Lipopolysaccharide binding protein inhibitory peptide protects against acetaminophen-induced hepatotoxicity

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Su GL, Hoesel LM, Bayliss J, Hemmila MR, Wang SC. Lipopolysaccharide binding protein inhibitory peptide protects against acetaminophen-induced hepatotoxicity. Am J Physiol Gastrointest Liver Physiol 299: G1319–G1325, 2010. First published September 16, 2010; doi:10.1152/ajpgi.00140.2010.—Acetaminophen (APAP)-induced liver injury remains the main cause of acute liver failure in the United States. Our previous work demonstrated that LPS binding protein (LBP) knockout mice are protected from APAP-induced hepatotoxicity. LBP is known to bind avidly to LPS, facilitating cellular activation. In this study, we sought to specifically inhibit the interaction between LBP and LPS to define the role of this interaction in APAP-induced liver injury. The peptide LBPK95A was able to inhibit LBP-mediated LPS activation of RAW 267.4 cells in a dose-dependent manner in vitro. In vivo, C57Bl/6 mice were treated with either LBPK95A or vehicle control concurrently with the administration of APAP (350 mg/kg). Mice treated with LBPK95A had significantly lower serum aspartate aminotransferase and alanine aminotransferase levels. Morphometric analysis of the liver tissue showed significantly less liver injury in mice treated with LBPK95A. To assess whether the LBPK95A altered glutathione depletion and APAP metabolism, we measured total glutathione levels in the liver after APAP. We found no difference in the glutathione levels and APAP-adduct formation between LBPK95A vs. vehicle control both at baseline and after APAP. In conclusion, our results support the hypothesis that LBP modulates APAP-induced liver injury through its ability to mediate activation by endogenous LPS. Our results suggest that blocking LBP-LPS interactions is a potential therapeutic avenue for the treatment of APAP-induced liver injury.

We have previously shown that lipopolysaccharide binding protein-deficient mice (LBP KO) are protected from APAP-induced hepatotoxicity and death (24). In this earlier study, we found that LBP KO mice had less liver injury and necrosis after a toxic dose of APAP compared with wild-type mice. LBP is an acute phase protein found in serum that is produced predominantly by hepatocytes (2, 17, 29, 32). The best known activity of LBP is its ability to augment responses to low and, thus, physiological levels of LPS. LBP binds with a high degree of specificity and affinity ($K_d \approx 10^{-9}$) to the lipid A portion of bacterial LPS (28) and accelerates binding to cellular LPS receptors such as CD14 (3) and TLR4/MD2, augmenting inflammatory cytokine production. Whether the effects of LBP on APAP-induced liver injury occur through its actions on LPS is not known. Prior studies on the role of LPS on APAP-induced liver injury have shown that pretreatment with LPS can attenuate liver injury from APAP (10). These studies utilized large exogenous doses of LPS, which can have an effect on altering cytochrome P-450 concentrations and thus alter APAP metabolism (12). Recent studies have pointed to a direct role for the innate immune system but the role of low concentrations endogenous LPS in APAP-induced liver injury remains unknown (14). In this study, we examined the hypothesis that LBP modulates APAP-induced liver injury through its effects on endogenous LPS. We sought to block binding of LPS to LBP by utilizing a synthetic peptide, LBPK95A, that mimics the lipid A binding site (1). We found that that LBPK95A was able to block cytokine production by LPS in vitro and decrease liver injury following APAP.

MATERIALS AND METHODS

Reagents. LPS from Escherichia coli (0111:B4) was obtained from Millipore (Billerica, MA). The LBP inhibitory peptide, LBPK95A, was synthesized by the University of Michigan Protein Structure Facility according to the instructions provided by Arana et al. (1). The peptide sequence RVQGRWKVRASSFK is identical to that reported from human LBP from amino acid 86–99 with the exception of a substitution of lysine 95 with alanine. The mouse LBP sequence is highly homologous to the human LBP and, as demonstrated in this study, the peptide is active against mouse LBP in vivo (1).

RAW cell assay. $5 \times 10^4$ RAW 264.7 cells (ATCC, Manassas, VA) were plated in 96-well opaque sterile tissue culture plates overnight so that they reached 75–90% confluence. They were then washed with serum-free medium (SFM) $\times 3$ prior to experimentation. Cells were stimulated with LPS in the presence and absence of serum with and without the addition of LBPK95A for 6 h prior to harvesting the supernatant. RAW cell activation was measured by the cumulative production of TNF-α. Mouse TNF-α levels were measured with a commercial kit from RD Systems (Minneapolis, MN) per manufacturer’s instructions. All conditions were plated in triplicate and the experiment was repeated at least twice. Serum was obtained from normal untreated C57Bl/6 mice from Harlan Laboratories (Oxford,
mi) or LBP KO mice. The mouse serum utilized was serum pooled from three or four mice. The average concentration of serum LBP in the wild-type mice was ~4 μg/ml. There was no detectable serum LBP in the LBP KO mice. LBP KO mice were a generous gift from Doug Golenbock (34). These animals had been backcrossed into the background C57Bl/6 strain at least 12 times before we acquired our colony. They were subsequently housed in a specific pathogen-free environment and allowed to breed.

**Animal model.** Age-matched 12- to 16-wk-old male C57Bl/6 mice (Harlan Laboratories, Oxford, MI) were used for all our experiments. Experiments were performed in accordance with National Institute of Health guidelines and prior approval was obtained from the University of Michigan Animal Care and Use Committee. After a 16-h fast, the mice were injected intraperitoneally with APAP (350 mg/kg; Sigma, St. Louis, MO) dissolved in sterile saline or sterile saline alone as the vehicle control. In addition to the APAP, mice were given in a separate intraperitoneal injection either LBPK95A (50 μg/500 μl) or vehicle control (same volume of saline). Depending on the experiment, the peptide or vehicle control was given either concurrently (time 0) or after the APAP. After injection with APAP, mice were fed ad libitum with standard chow. We have previously shown that the amount of feeding and activity was inversely related to the level of liver injury and illness.

**Serum aminotransferase measurements.** Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured at 37°C with a commercially available kit (Teco Diagnostics, Anaheim, CA).

**Histology.** Liver tissue was formalin fixed and paraffin embedded prior to sectioning. Liver sections were stained with hematoxylin and eosin. Quantitative analysis of the extent of tissue necrosis was performed as previously described (24). After digitally imaging three high-power fields per slide in a random and blinded fashion, areas of tissue necrosis or impending necrosis were identified by the presence of decreased eosinophilia, loss of cell architecture, vacuolization, cell disruption, and/or karyolysis. Areas of necrosis that had all the features, as well as areas of impending necrosis that had most of the features without karyolysis, were included in the quantitation of necrosis. The area of necrosis was highlighted and calculated by use of NIH Image 1.61 Software (National Institute of Health, Bethesda, MD).

**Measurement of GSH.** Total glutathione (GSH) was measured by using a commercially available kit from Oxford Biochemical Research (Oxford, MI) per the instructions from the manufacturer.

**Immunoblots of APAP adducts.** For detection of APAP protein adducts, immunoblots were performed with a rabbit anti-serum generously provided by Dr. Jack A. Hinson as previously described (15).

**Real-time RT-PCR.** Total RNA was isolated with the TRIzol reagent per manufacturer’s instructions (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was carried out using the IQ Sybr Green Supermix and the iCycler (Bio-Rad Laboratories, Hercules, CA). The following primers were utilized: 1FN-β 5′-AGCTCACAAGAAAGGAGCAGAACAT, 3′-GCCCTGTAGGTGAGGTTGATCT; interferon regulatory factor-7 (IRF-7) 5′-ACAGGGCGTTTTATCTTGCG, 3′-TCCAAGCTCCCGGCTAAGT; TNF-α 5′-GGGACAGTGACCTGGACTGT, 3′-CCATCCAATCGGTAGTAGCG. The comparative threshold cycle (Ct) or ΔCt method was used to determine the relative concentration of mRNA transcript between wild-type and SFM samples. The results are then expressed as “fold changes” relative to a calibrator. In all samples, the calibrator used was the concentration of RNA in the wild-type control groups at 2 h after APAP. The validity of the semiquantitative method was confirmed by a consistent log linear correlation of r2 > 0.95 between starting template RNA concentration and threshold cycle for all genes studied. All values are expressed as a ratio of target the ampiclon to the housekeeping gene (18S).

**Statistical analysis.** Analysis was performed using Statview software (SAS Institute, Cary, NC). Unless indicated otherwise, data are expressed as means ± SE. Analysis was performed by Student’s t-test and ANOVA with Fisher’s protected least significant difference post hoc analysis when more than two variables were compared. Statistical significance was assigned at P values <0.05.

**RESULTS**

**LBP peptide inhibits LPS activation of RAW 267.4 cells.** To assess the biological activity of our synthesized LBPK95A, we examined its ability to block LBP activity in vitro. Mouse macrophage cells (RAW 264.7 cell line) were stimulated with low concentrations of LPS (1 ng/ml) in the presence or absence of LBP from either LBPK95A or wild-type C57Bl/6 mice (Fig. 1). As expected (21), at low concentrations of LPS, little TNF-α was expressed in the absence of serum (SFM). Addition of 1% serum from wild-type mice, however, significantly increased TNF-α production whereas addition of 1% serum from LBPK95A wild-type mice did not (P < 0.0001). This is consistent with the previously described biological activity for LBP (34). The addition of LBPK95A to the wild-type serum groups inhibited TNF-α production in a dose-dependent manner (P < 0.0001). The addition of 10 μg/ml of LBPK95A reduced TNF-α compared with wild-type serum alone. Addition of higher doses of LBPK95A (40 μg/ml and 80 μg/ml) to wild-type serum reduced TNF-α to levels comparable to wild-type serum or SFM.

**APAP-induced liver injury is reduced in the presence of LBPK95A.** We examined the effect of LBPK95A administration on APAP-induced liver injury. LBP wild-type mice were given a single dose of APAP (350 mg/kg ip). The mice were then immediately administered either LBPK95A or an equal volume of the vehicle control (saline). At 6 h after treatment, the mice that received LBPK95A had significantly lower serum AST and ALT levels (Fig. 2) than the mice that received TGF-α production at 6 h by RAW cells 264.7 after stimulation with LPS (1 ng/ml) in the presence of either serum-free medium (SFM), 1% serum from C57Bl/6 mice (wild-type), 1% serum from LBPK-deficient mice (LBP KO), 1% serum from C57Bl/6 mice, and 10 μg/ml of the LBPK95A (wild-type + 10 μg/ml PEP), 1% serum from C57Bl/6 mice and 40 μg/ml LBPK95A (wild-type + 40 μg/ml PEP), 1% serum from C57Bl/6 mice and 80 μg/ml LBPK95A (wild-type + 80 μg/ml PEP). *P < 0.0001 compared with wild-type. #P < 0.05 compared with SFM.
vehicle controls. An additional control that was performed with heat-inactivated peptide (60°C for 30 min) administered concurrently with APAP showed that the inactivated peptide did not reduce liver injury (AST: 6,020 ± 1,779 IU/l; ALT: 5,433 ± 1,433 IU/l, n = 4 per group). Mice given only saline without any APAP had no liver injury whereas mice given peptide alone without any APAP only had minimal liver injury (Fig. 2).

Histological examination of the liver tissue from mice given LBPK95A, heat-inactivated LBPK95A, or vehicle control all showed evidence of centrilobular necrosis consistent with APAP-induced liver toxicity, but the area of necrosis and/or impending necrosis was significantly less in the mice given LBPK95A (Fig. 3). Quantitative analysis of the area of injury confirmed significantly less injury in mice given LBPK95A (Fig. 4).

Treatment with LBPK95A does not affect glutathione depletion after APAP. To assess a potential role for LBPK95A in modulating APAP metabolism and glutathione depletion, we examined the amount of total glutathione (GSH) in the liver after APAP at 2 h. Baseline GSH levels were similar in mice given either the vehicle control or LBPK95A. There was a significant (*P < 0.05) decrease in GSH levels of >95% by 2 h after APAP exposure, which is consistent with prior reports (Fig. 5). The early glutathione depletion results from the formation of NAPQI. Our results show that mice given either vehicle control vs. LBPK95A had the same decline in hepatic GSH levels, suggesting that the initial metabolism of APAP is similar despite treatment with LBPK95A.

Formation of APAP adducts is not different after administration of LBPK95A. To assess whether the reduction in liver injury that occurs after peptide delivery was due to differences in APAP bioactivation and protein adduct formation, immunoblots were performed on liver homogenates by using an anti-APAP antibody. C57Bl/6 mice were given APAP (350 mg/kg...
in the presence of either the LBPK95A or vehicle control saline and euthanized after 2 h (Fig. 6). A control mouse that did not receive any APAP did not develop any APAP adducts, as expected. In contrast, a wide array of protein adducts with different molecular weights was found in mice after APAP in both the peptide and vehicle control group. The pattern of protein adduct formation was similar in both groups, suggesting that there is no difference in APAP bioactivation and protein adduct formation despite treatment with the LBPK95A. This supports our hypothesis that the effects of LBP peptide on APAP-induced hepatotoxicity are not directly related to APAP metabolism.

**Administration of LBPK95A attenuated the APAP-induced increases in intrahepatic cytokine expression.** In previous studies (24), we found that the absence of LBP led to decreases in intrahepatic cytokine expression after APAP. We therefore examined the effect of LBPK95A on TNF-α and IFN-β expression after APAP. We found that by 6 h after APAP there was a significant increase in TNF-α and IFN-β steady-state mRNA levels (Fig. 7, A and B). However, the administration of LBPK95A completely inhibited this rise in cytokine expression. By contrast, IRF-7 RNA levels were unaffected by APAP (Fig. 7C).

**Delayed administration of LBPK95A effectively modulated APAP-induced liver injury.** To address the possibility using LBK95A as a therapeutic agent in APAP-induced liver injury, we examined the kinetics of LBPK95A on APAP-induced liver injury. We found that LBPK95A was most effective in reducing liver injury if given concurrently with the APAP (at time 0) (Fig. 8). However, delayed administration (1 and 3 h after APAP) also had a salutary effect on liver injury although the effectiveness of the agent was much less following delayed administration (Fig. 8).

**DISCUSSION**

In this study, we found that LBPK95A attenuates liver injury after APAP administration as evidenced by reductions in serum transaminase levels and decreased centrilobular necrosis. This is consistent with our prior studies in which LBP KO mice were protected from APAP-induced liver injury (24). Our previous studies showed that there was an elevation in portal LPS levels after APAP administration but there was no difference between LBP KO and wild-type mice, suggesting that LBP does not affect bacterial translocation and portal endotoxemia. Rather, it can modulate innate immune responses to endogenous LPS. A rise in portal LPS levels has been shown in other models of liver injury and is thought to be related to a combination of increased bacterial translocation and poor Kupffer cell function (7, 11, 30). Similar to our findings in APAP-induced liver injury, Lehnert et al. (11) also found that...
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Figure 8. Serum AST (A) and ALT (B) levels 6 h after APAP (350 mg/kg) in the presence of either the vehicle control saline or LBPK95A given either concurrently (0hr), 1 h after (1hr), or 3 h after (3hr) APAP. P < 0.001 by ANOVA compared with control; n = 4–6 mice per group.

Fig. 8. Serum AST (A) and ALT (B) levels 6 h after APAP (350 mg/kg) in the presence of either the vehicle control saline or LBPK95A given either concurrently (0hr), 1 h after (1hr), or 3 h after (3hr) APAP. P < 0.001 by ANOVA compared with control; n = 4–6 mice per group.

portal LPS levels rose after resuscitation in hemorrhagic shock but the amount of LPS did not differ between wild-type and LBP KO mice. Nevertheless, less liver injury was noted in the absence of LBP.

To examine whether LBP modulates liver injury through its ability to bind LPS, we sought to specifically inhibit the binding of LBP to LPS. Previous studies have shown that the NH2 terminus of LBP contains the binding site for LPS whereas the COOH terminus is responsible for binding to cellular receptors such as CD14 (4, 26). Synthetic peptides directed at human LBP amino acid region 86–99 are capable of inhibiting LBP activity by interfering with LBP-LPS interactions (19, 19). In particular, the amino acids Arg 94 (R) and Lys95 (K) are critical amino acids for the LPS binding function of LBP (9). Synthetic peptides that substitute the lysine in amino acid 95 with alanine result in more potent inhibition of LPS binding to LBP than synthetic peptides containing the native peptide sequence in this region (18). Our studies confirm that LBPK95A has potent ability to block LPS activation of mouse macrophage cells in vitro. We also found the LBPK95A was a potent inhibitor of APAP-induced hepatocyte injury.

A role for LBP has been suggested in many different models of organ injury including liver transplantation (30), common bile duct ligation (CBDL) (16), alcoholic liver injury (23, 25, 31), and hemorrhagic shock (11). These models differ from the APAP-induced liver injury in two ways. First, LBP levels are increased in most models of liver injury but in APAP-induced liver injury LBP levels decrease (data not shown). This is presumably due to the fact that LBP is an acute-phase protein produced by hepatocytes and thus would increase after any stress such as CBDL or liver transplantation. In APAP-induced liver injury, however, there is significant hepatocytes necrosis, which could result in decreased capacity to produce LBP. This, however, does not preclude a role for LBP in modulating LPS responses since the amount of LBP required for LBP bioactivity can be as low as 1% of what is found in serum. We have demonstrated this in our RAW 264.7 assays and other authors have also demonstrate a similar findings as 1–10% of serum concentrations of LBP is what is usually for most in vitro studies on LBP bioactivity (20). Secondly, the role of LPS in APAP-induced liver injury not as clear as it is in other models such as carbon tetrachloride and alcoholic liver injury in which injury is easily mitigated by the addition of nonabsorbable antibiotics, colectomy, or germ-free conditions (22). Blocking of LPS-activated cytokines, particularly TNF-α, also decreased liver injury in these other liver injury models (27). Such findings have not been demonstrated for APAP since usage of TNF KO mice and TNF antibodies did not result in decreased liver injury (5).

Liu et al. (13) showed that depletion of NK/NKT cells with anti-NK1.1 antibodies resulted in decreased liver injury after APAP, which was associated with a reduction in intrahepatic cytokine production. Similarly, mice deficient in cytokines such as IFN-γ were also protected from APAP-induced liver injury (6). A potential role for LPS was also demonstrated by the importance of TLR4 in modulating liver injury after APAP and ethanol exposure (35). Our data support a large contributory role for LPS since blocking LPS binding to LBP with LBPK95A resulted in significantly less liver injury. Although LPS is known to alter APAP metabolism at high doses, we did not see any gross changes in glutathione depletion and covalent binding of proteins by APAP to support this. Our data show that the effects of LBP are due not to altered metabolism of APAP but to its ability to modulate innate immune responses to LPS.

In our study, we found specific changes in intrahepatic cytokine expression. We found a rise in TNF-α and IFN-β levels after APAP, which was decreased in mice administered LBPK95A. TNF-α and IFN-β can be produced after stimulation of the TLR4 pathway by LPS. The former occurs through a MyD88-dependent pathway whereas the latter occurs through a MyD88-independent pathway. There is evidence that LBP may be important in mediating LPS activation through both for these pathways (8). It is not clear why IRF-7 mRNA, a key transcriptional regulator of type I interferons, was not increased after APAP, but the results suggest that there are specific signaling pathways changes that occur after APAP-induced liver injury rather than global induction of all pathways as a result of severe injury. Our results suggest that LBPK95A can alter immune responses to APAP-induced liver injury and that the likely mechanism is interference with LBP-LPS binding.

In our initial studies, we administered LBPK95A in conjunction with APAP; this resulted in the greatest reduction in
liver injury. In clinical practice, however, LBPK95A can only be a potential therapeutic agent if it is still effective when given after the initial APAP exposure. We therefore examined the effect of delayed LBPK95A administration and found that there was still significant improvement in liver injury even when LBPK95A is given 3 h post-APAP. It is important to recognize that the time course for APAP metabolism and liver injury is greatly accelerated in mice compared with humans. In humans, liver injury typically does not start until 24 h after APAP overdose and does not peak until 2–3 days after ingestion. By contrast, mice have peak liver injury usually within 6 h of APAP administration (5). The substantial benefit observed after a 3-h treatment delay in mice suggests that the time window for treatment in humans would be very feasible in the clinical setting.

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REFERENCES
