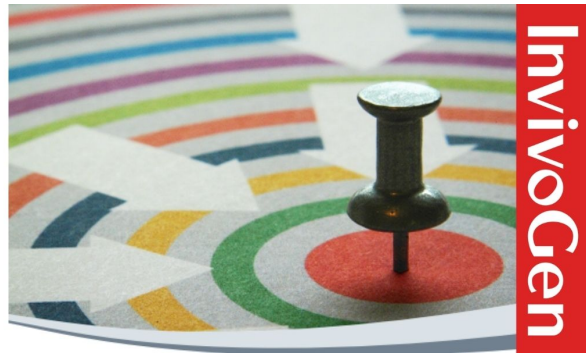


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**Role of Lipopolysaccharide-Binding Protein in Early Alcohol-Induced Liver Injury in Mice<sup>1</sup> **FREE****

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# Role of Lipopolysaccharide-Binding Protein in Early Alcohol-Induced Liver Injury in Mice<sup>1</sup>

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Cellular responses to endotoxins are enhanced markedly by LPS-binding protein (LBP). Furthermore, it has been demonstrated that endotoxins and proinflammatory cytokines such as TNF- $\alpha$  participate in early alcohol-induced liver injury. Therefore, in this study, a long-term intragastric ethanol feeding model was used to test the hypothesis that LBP is involved in alcoholic hepatitis by comparing LBP knockout and wild-type mice. Two-month-old female mice were fed a high-fat liquid diet with either ethanol or isocaloric maltose-dextrin as control continuously for 4 wk. There was no difference in mean urine alcohol concentrations between the groups fed ethanol. Dietary alcohol significantly increased liver to body weight ratios and serum alanine aminotransferase levels in wild-type mice ( $189 \pm 31$  U/L) over high-fat controls ( $24 \pm 7$  U/L), effects which were blunted significantly in LBP knockout mice ( $60 \pm 17$  U/L). Although no significant pathological changes were observed in high-fat controls, 4 wk of dietary ethanol caused steatosis, mild inflammation, and focal necrosis in wild-type animals as expected (pathology score,  $5.9 \pm 0.5$ ). These pathological changes were reduced significantly in LBP knockout mice fed ethanol (score,  $2.6 \pm 0.5$ ). Endotoxin levels in the portal vein were increased significantly after 4 wk in both groups fed ethanol. Moreover, ethanol increased TNF- $\alpha$  mRNA expression in wild-type, but not in LBP knockout mice. These data are consistent with the hypothesis that LBP plays an important role in early alcohol-induced liver injury by enhancing LPS-induced signal transduction, most likely in Kupffer cells. *The Journal of Immunology*, 2002, 168: 2963–2969.

Bacterial LPS (endotoxin), an abundant and essential lipid component of the outer membrane of Gram-negative bacteria, provokes a generalized proinflammatory response in the infected host that sometimes leads to septic shock and multiple organ failure (1, 2). Recently, the LPS signaling pathway has been elucidated in peripheral blood monocytes. Cellular responses to LPS are known to be enhanced markedly by LPS-binding protein (LBP),<sup>3</sup> a 60-kDa acute phase protein produced mainly by hepatocytes that catalytically transforms LPS from aggregates to monomers and facilitates interaction with membrane CD14 present on the surface of monocytes and macrophages (3–5). The binding of LPS-LBP complexes to CD14 mediates signal transduction, including NF- $\kappa$ B activation via Toll-like receptor 4 (TLR4) (6). Indeed, in the presence of LBP, TNF- $\alpha$  is released by monocytes at concentrations of LPS far below those required in the absence of LBP (7, 8). It has also been shown that LBP plays an important role in mediating Kupffer cell activation by LPS in the presence of a functional TLR4 (8). On the other hand, LBP neutralizes LPS when it is transferred to lipoproteins (9). Thus, LBP may either enhance or neutralize the biological activity of LPS.

Recently, considerable evidence has accumulated in support of the hypothesis that LPS and proinflammatory cytokines participate in mechanism of alcohol-induced liver injury (10–13). Chronic alcohol administration increases gut-derived LPS in the portal circulation and activates Kupffer cells to produce several proinflammatory cytokines such as TNF- $\alpha$  and IL-1 (10, 14). Indeed, intestinal sterilization with antibiotics (polymixin B and neomycin) (12) and displacement of Gram-negative bacteria with lactobacillus feeding (15) prevents alcohol-induced liver injury. Furthermore, recent studies with knockout and mutant mice showed that CD14 and TLR4 are indeed involved in early alcohol-induced liver injury (16, 17). Thus, it is clear that the LPS signaling pathway is involved in the mechanism of early alcohol-induced liver injury, but whether LBP is involved remains unknown. In the present study, the long-term intragastric ethanol feeding protocol developed by Tsukamoto et al. (18) for rats was adapted to mice to test the hypothesis that LBP is involved in early alcohol-induced liver injury by using LBP knockout mice.

## Materials and Methods

### Animals

Female wild-type (C57BL/6J) or LBP knockout mice (C57BL/6-Lbp<sup>tm1Buru</sup>) were obtained from The Jackson Laboratory (Bar Harbor, ME). Two-month-old mice (18–21 g) were used in this study and all animals received humane care in compliance with institutional guidelines. Mice had access to chow and water ad libitum prior to the study.

### Surgery

The surgical procedures used here were similar to methods described previously by Tsukamoto and French for rats (18), with modifications to accommodate the smaller size of mice (13). Briefly, mice were anesthetized by injection of pentobarbital sodium (50 mg/kg; Abbott Laboratories, North Chicago, IL), and laparotomy was performed under sterile surgical conditions. A PE90 polyethylene tube (BD Biosciences, Sparks, MD) was placed in the squamous part of the stomach, anchored to the stomach wall with dacron, and fixed to the abdominal wall. It was then tunneled s.c. to the dorsal aspect of the neck followed by closing of the abdominal wall

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<sup>3</sup> Abbreviations used in this paper: LBP, LPS-binding protein; ALT, alanine aminotransferase; CYP, cytochrome P450; TLR4, Toll-like receptor 4.

with 7-0 prolene sutures. The tube was then pulled through a 250P polysulfone attachment mouse button (Instech Laboratories, Plymouth Meeting, PA) and spring coil. The button was fixed under the skin with its metal spring coil outside of the body to protect the tube. The feeding tube was attached to an infusion pump by means of a swivel, allowing complete mobility of the mouse in a metabolic cage. Animals were allowed to recover for 1 wk with free access to chow diet and water before alcohol-containing or control high-fat liquid diets were infused.

### Diets

A liquid diet described by Thompson and Reitz (19) supplemented with lipotropes as described by Morimoto et al. (20) was used. The control diet (1.3 kcal/ml) contained corn oil as the source of fat (37% of total calories), protein (23%), carbohydrate (40%), plus minerals and vitamins. For the ethanol diet, dextrin-maltose was replaced isocalorically by ethanol (13). Throughout the experimental period of liquid diet delivery, mice had free access to cellulose pellets as a source of fiber (Harlan Teklad, Madison, WI).

### Experimental protocol

Wild-type and LBP knockout mice were randomly allocated to two experimental groups and were fed either an ethanol-containing or an isocaloric high-fat control diet. Animals received diets by infusion through an intragastric cannula for 4 wk as described previously (13). The liquid diet was fed continuously at the rate of 9–11 ml/day to achieve weight gain. Behavior was assessed using a 0–3 scoring system (0, normal; 1, sluggish movement; 2, loss of movement but still moving if stimulated; 3, loss of consciousness). Based on this score, alcohol administration was then adjusted carefully to prevent overdosing. Ethanol initially was delivered at 18 g/kg per day and was increased 1.5 g/kg per 2 days until the end of the second week and then 0.8 g/kg per 4 days until the end of the experiment. LBP knockout and wild-type mice were sacrificed after 4 wk and blood samples were collected via the inferior vena cava at necropsy. To avoid variation in parameters associated with peak and trough levels of alcohol in the enteral model (e.g., see Ref. 21), behavioral assessment of ethanol intoxication was used at sacrifice to avoid ethanol levels that are low or extremely high. Serum was stored at  $-80^{\circ}\text{C}$  until alanine aminotransferase (ALT) was analyzed by standard enzymatic procedures (22). Livers were removed and weighed and tissue samples were divided; some were fixed in Formalin, others were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### Urine collection and assay for ethanol

Concentrations of ethanol in urine are representative of blood alcohol levels (23). Mice were housed in metabolic cages that separated urine from feces and urine samples were collected over 24 h for each mouse. Mineral oil was used to prevent evaporation. Ethanol levels in urine were determined daily by measuring absorbance at 366 nm resulting from the reduction of nicotinamide adenine dinucleotide by alcohol dehydrogenase (22). The average urine alcohol concentration value from each individual animal over the course of the study was determined and these results were pooled to determine group means.

### Endotoxin assay

Blood collection and measurement of plasma endotoxin are described elsewhere (24). Blood samples collected from the portal vein were handled under pyrogen-free conditions and centrifuged at 1200 rpm for 10 min. Plasma was stored at  $-80^{\circ}\text{C}$  until measurement. Samples were diluted and heated before assay to minimize the effects of plasma inhibitors of endotoxin (25). Endotoxin was measured with a *Limulus* amoebocyte lysate test kit (Kinetic-QCL; BioWhittaker, Walkersville, MD) (26).

### Pathological evaluation

Formalin-fixed liver samples were embedded in paraffin and stained with H&E to assess steatosis, inflammation, and necrosis. Liver pathology was scored as described by Nanji et al. (27) as follows: steatosis (the percentage of liver cells containing fat):  $<25\% = 1+$ ;  $<50\% = 2+$ ;  $<75\% = 3+$ ;  $>75\% = 4+$ ; inflammation and necrosis: 1 focus per low-power field =  $1+$ ; 2 or more =  $2+$ . One point was given for each grade of severity of histological abnormality and a total score was calculated for each liver.

### RNase protection assay

Total RNA was isolated from hepatic tissue using RNA STAT 60 (Tel-Test, Friendswood, TX). RNase protection assays were performed using the RiboQuant multiprobe assay system (BD Pharmingen, San Diego, CA). Briefly,  $^{32}\text{P}$ -labeled RNA probes were transcribed with T7 polymerase us-

ing the multiprobe template set mCK-3. RNA ( $10\ \mu\text{g}$ ) was hybridized with  $4 \times 10^5$  cpm of probe overnight at  $56^{\circ}\text{C}$ . Samples were then digested with RNase followed by proteinase K treatment, phenol:chloroform extraction, and ethanol precipitation and resolved on 5% acrylamide-bisacrylamide (19:1) urea gels. After drying, gels were visualized by autoradiography.

### Western blotting for cytochrome P450 2E1

Mouse livers were immediately excised after sacrifice, placed into liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Tissue was subsequently homogenized in three volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM potassium chloride, 1 mM EDTA, and 0.25 M sucrose with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY), centrifuged at  $10,000 \times g$  for 30 min, and then  $9\ \mu\text{g}$  supernatant was immunoblotted after separation of proteins on a 9% polyacrylamide gel. Electrophoretic transfer of protein to nitrocellulose sheets was performed as previously described (28). Nitrocellulose sheets were blocked overnight in 3% (w/v) nonfat dry milk in TBS (10 mM Tris-HCl (pH 7.4) with 150 mM NaCl) at  $4^{\circ}\text{C}$ . Cytochrome P450 (CYP) 2E1 was detected using sheep anti-rabbit CYP2E1 Ab (1/5000) (OXIS International, Portland, OR), with rabbit anti-sheep HRP (1/5000), using ECL detection. Staining intensity of the complex was determined with a Bio-Rad GS-363 molecular imager (Bio-Rad, Richmond, CA) with a CH-imaging screen and Bio-Rad Molecular Analyst software.

### Statistics

Two-way ANOVA with Bonferroni's post hoc test was used for the determination of statistical significance. For comparison of pathological scores, the Mann-Whitney *U* rank sum test was used. Data are presented as mean  $\pm$  SEM. A  $p < 0.05$  was selected before the study as the level of significance.

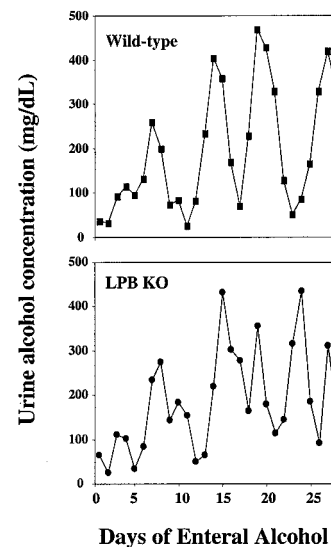
## Results

### Body weight gain

All animals survived surgery, and liquid diets were initiated after 1 wk to allow for complete recovery. Steady weight gains were obtained during 4 wk of continuous enteral feeding of liquid diets with or without ethanol, indicating adequate nutrition. There were no significant differences in weight gains among the groups studied.

### Urine alcohol concentration

As reported in previous studies in rats (29) and mice (13), urine alcohol levels fluctuated in a cyclic pattern from 0 to 500 mg/dl during enteral ethanol feeding (Fig. 1). This phenomenon was re-



**FIGURE 1.** Cycling of urine alcohol concentrations in mice. Animals were fed ethanol-containing diet as described in *Materials and Methods*. Cycling of urine alcohol in representative wild-type (A) and LBP knockout (B) mice are shown.

cently shown to be caused by hormones from the hypothalamic-pituitary-thyroid axis (30). Similar patterns were observed in wild-type and LBP knockout mice (Fig. 1). Mean urine alcohol concentrations over 4 wk were  $203 \pm 13$  mg/dl in wild-type mice and  $201 \pm 12$  mg/dl in LBP knockout mice; these values were not significantly different.

#### Liver:body weight ratios

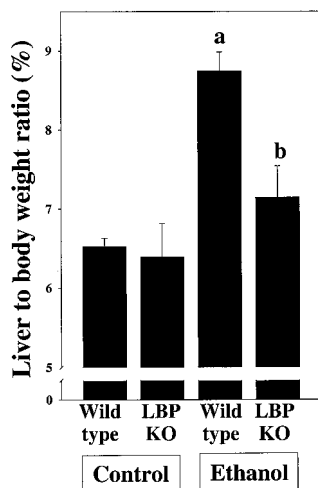
Four weeks of enteral ethanol treatment significantly increased liver:body weight ratios in wild-type mice fed ethanol ( $8.7 \pm 0.2\%$ ) over wild-type mice fed control diet ( $6.5 \pm 0.1\%$ ) (Fig. 2). This increase was blunted significantly in LBP knockout mice fed ethanol ( $7.1 \pm 0.4\%$ ). Thus, ethanol caused greater enlargement of livers in wild-type than in LBP knockout mice.

#### Serum transaminases

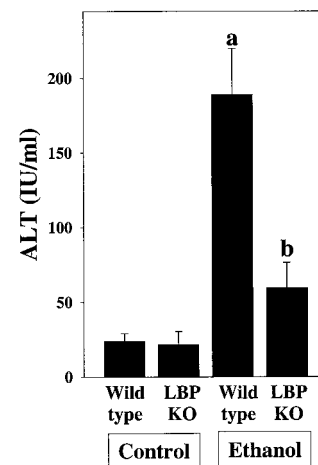
Serum ALT levels were around 25 U/L after 4 wk of high-fat control diet (Fig. 3). Four weeks of enteral ethanol treatment significantly increased serum ALT about 8-fold ( $189 \pm 31$  U/L) in wild-type mice. This increase was blunted significantly in LBP knockout mice fed ethanol ( $60 \pm 17$  U/L).

#### Pathological evaluation

Figure 4 shows representative photomicrographs of livers from wild-type and LBP knockout mice after 4 wk of enteral feeding with control or ethanol-containing diets. There were no pathological changes seen in control groups (Figs. 4, A and B and 5). However, moderate fatty accumulation and mild inflammation and necrosis were observed in wild-type mice fed ethanol (Fig. 4C), resulting in a total pathology score of  $5.9 \pm 0.5$  (Fig. 5). This value was significantly higher than in wild-type mice fed control diet. Steatosis in wild-type mice fed ethanol was observed mainly in pericentral to midzonal regions with a typical pattern of massive large droplets of fat. Furthermore, infiltrating inflammatory cells (Fig. 4E) and focal necrosis (Fig. 4F) were detected in wild-type mice fed ethanol. In contrast, these pathological changes were decreased significantly in livers from LBP knockout mice (Fig. 4, D, G, and H). The total pathology score of livers from LBP knockout



**FIGURE 2.** Effect of dietary ethanol on liver:body weight ratios in mice. Body and liver weights were determined at necropsy after 4 wk. Data represent means  $\pm$  SEM. High-fat control,  $n = 4$ ; high-fat ethanol,  $n = 6$ . a,  $p < 0.05$  compared with mice fed high-fat control diet; b,  $p < 0.05$  compared with wild-type mice fed ethanol by two-way ANOVA with Bonferroni's post hoc test. KO, knockout mice.



**FIGURE 3.** Effect of dietary ethanol on serum ALT levels. Blood samples were collected after 4 wk of high-fat diet with or without ethanol and ALT was determined as described in *Materials and Methods*. Data represent means  $\pm$  SEM. High-fat control,  $n = 4$ ; high-fat ethanol,  $n = 6$ , a,  $p < 0.05$  compared with mice fed high-fat control diet; b,  $p < 0.05$  compared with wild-type mice fed ethanol by two-way ANOVA with Bonferroni's post hoc test. KO, knockout mice.

mice fed ethanol was  $2.6 \pm 0.5$ , a value that was significantly lower than that of wild-type mice fed ethanol (Fig. 5).

#### Blood endotoxin

Four weeks of enteral ethanol treatment increased plasma endotoxin levels in the portal vein significantly (Fig. 6). However, there were no significant differences in portal endotoxin levels between wild-type ( $137 \pm 32$  pg/ml) and LBP knockout mice fed ethanol ( $124 \pm 46$  pg/ml).

#### Inflammatory cytokine mRNA expression in the liver

In wild-type mice fed ethanol for 4 wk, TNF- $\alpha$  mRNA expression was increased over high-fat controls (Fig. 7). This increase was blunted by 75% in LBP knockout mice fed ethanol for 4 wk. IL-6 was elevated as well in wild-type mice fed ethanol, but not in LBP knockout mice (Fig. 7). TGF- $\beta_1$  mRNA expression was similar in the groups studied.

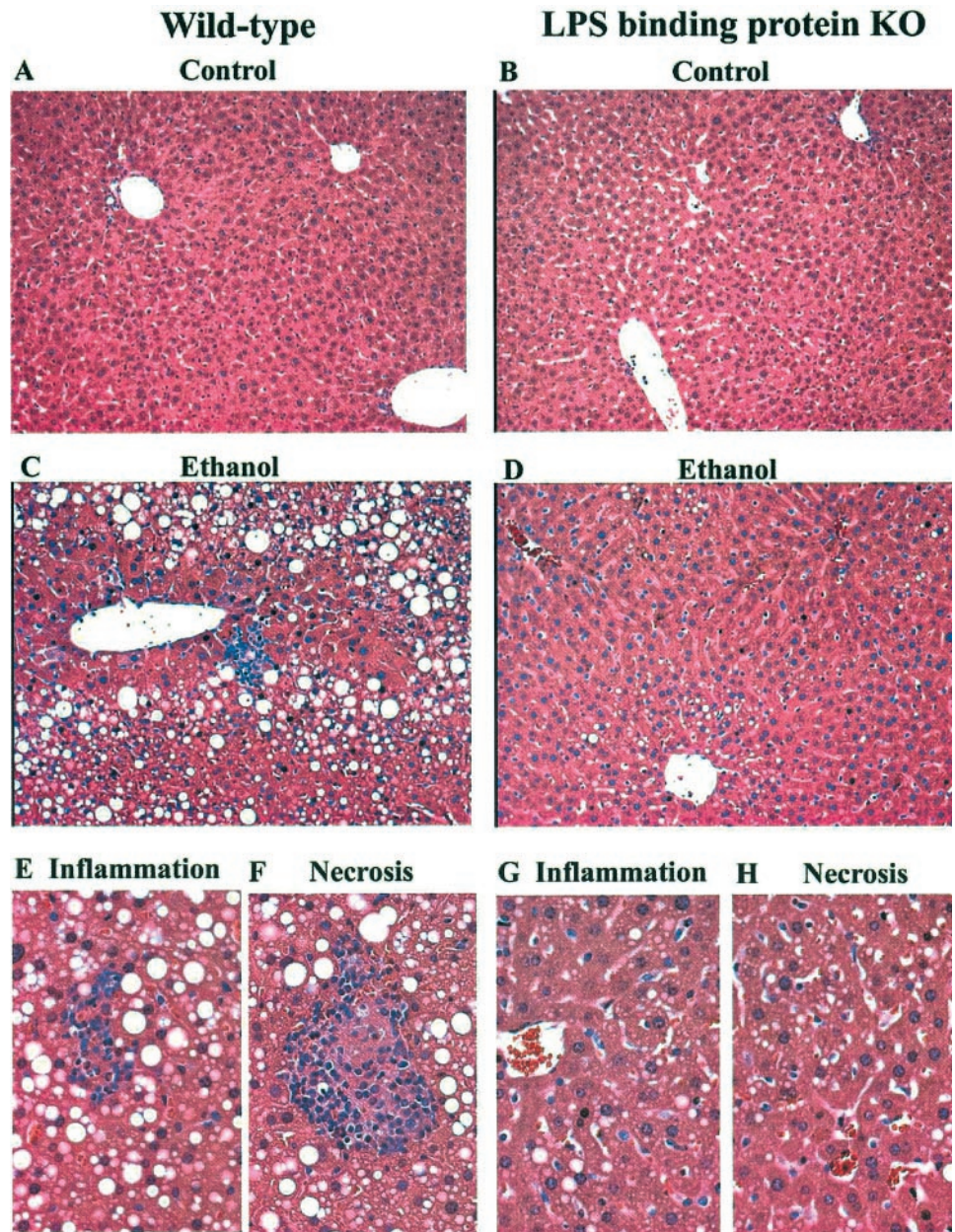
#### CYP2E1 expression in the liver

As reported in a previous study (31), CYP2E1 is increased in the liver by ethanol. Here, chronic alcohol administration increased expression of CYP2E1 equally in wild-type and LBP knockout mice (Fig. 8).

## Discussion

### Role of endotoxin in alcohol-induced liver injury

Numerous studies have shown that LPS from Gram-negative bacteria elicits a wide variety of host defense responses that lead to severe tissue injury, including liver injury in many models (1, 2). Furthermore, there are strong data supporting the hypothesis that LPS is involved in alcoholic liver injury (10–12). Excessive alcohol intake increases intestinal Gram-negative bacteria and gut permeability of normally nonabsorbed substances in both humans and animal models, resulting in increases of endotoxin in the portal vein (10, 11, 32). Moreover, reduction of Gram-negative bacteria prevents alcohol-induced liver injury (12, 15). The development of a mouse enteral alcohol model made it possible to study the effect of altering specific genes (e.g., knockout mice), such as the mice



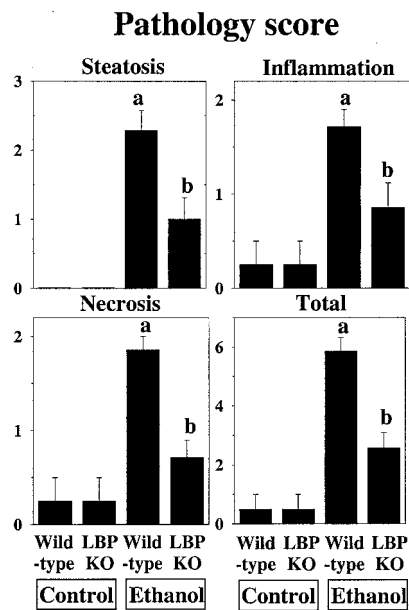
**FIGURE 4.** Representative photomicrographs of livers from wild-type and LBP knockout mice. Mice were treated as described in *Materials and Methods*. Livers from mice given high-fat control diet (A, wild-type, and B, LBP knockout (KO)), or high-fat ethanol-containing diet (C, wild-type, and D, LBP knockout) are shown. Original magnification,  $\times 100$ . With higher magnification ( $\times 200$ ), inflammation (E) and focal necrosis (F) are seen in livers from wild-type mice fed ethanol. No inflammation (G) or necrosis (H) is observed in LBP knockout mice fed ethanol. Typical photomicrographs.

studied here. It was reported very recently that alcohol-induced liver injury is blunted in TLR4-deficient and CD14 knockout mice (16, 17). Taken together, these data support the hypothesis that LPS plays an important role in alcohol-induced liver injury.

In the present study, 4 wk of enteral ethanol treatment increased plasma endotoxin levels in the portal vein significantly (Fig. 6). However, the levels to which portal LPS is increased are relatively low ( $<150$  pg/ml) after alcohol administration. It has been shown that liver damage due to low-level LPS is exacerbated by both acute and chronic alcohol administration (e.g., Refs. 14, 33, and 34), suggesting that ethanol sensitizes the liver to a LPS challenge. Indeed, Kupffer cells isolated from ethanol-treated animals have a more robust response to LPS *in vitro* than cells from control-treated animals (e.g., Ref. (14)). Taken together, these results suggest that ethanol causes sensitization to low, normally nontoxic, levels of LPS by priming the immune response in inflammatory cells (e.g., Kupffer cells). Importantly, the level of LBP in rodents is increased by chronic enteral alcohol (35), which may also play a role in increased sensitivity to LPS.

#### *LBP is critical in early alcohol-induced liver injury in mice*

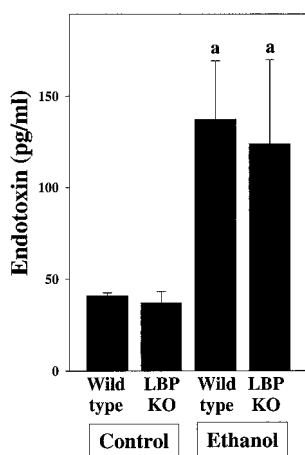
LBP is a critical component of innate immunity against bacterial infection. It serves to alert the host to the presence of minute amounts of toxins, such as LPS (7). Upon exposure to larger quantities of LPS, however, the amplification of LPS effects mediated by LBP may be detrimental to the host. For example, LBP in plasma enhances the LPS responsiveness of cells to produce proinflammatory cytokines *in vivo* and *in vitro* (8, 36). Indeed, LBP contributes to lethal effects of LPS toxicity and depletion of LBP with Abs that protect animals from lethal endotoxemia (4, 37). However, *in vivo* experiments performed in mice with a disruption of the *LBP* gene (i.e., LBP knockout mice) have yielded conflicting results. For example, one study showed that LBP knockout mice were resistant to endotoxemia (38), whereas another study did not (36). One possibility to explain this discrepancy is the dose of LPS administered. Indeed, LBP knockout mice were indeed protected when lower doses of LPS were used in the latter study (36). These results suggest that LBP may play an important role at low levels



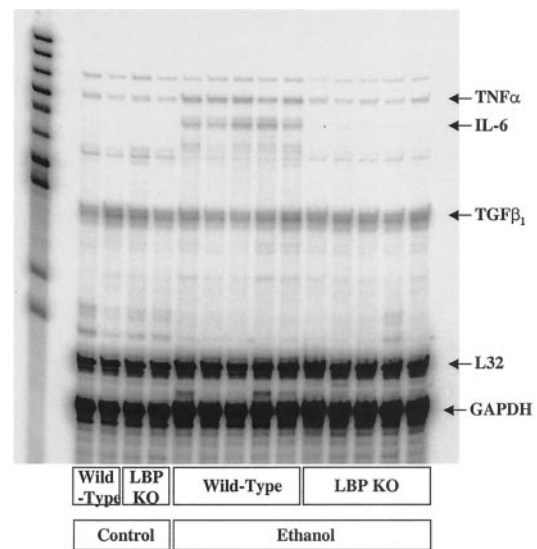
**FIGURE 5.** Hepatic pathology in wild-type and LBP knockout mice. Pathology was scored as described in *Materials and Methods*. Data represent means  $\pm$  SEM. High-fat control,  $n = 4$ ; high-fat ethanol,  $n = 6$ . a,  $p < 0.05$  compared with mice fed high-fat control diet; b,  $p < 0.05$  compared with wild-type mice fed ethanol using the Mann-Whitney  $U$  rank sum test. KO, knockout mice.

of LPS, but this protective effect is overridden during exposure to higher doses, which is consistent with *in vitro* findings (7).

In this study, alcohol increased portal endotoxin levels in both wild-type and LBP knockout mice to a similar extent. However, liver damage and increases in inflammatory cytokine expression were blunted in LBP knockout mice (Figs. 3–7). These results support the hypothesis that LBP plays a major role in the development of early alcohol-induced liver injury by enhancing the cellular response to the low levels of LPS caused by an increase in gut permeability due to ethanol (11). However, LBP is also known to bind to other small toxins, such as lipoteichoic acid from Gram-



**FIGURE 6.** Endotoxin levels in portal plasma. Plasma endotoxin levels in wild-type and LBP knockout mice (KO) given high-fat control or ethanol-containing diet were determined in the portal blood by the *limulus* amoebocyte lysate assay as described in *Materials and Methods*. Data are presented as mean  $\pm$  SEM. High-fat control,  $n = 4$ ; high-fat ethanol,  $n = 6$ . a,  $p < 0.05$  compared with mice fed high-fat control diet using two-way ANOVA with Bonferroni's post hoc test.

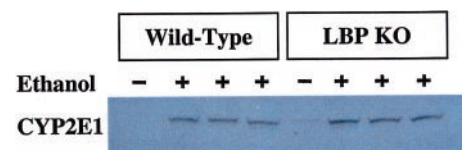


**FIGURE 7.** TNF- $\alpha$  mRNA in livers from wild-type and LBP knockout mice. Total mRNA was prepared from livers of wild-type or LBP knockout mice (KO) given high-fat control or ethanol-containing diet for 4 wk. RNase protection assays were performed to determine TNF- $\alpha$ , IL-6, and TGF- $\beta$ , mRNA with L32 and GAPDH as housekeeping genes as described in *Materials and Methods*. Typical experiment.

positive bacteria (39). The crossing of small non-LPS toxins from the gut could also be increased by alcohol; thus, knocking out of LBP would likely also be protective against liver damage due to these agents.

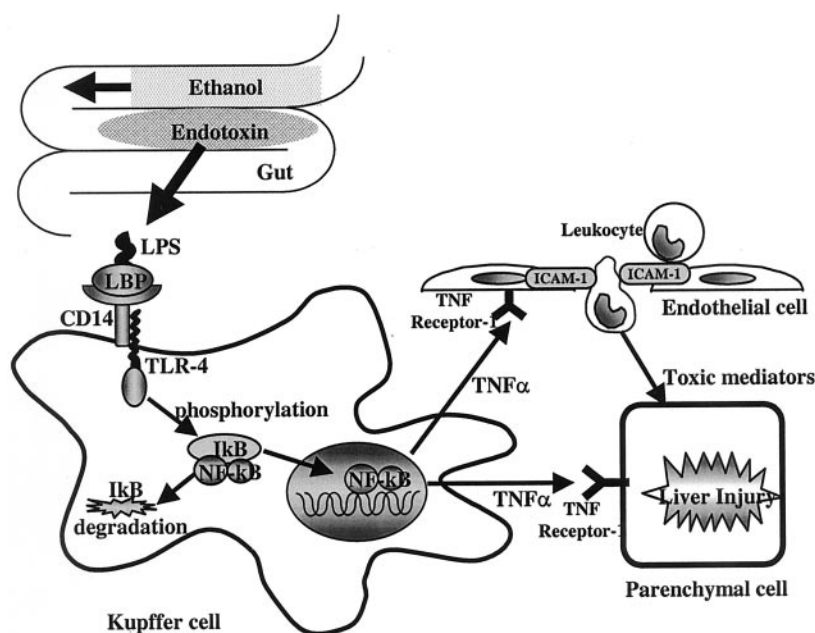
*Role of LBP in alcohol-induced inflammatory response*

Recent studies support the hypothesis that TNF- $\alpha$  from Kupffer cells plays an important role in early alcohol-induced liver injury (40). Indeed, alcohol-induced liver injury present in wild-type mice fed enteral ethanol chronically was nearly totally absent in TNFR1 knockout mice (13). In addition to direct toxic effects on hepatocytes, TNF- $\alpha$  can indirectly damage liver by activating endothelial cells and leukocytes to synthesize chemokines and adhesion molecules, which recruit leukocytes leading to inflammation in the liver (41). In the present study, dietary alcohol significantly increased expression of TNF- $\alpha$  mRNA in wild-type mice compared to high-fat controls, and these effects were blunted significantly in LBP knockout mice (Fig. 7). Under these conditions, liver injury was also prevented in LBP knockout mice despite similar levels of LPS in the portal blood (Figs. 4–7). These data suggest that LBP plays a major role in early alcohol-induced liver injury by mediating stimulation of inflammation caused by inflammatory cytokine production (e.g., TNF- $\alpha$ ). The finding that CYP2E1 induction after alcohol is similar in both wild-type and LBP knockout mice is interesting; these data suggest that the protective effect observed in LBP knockout mice is independent of



**FIGURE 8.** CYP2E1 in livers from wild-type and LBP knockout mice (KO). Proteins were prepared from livers of wild-type or LBP knockout mice given high-fat control or ethanol-containing diet for 4 wk. CYP2E1 protein was determined with anti-CYP2E1 Ab by Western blot as described in *Materials and Methods*. Typical experiment.

**FIGURE 9.** Working hypothesis. Chronic alcohol administration increases gut-derived endotoxin in the portal blood. LBP binds to LPS and facilitates interaction with membrane CD14 present on the surface of Kupffer cells (46). The binding of the LPS-LBP complex to CD14 mediates signal transduction, including NF- $\kappa$ B activation via TLR4 (8). This leads to production of toxic cytokines, including TNF- $\alpha$ . TNF- $\alpha$  stimulates endothelial cells and leukocytes to synthesize adhesion molecules (e.g., ICAM-1) that promote infiltration of leukocytes and contribute to inflammation and hepatocyte injury. Early alcohol-induced liver injury in this study was largely reduced in LBP knockout mice. Thus, it is concluded that LBP plays an important role in early alcohol-induced liver injury by mediating LPS-induced signal transduction.



CYP2E1 induction or that hepatotoxicity due to CYP2E1 is dependent on signals downstream from LBP. For example, cell death to HepG2 cells by TNF- $\alpha$  is enhanced by overexpressing CYP2E1 in the presence of ethanol (42).

Hepatic steatosis is one of the most earliest pathological changes found in humans consuming alcohol. In this study, hepatic steatosis was blunted significantly in LBP knockout mice fed ethanol (Figs. 4 and 5). LBP may be mediating fatty accumulation in liver both directly and indirectly. It is known that TNF- $\alpha$  stimulates lipid synthesis in the liver (43) and causes peripheral lipolysis that increases circulating levels of free fatty acids (44). Since the increase in TNF- $\alpha$  caused by ethanol was blunted in LBP knockouts (Fig. 7), LBP may be indirectly blocking this pathway. Furthermore, a role of LBP in lipid transport independent of LPS has been shown (45). Therefore, knocking out LBP may also directly decrease the delivery of lipid from the gut to the liver and thereby also prevent fat accumulation.

Figure 9 depicts a working hypothesis. First, alcohol increases the levels of circulating endotoxin in the portal blood (Fig. 6) (10). LBP binds to LPS and facilitates interaction with membrane CD14 present on the surface of Kupffer cells (3–5). The binding of LPS/LBP to CD14 mediates signal transduction, including NF- $\kappa$ B activation via TLR4 (8). Kupffer cell activation leads to production of toxic cytokines including TNF- $\alpha$  (Fig. 7). TNF- $\alpha$  can indirectly damage liver by increasing expression of ICAM-1 on endothelial cells, as well as increase production of chemoattractant molecules for inflammatory cells (46).

In conclusion, knocking out LBP protected against alcohol-induced liver injury in mice, most likely by blocking the effect of the increase in portal endotoxin from the gut. Therefore, pharmacological manipulation and targeting of the LPS-LBP complex could prove useful in this disease.

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