Lipopolysaccharide Binding Protein Expression in Primary Human Hepatocytes and HepG2 Hepatoma Cells*

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Lipopolysaccharide (LPS)-binding protein (LBP) is a normal plasma protein and an acute phase reactant important for host responses to Gram-negative bacteria and LPS. LBP forms high affinity complexes with LPS which bind to CD14, a monocyte surface protein, to initiate the release of inflammatory mediators. We found that human primary hepatocytes synthesize LBP and that the synthesis is up-regulated by interleukin (IL)-6. To examine this phenomenon in more detail, we evaluated the capacity of IL-6, IL-1, and tumor necrosis factor to induce LBP synthesis in HepG2 cells in the presence or absence of dexamethasone. IL-6 induced LBP synthesis. Dexamethasone, IL-1, and tumor necrosis factor had a synergistic effect when combined with IL-6, but demonstrated minimal effect independently. LBP biosynthesis was evaluated by immunoprecipitation of 35S-labeled LBP from HepG2 supernatants, measurement of steady-state LBP mRNA levels, and analysis of LBP-dependent LPS binding to CD14 positive cells. An 8-kDa protein was immunoprecipitated with anti-LBP antibodies from IL-6-stimulated HepG2 cell supernatants. Northern blot analysis of cellular RNA revealed an increase in LBP mRNA in IL-6-stimulated cells. CD14 expressing cells bound fluoresceinated LPS in the presence of supernatants from HepG2 cells treated with IL-6. These data provide the first information about specific cytokine and dexamethasone regulation of LBP expression in HepG2 cells. LBP behaves like a Type 1 acute phase protein.

The release of lipopolysaccharide (LPS)† from the outer cell wall of Gram-negative bacteria produces the acute systemic inflammatory response syndrome which in the most severe clinical scenario evolves into multiple organ failure and death (1). A variety of cytokines are released for host defense from cells of monocytic origin in response to challenge by LPS (2). In overwhelming sepsis, the inflammatory cascade may no longer be held in check, but may actually lead to an escalation of injury to host tissues by the very mediators initially released to preserve host function (3). TNF-α has emerged as one of the key mediators of shock (4). It is released from cells of monocytic origin in response to picomolar concentrations of circulating LPS (5).

The lipid A structure of LPS is involved in initiating a signal in the macrophage (MO) (6) through a novel receptor-dependent mechanism (7, 8). The lipid A moiety is bound to the circulating binding protein, lipopolysaccharide-binding protein (LBP) (9, 10). LBP delivers LPS to the MO receptor, CD14 (8). Although other mechanisms for LPS activation of monocytes are postulated, only LBP-dependent LPS binding to CD14 results in a 103 greater production of TNF-α (11). Depletion of LBP from serum suppresses TNF-α production (7). LBP also serves as an opsonin for Gram-negative bacteria and reduces the threshold concentration for LPS to induce cytokine release from the MO (12).

LBP and CD14 have emerged as the two central molecules for LPS activation of monocytes. The work herein is focused on the regulation of LBP expression. Elucidation of the regulatory mechanism for LBP synthesis is integral to understanding the physiologic changes of shock initiated by LPS. LBP is a circulating plasma protein present in normal individuals at a concentration of 0.1–5 μg/ml (13), which rise to ≥50 μg/ml in response to injury (14). LBP is known to be synthesized in the liver of rabbits constitutively (15) and in rat liver in several inflammatory models (16), but its synthesis in human hepatocytes has not been demonstrated. Moreover, nothing is known about the humoral mediators that regulate LBP synthesis.

Synthesis of acute phase proteins (APP) in the liver is regulated by IL-1 (17, 18), TNF-α (19), IL-6 (17, 20), IL-11 (21), interferon-γ (22), leukemia inhibitory factor (23), oncostatin M (24), transforming growth factor-β (25), and dexamethasone (26). Regulation of APP synthesis encompasses a spectrum of stimulatory, synergistic, and inhibitory responses (26, 27). We establish herein that primary human hepatocytes secrete LBP and that expression of LBP in the presence of IL-6 is elevated. The investigation of LBP synthesis in response to four humoral mediators, IL-1, TNF-α, IL-6 and dexamethasone, representative of the two principal APP regulatory classes (20) was undertaken in the HepG2 model utilized for elucidating the regulation of other cytokine-modulated hepatic APPs (28, 29).

Technical problems such as possible contaminating nonparenchymal cells in primary hepatocyte cultures (30) or the effect of primary hepatocyte culture techniques on their function (31) are circumvented by investigating APP regulation in hepatocyte cell lines derived from human hepatomas, such as the HepG2 cell line. LBP synthesis in HepG2 cells is stimu-
Hepatoma Cells—Primary hepatocytes were changed to Waymouth's J. Mathison (TSRI, La Jolla, CA) and cultured as described (35). Medium alone or with IL-1 after onset of stimulation; final supernatants were collected at 84 h.

Hemorrhage (SRI International Human Subjects Use Committee approval) was added at 1 h where indicated. Primary human hepatocytes were isolated by biopsy perfusion (32) of a non-transplanted liver from a donor who died from an intracranial hemorrhage (SRI International Human Subjects Use Committee approval case 616). Cells were plated 24 h after liver harvest. Initial cell viability was 85% by trypsin blue exclusion, and purity was greater than 90% by microscopy. Liver cells (1 x 10⁶ cells/35-mm collagen-coated plate) were cultured in 5% FCS in modified Waymouth's 752/5 medium (Life Technologies, Inc.) plus 2% dextran-coated FCS (7, 36). Cells were aspirated after 2 h to remove nonviable, unattached cells and replaced with fresh modified Waymouth's 752/5 without FCS. Cells were cultured between a "collagen sandwich" for 21 h prior to cytokine stimulation (34).

CHO-K1 cells stably transfected with human CD14 (CHO-hCD14) or vector (CHO-RSV) were generously supplied by J. D. Lee (TSRI, La Jolla, CA) and cultured as described (35).

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Media—**HepG2 cells (generously provided by Dr. G. Fey, The Scripps Research Institute (TSRI), La Jolla, CA) were cultured to confluence in minimal essential media (MEM, Flow, 11–10022, Irvine, CA) with 10% FCS heat-inactivated at 56 °C for 30 min. The culture medium was changed to 2% FCS plus MEM stimulatory media (SM) plus mediators for assessment of APP regulation (26). SM (cystine and methionine-free MEM, Select-amine Kit 300-9050AV, Life Technologies, Inc.) plus 2% dextran-coated FCS and [35S]methionine and [35S]cysteine (P5S]methionine, [35S]cysteine for 21 h. Cytolune concentrations are reported in the figure and table legends. Supernatants were collected and fresh stimulatory media applied at 12 and 39 h to remove cytokines. For metabolic labeling, the medium was changed to SMCYS-met alone or with IL-1 after onset of stimulation (34). For metabolic labeling, the medium was changed to 300 µl (Centricon™10, Amicon®, Beverly, MA).

**FITC-Re595 LPS Binding to CHO-hCD14 and CHO-RSV Cells—**FITC-Re595 LPS binding was determined by flow cytometry (FACS, Becton Dickinson). Cells for LBP mRNA—Total RNA from cytokine-stimulated HepG2 cells was extracted by the method of Chomczynski and Sacchi (41), formamide/formaldehyde denatured, electrophoresed, and transferred to Hybond-N+ (Amersham) (42). The blot was stained with methylene blue, hybridized with [α-32P]dCTP (Amersham PB 10205)-labeled hLBP cDNA (a generous gift of J. Han, TSRI), and autoradiographed (Hyperfilm™MP, Amersham, Sweden).

**ELISA for LBP, Fibrinogen, and α1-Acid Glycoprotein—**Goat anti-recombinant human LBP (gt-α-LBP), biotinylated-gt-α-LBP and hLBP were generously provided by L. Hatlen (TSRI). Microtiter plates were coated with gt-α-LBP (75 µg/ml) in carbonate-bicarbonate buffer overnight at 4 °C, washed with phosphate-buffered saline + 0.1% Tween (all washes), blocked for 1 h at 37 °C with 10% milk powder in phosphate-buffered saline. Plates were incubated with hLBP, primary human hepatocyte, and HepG2 supernatants for 2 h at 37 °C followed by biotinylated-gt-α-LBP (75 µg/ml) for 1 h at 37 °C, horseradish peroxidase streptavidin (HRP-Streptavidin 43-4323, Zymed Laboratories Inc., San Francisco, CA.) for 30 min at 37 °C and o-phenylenediamine (DAKO S2000, Denmark) for 20 min at room temperature. The reaction was terminated with 4 n H2SO4 and read at 490 nm.

Human fibrinogen (hFIB) and α1-acid glycoprotein (hAGP) were quantitated as above using the p-nitrophenylphosphate chromogen in diethanolamine buffer (Sigma 104–105) (36). Plates were coated with gt-α-hFIB (Cappel 55123, Durham, NC) or gt-α-hAGP (Calbiochem 122163, La Jolla, CA), incubated with hFIB (Sigma 850–80) or hAGP (Calbiochem 112251) and HepG2 supernatants followed by rb-α-hFIB (Calbiochem 341552) or rb-α-hAGP (Accurate Chemical & Scientific Corp. 2357096, Westbury, NY) and detected with alkaline phosphatase-conjugated gt-α-rib-IgG (Tago 8500, Burlingame, CA).

**Total Cellular RNA, DNA, and Protein—**Total RNA, DNA, and protein were precipitated from cytokine-stimulated HepG2 cells disrupted in 5 ml of phosphate-buffered saline for 30 s at 4 °C (Microson Ultrasonic Cell Disruptor XI) according to the modified Ogur-Rosen Procedure (37). RNA was assayed by the orcinol reaction (Orcinol 01875 and RNA Type XI R-6750, Sigma) (38), DNA by the diphenylamine reaction (diphenylamine D-2385 and DNA D-3684, Sigma) (39) and protein by the BCA assay (Pierce Chemical Co., 23225).

**Analysis of Steady State Total RNA from Cytokine-stimulated HepG2 Cells for LBP mRNA—**Total RNA from cytokine-stimulated HepG2 cells was extracted by the method of Chomczynski and Sacchi (41), formamide/formaldehyde denatured, electrophoresed, and transferred to Hybond-N+ (Amersham) (42). The blot was stained with methylene blue, hybridized with [α-32P]dCTP (Amersham PB 10205)-labeled hLBP cDNA (a generous gift of J. Han, TSRI), and autoradiographed (Hyperfilm™MP, Amersham, Sweden).

**Re595 LPS Binding to CHO-hCD14 and CHO-RSV Cells—**Re595 LPS binding was determined by flow cytometry (FACS, Becton Dickinson).

**RESULTS**

**LBP Expression in Primary Human Hepatocytes and HepG2 Hepatoma Cells—**In order to determine if LBP is synthesized by human liver cells, the supernatants of primary cultured human hepatocytes were assayed for the secretion of LBP as a function of time after stimulation and compared with the secretion of the control hepatic acute phase protein, AGP. The results are shown in Table I. LBP is expressed by primary human hepatocytes. Primary hepatocytes in control medium alone roughly halved their rate of LBP and AGP output by 39 h, and a similar effect was seen when the hepatocytes were cul-
up-regulation by a factor of 10 on Day 1. IL-1 and TNF alone or in combination do not show any effect on LBP secretion on Day 1 and >100-fold on Day 2. DEX further enhances IL-6 stimulation. LBP synthesis by IL-6 is >10-fold above baseline level. Thus, primary human hepatocytes synthesize LBP, and LBP expression by these cells is increased in the presence of IL-6. The limited quantities of freshly isolated human hepatocytes precluded their extensive use for additional stimulatory studies, which were continued with HepG2 cells.

Thus, we studied the human hepatoma HepG2 cell line as a model for LBP secretion as others have done for a variety of APPs (18). HepG2 cells were stimulated with IL-1, TNF, IL-6, and DEX. The induction of LBP synthesis by the individual and combined mediators was evaluated as a function of time after stimulation. LBP synthesis by IL-6 is >10-fold above base line on Day 1 and >100-fold on Day 2. DEX further enhances IL-6 up-regulation by a factor of 10 on Day 1. IL-1 and TNF alone or in combination do not show any effect on LBP secretion on Day 1. There is a slight increase in LBP secretion in the presence of IL-1 and TNF when combined with DEX on Day 2, but this is more than two orders of magnitude less than that detected with IL-6 and DEX. Therefore, LBP expression is IL-6 dependent and is maximally augmented by a combination of DEX, IL-1, and TNF.

In order to confirm that the HepG2 cells used in these studies respond to cytokines as previously reported and do not possess a defective IL-1β regulatory site (45), we examined the synthesis of α1-AGP as representative of a Type 1 and FIB as representative of a Type 2 APP. The concentration of LBP, α1-AGP, and FIB was normalized to total cellular DNA after 48 h of treatment (Table II). FIB secretion was increased by IL-6 and inhibited by IL-1 or TNF, while α1-AGP was maximally stimulated by IL-1, IL-6, and DEX (Table II) confirming the ability of these particular HepG2 cells to respond to cytokines according to established patterns of regulation (26, 27). LBP production is up-regulated by IL-6 and is synergistically augmented by DEX, IL-1, and TNF, corresponding to the pattern of induction seen for α1-AGP, a Type 1 APP.

De Novo Synthesis of LBP by Cytokine-induced HepG2 Cells—Immunoprecipitation of metabolically labeled proteins from HepG2 supernatants was performed to determine if the LBP secreted into the supernatant is due to de novo synthesis. The results are shown in Fig. 1. Neither DEX, IL-1, or TNF induced the production of radioiodinated LBP. Only IL-6-stimulated HepG2 cells secreted a 35S-labeled protein with an approximate molecular mass of 60 kDa, characteristic of hLBP (9). Thus, IL-6 induces the production of newly synthesized LBP.

Steady State Determination of mRNA for LBP from Cytokine-stimulated HepG2 Cells—We next asked whether the expression of LBP was reflected by elevated levels of steady state mRNA for LBP. Cellular RNA was isolated from HepG2 cells stimulated with individual cytokines or combinations of mediators. Methylene blue staining for total RNA in each lane revealed comparable concentrations of 18 S and 28 S RNA in each treatment group (Fig. 2a). Northern blot analysis of total RNA for LBP mRNA demonstrated a single band just above the 18 S RNA in the HepG2 cells stimulated with IL-6 alone (Fig. 2b). In addition, there is a synergistic increase in LBP mRNA when IL-6 is present in combination with IL-1 and TNF, reflecting the pattern of cytokine-induced LBP secretion. There was no detectable signal with IL-1, TNF, or DEX alone. In addition, no signal could be detected in Northern blot analysis of total RNA from HepG2 cells stimulated with lower concentrations of IL-6 (102, 10, and 1 unit/ml) or with IL-1 and TNF, reflecting the pattern of cytokine-induced LBP secretion. There was no detectable signal with IL-1, TNF, or DEX alone. In addition, no signal could be detected in Northern blot analysis of total RNA from HepG2 cells stimulated with lower concentrations of IL-6 (102, 10, and 1 unit/ml) or with IL-1 and TNF, reflecting the pattern of cytokine-induced LBP secretion. There was no detectable signal with IL-1, TNF, or DEX alone. Therefore, the steady state level of mRNA for LBP is elevated in IL-6-stimulated cells, and this effect is synergistically augmented by IL-1 and TNF.

Functional Properties of HepG2 LBP—To confirm that the 60-kDa protein expressed by IL-6-stimulated HepG2 cells possessed the characteristic property of hLBP, namely to facilitate the binding of LPS to CD14, we evaluated the capacity of CD14 positive cells to bind FITC-Re595 LPS in the presence of diluted human serum or supernatants from cytokine-stimulated HepG2 cells. There was minimal binding of FITC-Re595 LPS to CHO-hCD14 cells in the presence of supernatant from HepG2

![Fig. 1. Detection of 35S-labeled LBP by immunoprecipitation from cytokine-stimulated HepG2 supernatants.](image-url)

### Table II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>APP supernatant</th>
<th>(-)Dexamethasone</th>
<th>(+)Dexamethasone</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ng LBP/μg DNA</td>
<td>ng Fib/μg DNA</td>
<td>ng AGP/μg DNA</td>
</tr>
<tr>
<td>10² units/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No cytokines</td>
<td>0.2</td>
<td>0.7</td>
<td>19.3</td>
</tr>
<tr>
<td>IL-1</td>
<td>0.4</td>
<td>0.1</td>
<td>23.5</td>
</tr>
<tr>
<td>TNF</td>
<td>0.1</td>
<td>0.2</td>
<td>8.5</td>
</tr>
<tr>
<td>IL-1 + TNF</td>
<td>0.3</td>
<td>0.1</td>
<td>14.9</td>
</tr>
<tr>
<td>IL-6</td>
<td>52.0</td>
<td>477.5</td>
<td>23.0</td>
</tr>
<tr>
<td>IL-6 + IL-1</td>
<td>92.0</td>
<td>104.7</td>
<td>143.4</td>
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<tr>
<td>IL-6 + TNF</td>
<td>74.2</td>
<td>134.5</td>
<td>25.1</td>
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<tr>
<td>IL-6 + IL-1 + TNF</td>
<td>122.6</td>
<td>51.0</td>
<td>181.7</td>
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* Conversion to (ng/μg RNA) × 0.28.
* Conversion to (ng/μg cell protein) × 0.06.
* Conversion to (ng/μg RNA) × 0.31.
* Conversion to (ng/μg cell protein) × 0.06.
cells cultured in 2% MEM alone (Fig. 3a) or 2% MEM + DEX (not shown). FITC-Re595 LPS binding to CHO-hCD14 cells in the presence of IL-6 induced supernatants (Fig. 3e) displayed a positive shift in mean fluorescence as does FITC-Re595 LPS binding to CHO-hCD14 cells in the presence of diluted human serum (Fig. 3b). There is no shift in mean fluorescence when FITC-Re595 LPS is presented to CHO-hCD14 cells in the presence of diluted normal human serum or IL-6-stimulated HepG2 supernatants (not shown), same conditions as for IL-1.

This suggested that the IL-6-induced, 60-kDa protein demonstrated by ELISA and immunoprecipitation is in fact functional LBP. To confirm that LBP was responsible for enhanced binding of FITC-LPS to CHO-hCD14 cells, diluted human serum and the IL-6-induced HepG2 supernatant was incubated with polyclonal goat anti-rhLBP IgG or preimmune polyclonal goat IgG. The binding of FITC-Re595 LPS to CD14 positive cells in the presence of diluted human serum or IL-6-stimulated HepG2 cell supernatant was neutralized by preincubation with polyclonal goat anti-rhLBP antibody (Fig. 3, d and g), but not with preimmune goat IgG (Fig. 3, c and f).

In studies not shown, we established that no binding of FITC-Re595 LPS was detected in the presence of the cytokine-stimulated supernatants using the control transfected line CHO-RSV. We also determined that anti-CD14 monoclonal antibody abolished the ability of LPS to bind to CHO-hCD14 cells in the presence of hLBP or IL-6-stimulated HepG2 supernatants (not shown). These data show that the 60-kDa, immunoprecipitable LBP also possesses the functional capacity of LBP to bind LPS to CD14.

To determine if the synergy between IL-6 and DEX, IL-1, and TNF observed by ELISA was also manifested in the functional assay, the mean fluorescence of CD14 cells in the presence of FITC-Re595 LPS and HepG2 supernatants from cells stimulated with 2% MEM + DEX with either IL-6 + IL-1, or IL-6 + TNF, or IL-6 + IL-1 + TNF was evaluated. The results are shown in Table III. The functional response is also enhanced by DEX, IL-1, and TNF. Thus, the ELISA assays of LBP mass are confirmed by a similar pattern in the functional assays of LBP activity.

**DISCUSSION**

The goal of this study was to determine if human LBP, the plasma-binding protein essential for the delivery of bacterial endotoxin (LPS) to the MØ and for activation of cytokine release in shock is synthesized by human hepatocytes. We have demonstrated here that LBP is synthesized by primary human hepatocytes. The initial level of LBP secretion after explantation is 50 ng/h/10⁶ cells and stabilizes at approximately 22-25 ng/h/10⁶ primary human hepatocytes. The decrease in LBP secretion by control cells between 12 and 39 h may be due to adaptation in the culture environment (31) or to the disappear-
HepG2 cell supernatants are as described in Table II. FACs® assay is used to assess the transfection of HepG2-LPS binding to CHO-hCD14 cells. The volume of HepG2 supernatant used was 10 μl.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean fluorescence*</th>
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<tbody>
<tr>
<td></td>
<td>(d)-Dexamethasone</td>
</tr>
<tr>
<td>pro-&lt;i&gt;g&lt;/i&gt;-LBP</td>
<td>gi-o-rhLBP</td>
</tr>
<tr>
<td>No cytokines</td>
<td>1.16 0.41</td>
</tr>
<tr>
<td>IL-1</td>
<td>0.44 0.23</td>
</tr>
<tr>
<td>TNF</td>
<td>0.77 0.70</td>
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<tr>
<td>IL-1 + TNF</td>
<td>0.39 0.20</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.23 0.18</td>
</tr>
<tr>
<td>IL-6 + IL-1</td>
<td>4.42 0.18</td>
</tr>
<tr>
<td>IL-6 + TNF</td>
<td>4.49 0.18</td>
</tr>
<tr>
<td>IL-6 + IL-1 + TNF</td>
<td>5.18 0.18</td>
</tr>
</tbody>
</table>

* Data reported as mean fluorescence for CHO-hCD14 minus CHO-RSV.

The fact that LBP is both an IL-6-dependent APP and an enhancer of acute phase responses, by enabling cells to respond to LPS and secrete cytokines including IL-6, suggests that in some circumstances such as septic shock, LBP may participate in an autocalytic inflammatory response. It is known that IL-6 levels rise in the peripheral circulation during infections (5–70 ng/ml) (48) and that intravenously administered IL-6 is cleared by the liver (49). The train of events would be that LPS reacts with base-line LBP, the LPS-LBP complexes stimulate MØ and other cells to release IL-6, the IL-6 induces hepatic synthesis of LBP, and the cycle starts over again. With the initial inflammatory response there is an increase in vascular permeability which leads to extravasation of serum proteins, including LBP. Sequestration of LBP in the extravascular space enables macrophages, epithelial cells (50), and smooth muscle cells, to respond to LPS and secrete mediators that could enhance the spread of inflammation locally. If the LPS source is not removed, or if the inflammation becomes sustained by virtue of TNF-α and IL-1 expression, progression to septic shock and multiple organ failure could easily occur.

Since TNF-α appears to be one of the key mediators of endotoxic shock and its release from the MØ is dependent on the delivery of LPS to MØ by LBP, inhibition of IL-6-dependent LBP synthesis may potentially diminish the hyper-responsive inflammatory state. Further elucidation of the molecular mechanisms of LBP regulation may ultimately permit the development of therapeutic interventions to modulate the LPS-LBP-CD14 pathway and attenuate septic shock.

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Human LBP Expression