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Role of Lipopolysaccharide (LPS), Interleukin-1, Interleukin-6, Tumor Necrosis Factor, and Dexamethasone in Regulation of LPS-Binding Protein Expression in Normal Hepatocytes and Hepatocytes from LPS-Treated Rats

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Lipopolysaccharide (LPS)-binding protein (LBP) has been reported to be an acute-phase protein. LBP binds to LPS with a high affinity; LPS-LBP complexes then interact with the receptor CD14, resulting in increased expression of LPS-inducible genes. Hepatocytes represent a major source of LBP, but little is known about the regulation of rodent hepatocyte LBP synthesis. In these studies, undertaken to characterize hepatocyte LBP expression, we show that greater-than-20-fold increases in LBP mRNA levels in hepatocytes occurred following injection of LPS or turpentine in rats. In primary cultures of rat hepatocytes, the addition of interleukin-6 (IL-6) and LPS led to 4.5- and 3.2-fold stimulation in LBP mRNA levels, respectively. The induction of LBP by IL-6 or LPS was attenuated by dexamethasone. In contrast to IL-6 and LPS, in the presence of 10^{-6} M dexamethasone, IL-1 and tumor necrosis factor (TNF) led to maximal LBP mRNA induction levels, 4.7- and 3.8-fold, respectively, suggesting that IL-6 and LPS stimulate LBP expression by mechanisms different from those of IL-1 and TNF. Similar induction levels of LBP mRNA were seen in rat H35 hepatoma cells for all four stimuli, and dexamethasone inhibited these responses. Dexamethasone alone increased the spontaneous induction in primary hepatocytes at early time points but suppressed induction at later time points. Furthermore, hepatocytes from rats treated with LPS in vivo exhibited a >10-fold increase in mRNA expression in response to LPS and enhanced responses to TNF and IL-1. As with the normal hepatocytes, dexamethasone inhibited the LPS-dependent induction in the LPS-treated rat hepatocytes. These data suggest that LBP synthesis by hepatocytes is under the control of LPS, IL-1, TNF, IL-6, and glucocorticoids and that the LPS treatment primes hepatocytes for subsequent responses to LPS, TNF, and IL-1 for LBP synthesis.

Lipopolysaccharide (LPS)-binding protein (LBP) is a part of the LPS recognition system of mammals first identified and purified in rabbits and humans (34–36). LBP is synthesized as a 50-kDa protein which is glycosylated and secreted as a 60-kDa product of hepatocytes as well as other cells outside the liver (29, 33). High homology between LBP and bactericidal permeability-increasing protein, cholesterol ester transport protein, and phospholipid transfer protein (PLTP) has been described (10, 11, 16). Both LBP and bactericidal permeability-increasing protein bind LPS with high affinity. LBP-LPS complexes are recognized by the cell surface receptor CD14, and the interactions of these complexes with CD14 results in the activation of macrophages for cytokine synthesis (15, 28, 41). Endothelial cells can be activated for adhesion molecule expression and cytokine synthesis by soluble CD14 following interaction of LBP-LPS complexes (18, 27).

Because levels of circulating LBP increase by up to 100-fold following administration of silver nitrate in rabbits, LBP has been called an acute-phase protein (35). We have reported previously that LBP mRNA levels in rat hepatocytes increase up to 20-fold following in vivo treatment with LPS, turpentine, or *Corynebacterium parvum*, supporting the acute-phase protein designation for LBP (14). Regulation of the synthesis of other acute-phase reactants has been shown to be under the control of cytokines (especially interleukin-6 [IL-6], IL-1, and tumor necrosis factor [TNF]) and glucocorticoids (2, 13, 20, 24). Human hepatocytes and HepG2 hepatoma cells were shown recently to respond to IL-6 by increased LBP expression (17). It is unknown whether LBP synthesis is under similar regulation in rodent hepatocytes. We postulate that because of the critical role of LBP in the recognition and clearance of circulating LPS and gram-negative bacteria, synthesis should be rapid and sensitive to low levels of stimuli. In these studies, we first characterized the time course for in vivo induction of LBP in hepatocytes following LPS or turpentine treatment in rats. We then established in vitro conditions for the study of the regulation of hepatocyte LBP mRNA expression as well as the cytokine, LPS, and glucocorticoid regulation of LBP expression in primary cultures of hepatocytes from normal rats, hepatocytes from rats pretreated with LPS in vivo, as well as the H35 rat hepatoma cell line.

MATERIALS AND METHODS

Reagents. LPS (*Escherichia coli* O111:B4) was purchased from Sigma Chemical Co. (St. Louis, Mo.), Williams medium E was purchased from Gibco (Grand Island, N.Y.), recombinant human IL-6 and recombinant murine TNF alpha (TNF-α) were purchased from Genzyme (Boston, Mass.), recombinant human IL-1β was obtained from the National Cancer Institute (Craig Reynolds), fetal bovine serum (FBS) was purchased from Hyclone Labs (Logan, Utah), all plates for cell culture were purchased from Corning Co. (Corning, N.Y.), and turpentine was purchased from Parks Corp. (Somerset, Mass.). Sulfosuccinimidyl-2-(*p*-azidosalicylamido)1,3′-dithiopropionate (SASD) was purchased from Pierce Chemicals (Rockford, Ill.), and prestained molecular weight standards and other materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories (Richmond, Calif.). $Na^{125}I$ was purchased from NEN (Boston, Mass.).

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Animals. Male Sprague-Dawley rats weighing 200 to 250 g were purchased from Harlan Sprague-Dawley (Madison, Wis.). Rats were injected with LPS (10 mg/kg of body weight intraperitoneally) or with turpentine (2.5 ml/kg intramuscularly) in the right hind limb. Hepatocytes were isolated 3 to 48 h following LPS or turpentine injection as well as from normal (control) rats.

Hepatocyte isolation. Hepatocytes were isolated from normal and LPS-stimulated rats by an in situ collagenase perfusion technique, modified as described previously (31, 39). Hepatocytes were separated from the nonparenchymal cells by two cycles of differential centrifugation (50 g for 2 min) and further purified over a 30% Percoll gradient. Hepatocyte purity exceeded 98% by light microscopy by this procedure, and viability ranged from 89 to 99% by trypan blue exclusion.

Cell culture and treatment. Hepatocytes (5×10^6) were placed onto 100-mm-diameter gelatin-coated petri dishes in 5 ml of culture medium. Medium consisted of Williams medium E supplemented with insulin (10^{-6} M), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (15 mM), L-glutamine (2 mM), penicillin, streptomycin, and 10% FBS. Hepatocytes were incubated with 5% CO_2 at 37°C for 24 h. The cells were washed four times with 20 ml of Williams medium E without additives or FBS; the medium was then replaced with Williams medium E supplemented as described above except that no FBS was added. Various test signals (IL-6, IL-1β, TNF-α, LPS, and dexamethasone) were added to the cultures. At various time points, total RNA was extracted for Northern (RNA) blot analysis from stimulated or unstimulated cells. In some experiments, the supernatants were removed after 18 h, the cells were washed to remove cytokines and/or LPS, and fresh supernatant was added and then collected 8 h later. This supernatant was then analyzed for LBP bioactivity and LPS-binding activity (see below).

H35 rat hepatoma cells (kindly provided by Heinz Baumann, Roswell Park Cancer Institute, Buffalo, N.Y.) were grown in Dulbecco's modified Eagle's medium (DMEM; Whittaker M. A. Bioproducts) supplemented with insulin (10^{-6} M), HEPES buffer (15 mM), L-glutamine (2 mM), penicillin, streptomycin, and 10% FBS. The cells were washed four times with DMEM to remove serum; the medium was then replaced with DMEM supplemented as described above except that no FBS was added. Various test signals (IL-6, IL-1β, TNF-α, LPS, and dexamethasone) were added to the cultures. After 24 h, total RNA was extracted for Northern blot analysis from stimulated or unstimulated cells.

RNA isolation and Northern blot analysis. Total RNA was isolated by standard methods with RNAzol B (Biotec Laboratories, Houston, Tex.) (9). RNA concentrations were determined by measuring the A_{260}. Total RNA (20 μg) from each sample was electrophoresed on a 1% agarose gel containing 3% formaldehyde prior to transfer to the gene screen membrane. The 650-bp cDNA for rat LBP was cloned from a rat hepatocyte cDNA library in our laboratory (33). cDNA probes for rat acid-1-glycoprotein (AGP) and fibrinogen were obtained from Heinz Baumann (Roswell Park Cancer Institute) and David Samols (Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio), respectively. cDNA probes were labeled with [^{32}P]dCTP by random priming. Hybridizations were carried out overnight at 43°C in a solution containing 50% deionized formamide, 0.25 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 1 mM EDTA, 7% SDS, and 100 μg of denatured salmon sperm DNA per ml. The hybridized filters were washed successively at 53°C in 2× SSC–0.1% SDS, 25 mM $NaHPO_4$–1 mM EDTA–0.1% SDS, and 25 mM $NaHPO_4$–1 mM EDTA–1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). All Northern blot membranes probed for rat LBP, AGP, or fibrinogen were stripped for rehybridization with 18s rRNA as equal loading controls. Fold increases always refer to the increase in mRNA levels of the treatment group over those of untreated cells (control) cultured for the same time period.

Isolation of peritoneal macrophages. Peritoneal macrophages were harvested from normal Sprague-Dawley rats. Animals were euthanized with CO_2, and the skin was incised by sterile technique. The peritoneum was lavaged with 50 ml of ice-cold, supplemented Williams medium E without FBS. The macrophages were isolated by centrifugation and placed on 35-mm-diameter plastic tissue culture wells at 10^6 cells per ml in supplemented Williams medium E. Cells were incubated at 37°C overnight prior to use.

Assay of LBP bioactivity. LBP-like activity was measured by determining the capacity of the hepatocyte supernatants (containing secreted LBP) to upregulate the production of TNF by cultured peritoneal macrophages in response to LPS (1 ng/ml) (30). Macrophages were isolated and placed in tissue culture wells as described above. After 18 h of incubation, LPS (1 ng/ml) and hepatocyte supernatants (1%) were added to the wells. Macrophage supernatants were collected at 4 h for the TNF assay. TNF was measured in the macrophage supernatants by assessing lysis of the L929 fibroblasts in the presence of actinomycin D (1).

Photoaffinity labeling. *E. coli* O111:B4 LPS was first sonicated and then coupled to SASD, as described previously (40). The resulting LPS-ASD complex was radiolabeled with $Na^{125}I$ by use of Chloramine T, as described previously (40). The product typically had a specific activity of 6×10^9 cpm/mg of LPS. ^{125}I-ASD-LPS (0.5 μCi) containing 0.2 μg of LPS was then incubated with hepatocyte supernatants collected following various treatments (protein, 100 μg) in borosilicate glass tubes for 10 min at 37°C. Cross-linking of the ligand to the protein was accomplished by photolysis for 10 min at room temperature with short-wave UV irradiation (Hoefer Scientific Instruments, San Francisco, Calif.; UVC 1000 cross-linker, maximal emission at 254 nm). After cross-linking, samples were concentrated with a speed vacuum.

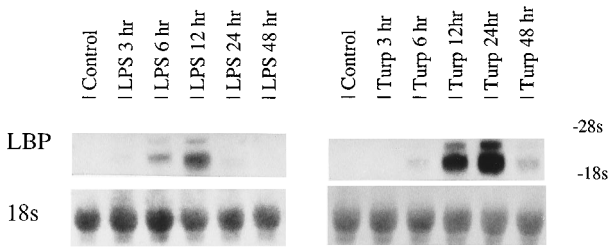

FIG. 1. Time course of hepatocyte LBP induction following LPS or turpentine (Turp) injection in vivo. Male Sprague-Dawley rats (200 to 250 g) were injected intraperitoneally with LPS (10 mg/kg) or intramuscularly with turpentine (2.5 ml/kg). Hepatocytes were isolated at 3, 6, 12, 24, and 48 h from LPS- and turpentine-injected rats and from control (untreated) rats. Total RNA was extracted from freshly isolated hepatocytes, and Northern blot analysis was performed for LBP mRNA. Membranes were rehybridized with probe for 18s rRNA. Each blot shown is representative of two identical experiments.

SDS-PAGE. SDS-PAGE was carried out with the buffer system of Laemmli (23) with a 10% resolving gel and a 4% stacking gel. The protein bands were visualized by autoradiography. The relative mobilities of proteins were determined with prestained low-range molecular mass protein standards. When autoradiography was performed, gels were dried on a slab gel drier (Hoefer) and Kodak X-Omat XAR-5 film was used with the aid of a DuPont Cronex Lighting Plus Intensifier Screen (X-Ray of Georgia, Norcross). Gels were kept at −70°C until the desired intensity was obtained (5 to 24 h). All photoaffinity labeling and SDS-PAGE experiments were performed three times.

RESULTS

Time course of LBP mRNA induction by LPS and turpentine in the livers of treated rats. Experiments were designed to determine the time course of hepatocyte LBP mRNA expression in vivo in models of endotoxemia and the acute-phase response. Rats were injected with LPS (10 mg/kg intraperitoneally) or turpentine (2.5 ml/kg intramuscularly), and total RNA was extracted from freshly isolated hepatocytes at the time points indicated in Fig. 1. Northern blot analysis shows that LBP mRNA levels in control hepatocytes were barely detectable. In contrast, LBP mRNA levels increased as early as 3 h after in vivo LPS injection, reached a maximum at 12 h, and rapidly decreased to baseline by 48 h. Increased LBP mRNA levels in hepatocytes from turpentine-treated rats were detected at 6 h postinjection; LBP mRNA levels peaked at 24 h and subsequently declined but remained elevated until 48 h. Maximal mRNA levels occurred 12 h later in the turpentine-treated group than in the LPS-treated group. However, turpentine injection resulted in a greater induction of LBP mRNA. After probing for LBP, membranes were stripped and rehybridized with probe for 18s rRNA. Scanning densitometry of autoradiographs standardized relative to 18s rRNA in each lane revealed a 24-fold increase in peak LBP mRNA levels in the LPS-treated rats and a 27-fold increase in the turpentine-treated group (data not shown).

Spontaneous induction of LBP mRNA under various conditions. To better understand the regulation of hepatocyte LBP synthesis, it was necessary to determine the influence of in vitro conditions on hepatocyte LBP synthesis. To this end, rat hepatocytes were placed in culture for 2 h in either the presence or absence of dexamethasone, and LBP mRNA levels were determined at various time points. Figure 2A shows that the processes of liver perfusion and hepatocyte isolation did not increase LBP mRNA expression compared with the levels obtained from the whole liver immediately prior to the cell isolation procedure. Hepatocytes simply placed in culture exhibited a greater-than-10-fold increase in spontaneous induction of LBP mRNA expression compared with the minimal levels

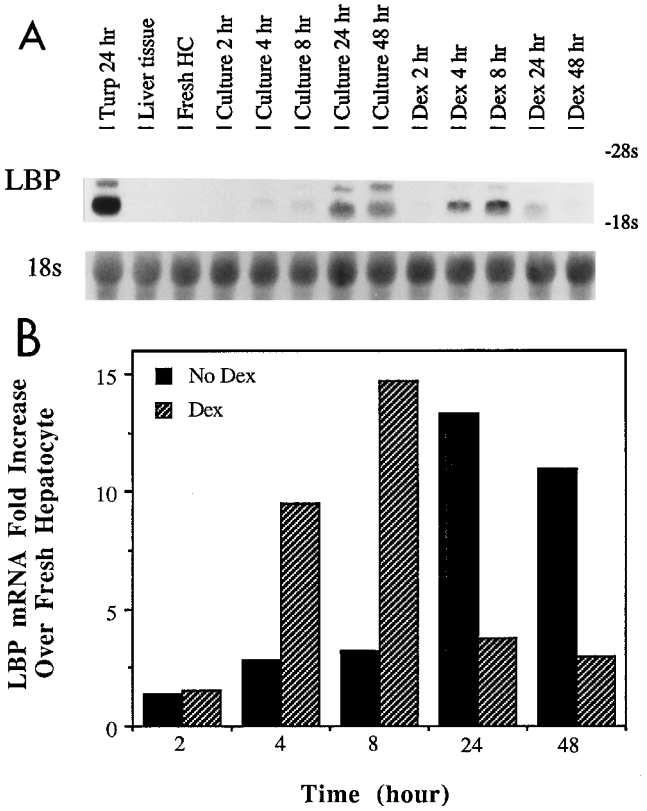

FIG. 2. Spontaneous induction of LBP mRNA under various conditions. (A) LBP mRNA levels are shown for the whole liver prior to collagenase perfusion (liver tissue), isolated unplated hepatocytes (fresh HC), and hepatocytes plated in the absence (culture) or presence (Dex) of dexamethasone (10^{-6} M). For comparison, LBP mRNA levels in hepatocytes from turpentine (Turp)-injected rats show a high-level control. (B) Autoradiograph mRNA signals from cultured hepatocytes in the absence or presence of dexamethasone were quantitated by scanning densitometry and corrected relative to 18s rRNA.

seen in whole liver or freshly isolated hepatocytes. This increase in spontaneous induction occurred after 6 h in culture, peaked at 24 h after plating, and had declined only slightly by 48 h. As demonstrated in Fig. 2A, the spontaneous increase in LBP mRNA never reached the maximal degree of induction achieved in vivo following turpentine injection.

Dexamethasone, a glucocorticoid, is known to enhance the in vitro induction of some rat acute-phase reactant syntheses by cytokines (26). When dexamethasone was added to cultured hepatocytes, a fivefold increase in LBP mRNA levels was seen in hepatocytes cultured during the first 24 h. However, in hepatocytes cultured for longer than 24 h, dexamethasone suppressed the spontaneous increases in hepatocyte LBP mRNA levels (Fig. 2B).

We also tested for the influence of rat tail collagen as a surface matrix instead of gelatin as well as for the effect of the presence or absence of calf serum or insulin on spontaneous LBP expression. No differences in LBP mRNA levels were seen between hepatocyte cultures with commercially available gelatin and those with rat tail collagen. The presence or absence of insulin or serum also had no effects (data not shown).

Time course of hepatocyte LBP induction following LPS, IL-1, TNF, or IL-6 exposure in vitro. LPS injection in vivo increased LBP expression. To determine the direct effects of LPS, 10 μg of LPS per ml was added to cultured hepatocytes and total RNA was extracted at the time points indicated in Fig. 3. The addition of LPS stimulated a small (threefold) increase in LBP mRNA levels, with maximal stimulation occurring after 24 h, followed by a subsequent decrease.

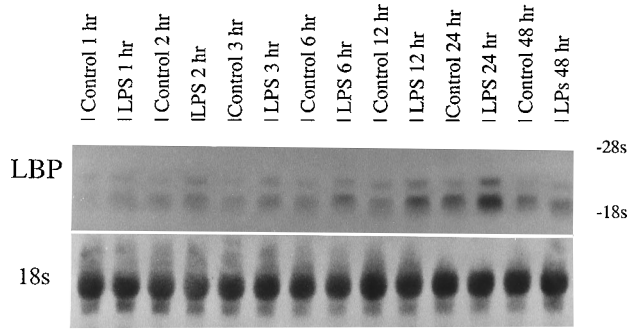

FIG. 3. Time course of hepatocyte LBP induction following LPS exposure in vitro. RNA was isolated from hepatocytes following LPS (10 μg/ml) stimulation for the times indicated or from unstimulated (control) cells. The results represent one of three similar experiments.

LPS elicits the synthesis of multiple inflammatory mediators which are known to regulate the hepatic acute-phase response. IL-1, IL-6, and TNF are among the mediators which are known to enhance hepatocyte synthesis of acute-phase reactants. To determine whether LBP expression was increased with stimulation by IL-1, TNF, or IL-6, cultured hepatocytes were exposed to these cytokines and LBP mRNA levels were determined at 6, 24, and 48 h. As shown in Fig. 4, LBP mRNA levels were increased by all three cytokines, and maximal mRNA levels were obtained at 24 h.

The concentration-dependent effects of IL-1, IL-6, TNF, and LPS on LBP mRNA expression were determined. The capacity of dexamethasone to modulate IL-1, IL-6, TNF, or LPS-induced changes in LBP mRNA levels was also determined. On the basis of the data in Fig. 4, the 24-h time point was used in these experiments. Figure 5 shows that IL-6 and LPS upregulated LBP mRNA levels in a dose-dependent manner, with increases of 4.5-fold and 3.2-fold, respectively, at the highest concentration. These IL-6- and LPS-dependent in-

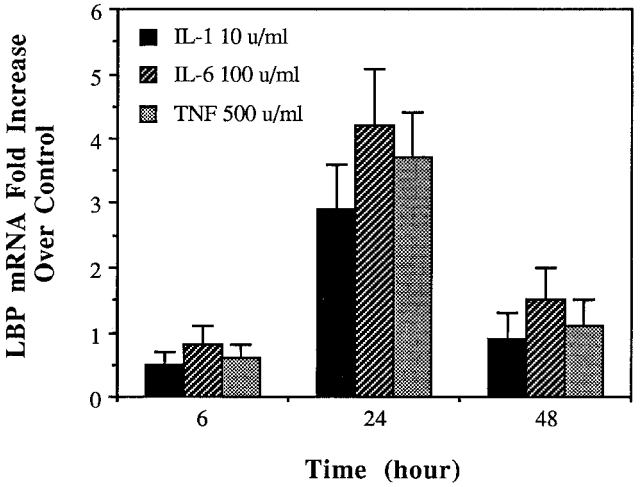

FIG. 4. Time course of hepatocyte LBP induction following IL-1, TNF, and IL-6 exposure in vitro. RNA was isolated from hepatocytes following IL-1 (10 U/ml), TNF (500 U/ml), and IL-6 (100 U/ml) stimulation for the times indicated or from unstimulated cells (control). The results represent one of three similar experiments.

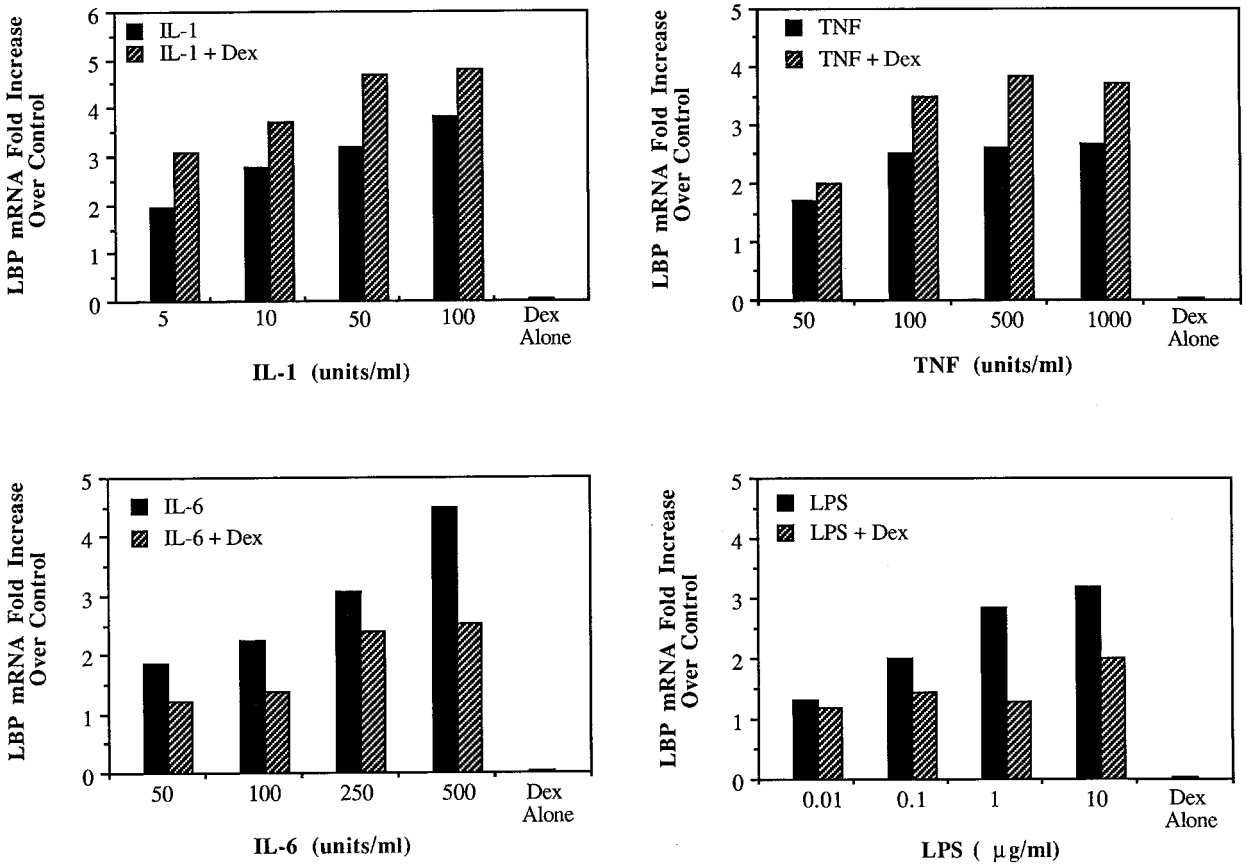

FIG. 5. Concentration-dependent effects of IL-6, IL-1β, TNF-α, and LPS, with and without dexamethasone (Dex), on rat hepatocyte LBP mRNA levels. Hepatocytes were stimulated for 24 h with IL-6, IL-1β, TNF-α, and LPS at the concentrations indicated in the presence or absence of 10^{-6} M dexamethasone (Dex). Total RNA was isolated, Northern blot hybridization was performed, and LBP mRNA levels were determined by scanning densitometry. Results represent fold increases in mRNA levels over those of unstimulated hepatocytes (control).

creases in LBP mRNA were attenuated by dexamethasone. In contrast to the results with IL-6 and LPS, the combination of dexamethasone with IL-1β or with TNF-α led to maximal LBP mRNA induction, 4.7- and 3.8-fold, respectively, at 500 U of TNF-α per ml and 50 U of IL-1β per ml. Combinations of IL-1β and TNF-α, or IL-1β and IL-6, exhibited no greater increases than those seen when the cytokines were added alone (data not shown). Dexamethasone alone had a minimal effect on LBP expression at this time point.

To ensure that our primary hepatocyte cultures exhibited the described induction of other more-characterized acute-phase reactants, AGP and fibrinogen mRNA levels were compared with LBP mRNA levels (Fig. 6). In agreement with previous reports (12), dexamethasone treatment markedly increased AGP mRNA levels, which were further increased by the addition of IL-1 with dexamethasone. IL-6 alone increased fibrinogen expression, which was further increased with the addition of dexamethasone. Thus, the hepatocytes used in our experiments exhibited the expected response to IL-1, IL-6, and dexamethasone for the expression of the acute-phase reactants AGP and fibrinogen.

Comparison of levels of induction of LBP mRNA by IL-1β, IL-6, TNF-α, and LPS in normal rat hepatocytes and hepatocytes from LPS-injected rats. Since the primary role of LBP is to recognize and bind LPS, it was of interest to know how previous LPS treatment influenced subsequent LBP synthesis. To determine whether endotoxemia alters hepatocyte LBP synthesis to cytokines, dexamethasone, or LPS in vitro, hepatocytes were isolated from rats injected with LPS (10 mg/kg) in vivo and then stimulated as described above in vitro. Hepatocytes were isolated 24 h after the injection of rats with either LPS (primed cells) or saline (control cells) and placed in culture for 24 h. These cells were then stimulated for 24 h in vitro with IL-1β, IL-6, TNF-α, and LPS at concentrations found to be optimal in cells from normal rats. Figure 7 reveals the results of Northern blot analysis for LBP mRNA following cytokine and LPS stimulation of control versus in vivo-primed hepatocytes. As described above, normal hepatocytes exhibited only two- to fourfold increases in response to IL-1, TNF, IL-6, or LPS. In contrast, basal expression of LBP mRNA was higher in hepatocytes from LPS-injected rats, and much greater increases in LBP mRNA levels were seen with in vitro stimulation in the LPS-primed group. The greatest induction was seen with LPS, followed by that with TNF-α and that with IL-1β.

Concentration-dependent effects on LBP expression by LPS-primed rat hepatocytes. We demonstrated that LPS was the most potent stimulus for LBP expression in LPS-primed hepatocytes. This finding suggests that the endotoxemia alters the hepatocytes to respond to subsequent LPS stimulation. The capacity of hepatocytes to respond to a wide range of LPS concentrations was tested. A greater-than-two-fold increase in LBP mRNA levels was seen with 1 ng of LPS per ml, with concentration-dependent increases seen at LPS concentrations up to 10 μg/ml (Fig. 8). Higher concentrations (100 μg/ml)

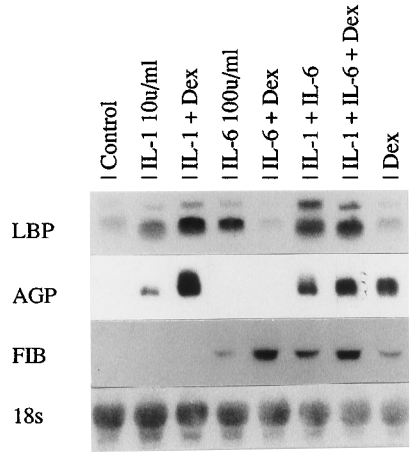

FIG. 6. Comparison of responses of cultured normal hepatocytes to cytokines, with or without dexamethasone (Dex), in changes in mRNA levels for LBP, AGP, and fibrinogen (FIB). Hepatocytes were isolated from normal rats and placed in culture for 24 h with 10% serum. The cells were washed four times to remove serum before the indicated signals were added. Total RNA was extracted and hybridized with ^{32}P-labeled cDNA probes for LBP, AGP, and fibrinogen.

resulted in no further increase (data not shown). Similar to the results with the nonprimed hepatocytes, dexamethasone at 10^{-6} M attenuated the increase in LBP mRNA levels in LPS-primed hepatocytes.

Assay of LBP bioactivity. LBP bioactivity was determined by assessing the capacity of hepatocyte supernatants to upregulate cultured macrophage TNF production in response to added LPS. Since Northern blot analysis showed that LPS was the most potent stimulus for the induction of LBP mRNA in hepatocytes from LPS-treated rats, supernatants from cultured normal hepatocytes or hepatocytes from LPS-treated rats following LPS stimulation in vitro were collected for LBP bioactivity. In these experiments, hepatocytes from normal rats or rats pretreated with LPS were placed in culture and exposed to LPS in vitro. After 18 h, the cells were washed four times to remove all LPS, and new medium without serum was added. Eight hours later, the supernatants were removed and tested for LBP bioactivity.

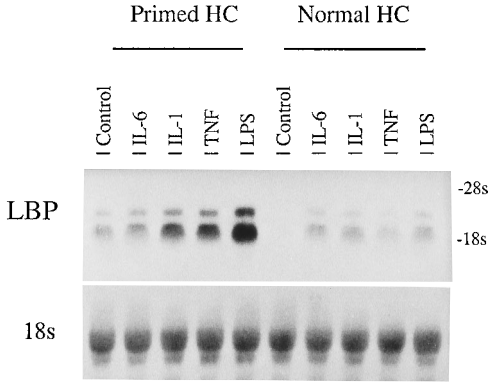

FIG. 7. Comparison of levels of induction of LBP mRNA by IL-6, IL-1β, TNF-α, and LPS in normal rat hepatocytes (normal HC) and hepatocytes from rats injected with LPS (10 mg/kg; primed HC). Hepatocytes were isolated at 24 h from LPS-injected rats and normal rats and placed in culture. Cultured rat hepatocytes were stimulated for 24 h with IL-6 (200 U/ml), IL-1β (10 U/ml), TNF-α (500 U/ml), and LPS (10 μg/ml).

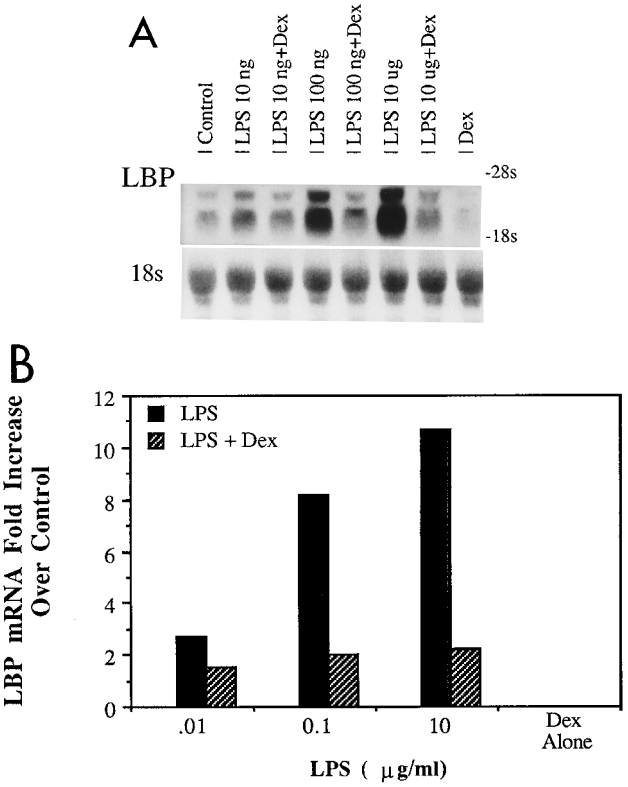

FIG. 8. Concentration-dependent effects of LPS on LBP mRNA levels in hepatocytes from LPS-treated rats. Rats were injected intraperitoneally with LPS (10 mg/kg), and after 24 h, hepatocytes were isolated from animals and placed in culture. Hepatocytes that had been in culture for 24 h were stimulated for the subsequent 24 h with LPS, at the concentrations indicated, in the presence or absence of dexamethasone (Dex; 10^{-6} M). (A) Northern blot; (B) fold increase in LBP mRNA of stimulated hepatocytes over that of unstimulated hepatocytes (control) based on quantitation by scanning densitometry with correction to 18s. The figure is representative of three experiments.

Peritoneal macrophages were harvested by lavage and placed in culture with serum-free medium. After 18 h, serum-free hepatocyte supernatants (1% [vol/vol]) were added to the macrophage cultures after washing with serum-free medium. LPS was then added at 1 ng/ml. After 4 h, an aliquot of supernatant was removed for the determination of TNF levels. Studies showed that macrophages cultured in serum-free medium did not respond to LPS with an increase in TNF synthesis (data not shown). When the macrophages were exposed to only the hepatocyte supernatant in the absence of LPS, no increase in TNF production was seen (Fig. 9, solid bars). The addition of 1% hepatocyte supernatant with LPS resulted in a marked increase in TNF synthesis that was greater when supernatant from hepatocytes isolated from LPS-treated rats was used. This effect was even greater when the primed hepatocytes were treated with LPS in vitro, indicating that more LBP-like activity was present in these supernatants. The minimal effect of in vitro LPS on hepatocytes from normal rats suggests that the LPS effect on the primed cells was not due to carryover of LPS.

Supernatant LPS-binding activity. To identify LPS-binding activity expressed by hepatocytes, aliquots from supernatant from hepatocyte cultures were incubated with ^{125}I-ASD-LPS, cross-linked by exposing the sample to UV light, and analyzed by subjecting the sample to electrophoresis and autoradiography. As can be seen in Fig. 10, LPS-binding proteins with

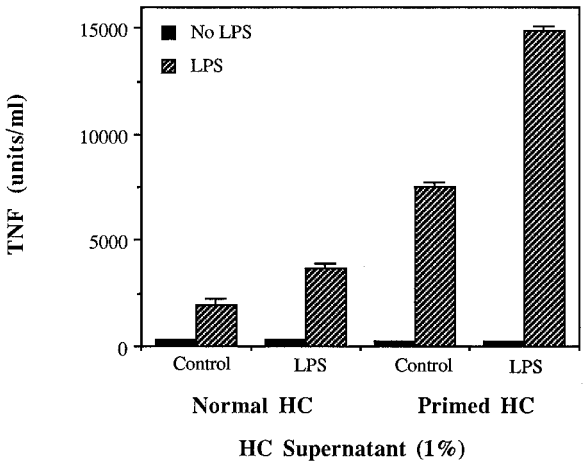

FIG. 9. Assay for LBP bioactivity. Hepatocyte LBP bioactivity was determined by measuring the capacity of 1% hepatocyte supernatant to upregulate production of TNF from rat peritoneal macrophages in response to LPS. Hepatocytes from normal rats (normal HC) or LPS-pretreated rats (primed HC) were exposed to medium or LPS (10 μg/ml) for 18 h. The cells were then washed, and fresh medium was added for 8 h. This supernatant was added to macrophages with or without LPS (1 ng/ml). After 3 h, macrophage supernatant was assayed for TNF levels by an L929 fibroblast lysis assay. The results show the means ± standard errors of the means SEM of duplicate cultures.

apparent molecular masses of 30, 40, and 60 kDa were observed in all lanes. However, only lane 4 shows the presence of an intensely labeled 60-kDa protein. This demonstrates that hepatocytes obtained from LPS-injected rats, treated in vitro with LPS, show increased expression of a 60-kDa LPS-binding protein. An LPS-binding protein with a similar molecular mass

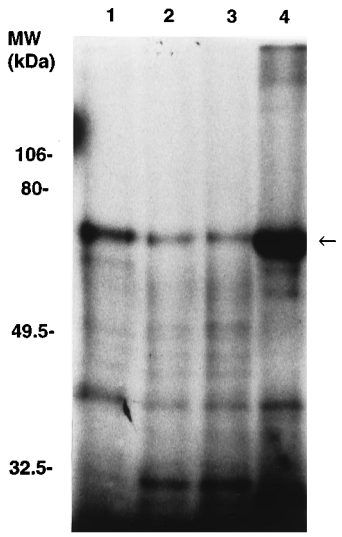

FIG. 10. Photoaffinity labeling of hepatocyte supernatants with ^{125}I-ASD-LPS. Hepatocyte supernatants from normal and LPS-injected rats were treated in culture for 18 h in the absence and presence of LPS (10 μg/ml). The cells were washed, and fresh medium was added for 8 h. Supernatants from these cells were incubated with ^{125}I-ASD-LPS and photoactivated. The incubation mixture was electrophoresed on an SDS–10% polyacrylamide gel, and autoradiography was performed. Lanes: 1 and 2, supernatants of hepatocytes from saline-treated rats treated in vitro without or with LPS, respectively; 3 and 4, supernatants of hepatocytes isolated from LPS-injected rats treated in vitro without or with LPS, respectively. Prestained molecular mass markers (in kilodaltons) of protein standards are indicated on the left. An arrow indicates the 60-kDa LBP of interest.

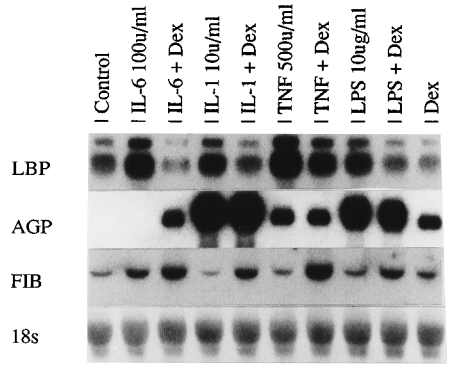

FIG. 11. Changes in mRNA levels of LBP, AGP, and fibrinogen (FIB) induced by IL-6, IL-1, TNF, and LPS in the presence or absence of dexamethasone (Dex). H35 cells in 100-mm-diameter petri dishes were treated for 24 h with serum-free medium containing the indicated signals. Total RNA was extracted, and 10 μg of each preparation was analyzed by Northern blot hybridization for LBP, AGP, and fibrinogen mRNA levels.

has been identified previously and characterized in rabbit, human, and bovine sera (21, 30, 35).

LBP, AGP, and fibrinogen expression in H35 cells. The responses of the normal and primed hepatocytes to glucocorticoids for LBP expression were atypical for many other acute-phase reactants and could potentially be explained by the action of the steroids on a few contaminating macrophages. To address this issue, the responses of a rat hepatoma cell line to dexamethasone, IL-1, IL-6, and TNF were tested. The acute-phase response has been well characterized in these cells (5, 7) and is similar to that of primary rat hepatocytes. As shown in Fig. 11, IL-1, IL-6, TNF, and LPS alone all increased LBP mRNA levels at 24 h. The inclusion of dexamethasone prevented the increase when added with IL-1, IL-6, and LPS and attenuated the rise seen with TNF. The expected responses to IL-1 and dexamethasone and to TNF and dexamethasone (6) were seen in the AGP mRNA expression levels for H35 cells. Likewise, fibrinogen was induced by IL-6 alone and by IL-1, TNF, and LPS with dexamethasone, again confirming the expected acute-phase response from these cells (7, 8). These results confirm the negative effect of dexamethasone on IL-6 and LPS in LBP mRNA expression seen in normal and primed hepatocytes. However, unlike the results with the normal hepatocytes, where dexamethasone increased IL-1- and TNF-induced LBP mRNA expression, dexamethasone reduced H35 cell LBP mRNA expression levels.

DISCUSSION

LBP is a plasma protein which plays a crucial role in LPS recognition. Complexes are formed when LBP binds to the lipid A region of LPS. These complexes are then recognized by CD14 on the surface of LPS-responsive cells such as macrophages (41) and neutrophils (19, 37) or by soluble CD14, which interacts with endothelial cells (18, 27). In macrophages, this recognition initiates a cell-signaling process which involves activation of a protein tyrosine kinase, resulting in cytokine synthesis (32). LBP is a 50-kDa polypeptide that is glycosylated and secreted as a 60-kDa glycoprotein by hepatocytes (29). Plasma levels of LBP increase severalfold following injection of silver nitrite in rabbits. We have shown up to a 20-fold increase in LBP mRNA levels in hepatocytes isolated from rats injected with LPS, turpentine, or *C. parvum* (14). As a result of these observations, LBP has been designated an acute-phase

protein (29). We have also shown that extrahepatic sites of LBP synthesis exist (33). In this study, we sought to determine the signals responsible for the stimulation of LBP synthesis in cultured rat hepatocytes. As an acute-phase reactant, LBP expression was expected to be under the control of the cytokines IL-1, TNF, and IL-6 as well as glucocorticoids. Our findings demonstrated the following. (i) LBP mRNA levels peak in hepatocytes in vivo at 12 h following an intraperitoneal bolus of LPS and 24 h following intramuscular turpentine treatment. (ii) LBP mRNA levels increase spontaneously in cell culture, and this response is accelerated by dexamethasone early but suppressed by dexamethasone beyond 24 h. (iii) LPS, IL-1, TNF, and IL-6 each can stimulate two- to fourfold increases in LBP mRNA levels in primary rat hepatocyte cultures. Dexamethasone suppresses the response to IL-6 and LPS but increases the response of hepatocytes to IL-1 and TNF. (iv) Hepatocytes isolated from rats previously injected with LPS exhibit a markedly enhanced response to LPS, TNF, and IL-1. The 10-fold increase in LBP mRNA seen in LPS-primed hepatocytes in response to LPS in vitro is suppressed by the addition of dexamethasone. (v) The increased LBP mRNA levels were associated with significant increases in the release of LBP activity and LPS binding to a 60-kDa protein. (vi) In H35 rat hepatoma cells, dexamethasone inhibits the induction of LBP by IL-1, IL-6, TNF, and LPS.

The synthesis of other acute-phase proteins by hepatocytes is part of an adaptive response to tissue injury and infection under the control of cytokines and glucocorticoids, which are elevated after these insults. Acute-phase reactants are grouped typically into those that are stimulated by IL-1 and/or TNF (the so-called early-response cytokines) and those that are stimulated by IL-6 or cytokines which act via the IL-6 receptor (4, 22). The synthesis of some acute-phase reactants is stimulated primarily by glucocorticoids, while glucocorticoids enhance the synthesis induced by cytokines, especially IL-6. Although marked increases in LBP mRNA caused by stimuli which mimic gram-negative bacterial sepsis (LPS injection) or remote tissue injury (turpentine injection) are seen in vivo, only modest increases in LBP mRNA levels are caused by exposure of normal hepatocytes to the acute-phase cytokines in vitro. Combinations of IL-1, TNF, and IL-6 were no more effective than other cytokines alone (data not shown). Of note, LPS was as effective as the cytokines tested in stimulating increases in LBP mRNA. It would appear that LPS has the capacity to stimulate the synthesis of LBP. This finding is not entirely surprising since LBP has the role of recognizing LPS. The uniform responses to IL-1, TNF, and IL-6 as well as the similar response to LPS and the spontaneous increases in LBP mRNA levels in cultured hepatocytes suggest that the synthesis of increased LBP by rat hepatocytes results from any one of these stimulations and requires only minor perturbations. This is consistent with the notion that the synthesis of LBP is part of an early-alarm system aimed at recognizing and binding to LPS and thus enhancing the activation of the immune system to combat the invading gram-negative bacteria. Recent observations by Grube and coworkers (17), showing that human hepatocytes and HepG2 human hepatoma cells are responsive only to IL-6, suggest that important species differences in the synthesis of LBP are likely to exist.

Like the responses to cytokines and LPS, the effects of dexamethasone were somewhat atypical compared with those of other acute-phase reactants. Dexamethasone increased spontaneous induction at early time points but suppressed it at later time points. A mild enhancing effect was seen with IL-1 and TNF. Thus, the primary role of glucocorticoids may be to enhance LBP synthesis to these early-response cytokines. Dexamethasone suppressed the response to IL-6 and LPS. Since dexamethasone can inhibit cytokine synthesis, it is conceivable that this was an indirect effect from inhibiting IL-1 or TNF synthesis in the cultures. To address this point, we examined the responses of a rat hepatoma cell line and showed that dexamethasone inhibited LBP mRNA expression in response to all three cytokines and LPS while having the expected synergistic effect on AGP and fibrinogen expression in response to the cytokines. In addition, anti-TNF antibodies and IL-1 receptor antagonist failed to block the LPS responses of primed hepatocytes (data not shown). Therefore, these data indicate a direct effect of dexamethasone and suggest that IL-6 and LPS stimulate LBP expression by mechanisms different from those of TNF and IL-1 in primary hepatocyte cultures. This finding may not be unexpected since IL-6 stimulates gene expression via transcriptional factors different from those of IL-1 or TNF (4). It is likely that the differences between the primary hepatocytes and the H35 hepatoma cells in response to IL-1 and TNF or dexamethasone are due to basic differences in how the primary cells and cell line respond to stimuli.

The levels of gene expression induced by IL-1, TNF, IL-6, and dexamethasone are relatively minor compared with the greater-than-20-fold induction seen following in vivo stimulation. This finding suggests that primary hepatocytes in culture exhibit an altered response pattern compared with that of cells in the intact liver or that other stimuli not tested here contribute to the induction. We observe here that hepatocytes from rats treated with LPS in vivo exhibit marked increases in response to LPS, TNF, and IL-1, with up to 10-fold increases in mRNA levels. The induction was seen at LPS concentrations even as low as 1 ng/ml. This increase in response was also seen when hepatocytes were obtained from rats injected with turpentine 24 h previously (data not shown). This response in cell culture takes place even after the cells have been in culture for 24 h, indicating that the changes in the hepatocytes persist in culture and that large increases in LBP synthesis can take place in response to LPS and the cytokines in vitro. As with the normal hepatocytes, dexamethasone inhibited the induction by LPS. Others have shown enhanced synthesis of nitric oxide by hepatocytes in response to LPS or TNF in vitro following in vivo infusion of LPS for 30 h (25). Taken together, these data indicate that hepatocytes can be primed in vivo for subsequent response to LPS or certain cytokines. The mechanisms of the priming and enhanced responses are unclear and may involve any level of LPS or cytokine regulation of gene expression. This effect may be a mechanism by which hepatocytes are primed by an initial insult to respond more rapidly and vigorously to subsequent challenges and could represent a mechanism to increase nonspecific resistance to subsequent infection. However, endotoxin tolerance where animals previously treated with endotoxin become less responsive to an endotoxin challenge (3) is also known to occur. In addition, an initial LPS treatment can render macrophages less responsive to a subsequent LPS challenge (38). How the enhanced response of hepatocytes to LPS in vitro following in vivo LPS preexposure relates to in vivo LPS tolerance is unclear. Our data would suggest that the capacity to produce LBP, which binds LPS, is increased following an initial septic insult. This may not have an influence on subsequent macrophage activation while still increasing the LPS-binding capacity, since macrophage activation will be determined by other factors such as CD14 expression. It is also important to point out that it is not known if hepatocyte priming can be demonstrated in vivo.

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