs. WT.
LBP-deficient mice compared with wild-type mice, although portal endotoxin levels are not different in the two mouse strains. After alcohol, TNF-α formation is also less in LBP deficient, consistent with a role for LBP in promoting LPS-dependent TNF-α by Kupffer cells after alcohol exposure (46).

Hemorrhage/resuscitation leads to the release and wash out of gut-derived endotoxin at the time of resuscitation. In contrast to many models employing injected LPS, endotoxin released after resuscitation acts on hepatic tissue already compromised and possibly primed by the effects of the preceding hemorrhage. Thus tissue compromised by the hypoxic stress of hemorrhage may be more prone to the injurious effects of LPS and related inflammatory reactions.

LBP binds LPS and then interacts with CD14 and TLR4 to initiate the phosphorylation of MAPKs (2, 5). After hemorrhage/resuscitation in our mouse model, these pathways were activated, as shown by phosphorylation of ERK and c-Jun, with the latter being a component of AP-1 and a substrate of JNK (Fig. 5). In LBP-deficient mice, phosphorylation of ERK and c-Jun after hemorrhage/resuscitation was decreased compared with wild-type mice, but p38 phosphorylation was not different. Because results were obtained from the whole liver, MAPK activation cannot be attributed to a specific cell type. JNK and ERK participate in inflammatory responses after ischemia-reperfusion and hemorrhage/resuscitation. JNK activation leads to c-Jun phosphorylation with a consequent activation of the transcription factor AP-1 (24, 25, 38). Selective inhibition of JNK results in decreased phosphorylation of c-JUN and confers protection in a model of rat liver transplantation (45). Antioxidants that block the activation of AP-1 prevent organ damage after hemorrhage/resuscitation (38, 43).

Although ERK is a survival signaling molecule in some cell types, ERK activation also appears to contribute to organ dysfunction after trauma/hemorrhage, and prevention of ERK phosphorylation by tyrosine kinase inhibition improves cardiac output and hepatocellular function (9, 25). The activation of ERK is at least partially redox sensitive (37), and the present findings in mice confirm the association of MAPK activation with proinflammatory cell signaling, oxidant stress, and organ injury after hemorrhage/resuscitation.

Previous work (6, 20, 21, 26) has shown a role of Kupffer cells in inflammatory responses after hemorrhage, trauma, and sepsis. After hemorrhage, Kupffer cells increase their sensitivity to endotoxin to produce TNF-α and IL-1 (6, 26). Hemorrhage also enhances the cytotoxicity of Kupffer cells (6). When sepsis is combined with trauma in rats, mortality increases, an event associated with exaggerated nitric oxide and superoxide formation by Kupffer cells (21). Even mild hemorrhage enhances phorbol ester-stimulated superoxide production by Kupffer cells (20). Because Kupffer cell responses to LPS are magnified by LBP (44), Kupffer cells potentially mediate, at least in part, liver damage after hemorrhage/resuscitation. Future studies will be needed to determine the specific role of Kupffer cells in the LBP dependency of liver injury after hemorrhage/resuscitation.

In conclusion, the findings of the present study provide evidence that LBP contributes to the pathogenesis of liver injury after hemorrhage/resuscitation via the promotion of proinflammatory signaling and oxygen free radical production. Future studies will be required to better characterize the specific cell type(s) mediating the effects observed to develop clinically relevant interventional strategies to ameliorate the harmful LBP-dependent consequences of hemorrhage/resuscitation.

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