Lipopolysaccharide signaling induces serum amyloid A (SAA) synthesis in human hepatocytes in vitro

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Abstract To investigate the role of lipopolysaccharide (LPS) in hepatocyte activation, we examined the expression of Toll-like receptor 4 (TLR4), the putative receptor for LPS in human hepatocytes. TLR4 mRNA and protein expression was confirmed in human hepatocytes. Stimulation of human hepatocytes with LPS results in rapid degradation of IkappaB-α and mitogen activated protein kinase activation. Human hepatocytes stimulated by LPS produced serum amyloid A protein. Our data suggest that human hepatocytes utilize components of TLR4 signal transduction pathways in response to LPS and these direct LPS-mediated effects on hepatocytes may contribute to liver inflammation and injury.

Keywords: Hepatocyte; Lipopolysaccharide; Toll-like receptor; Serum amyloid A protein

1. Introduction

Bacterial lipopolysaccharide (LPS), an essential component of the outer membrane of gram-negative bacteria, provokes a generalized proinflammatory response in the infected host that leads to septic shock and multiple organ failure [1]. LPS also causes liver injury [2]. The liver is an immunocompetent organ that plays a key role in the innate immune responses to pathogens [3]. The liver produces both inflammatory mediators and acute-phase reactants and functions to remove pathogens and microbial products from the blood [4]. Although hepatocytes have been reported to respond to LPS, the mechanisms by which LPS stimulates human hepatocytes remain uncertain. It has been suggested that Kupffer cells, as well as other macrophage populations, are responsive to LPS and produce TNF-α and IL-1 that activate hepatocytes [5]. Recently, Toll-like receptors (TLRs), several mammalian *Toll* homologues, have been identified and shown to play important roles in the recognition of various bacterial components [6]. In humans, the TLR family consists of 10 members, all of which are involved in the recognition of pathogen-associated molecular patterns [7]. Among these, Toll-like receptor 4 (TLR4) has been shown to be critical for LPS and endotoxin signaling [8]. TLR receptors have been identified on monocytes, macrophages, Kupffer cells and dendritic cells, and LPS binds to Kupffer cells via TLR4 [9]. LPS activation of Kupffer cells leads to upregulation of inflammatory cytokines, which are involved in liver damage [10]. Therefore, the general view is that the Kupffer cell is the major target of LPS in the liver.

Serum amyloid A protein (SAA) is a major acute-phase protein (APP) produced in the liver after various insults such as infection and inflammation [11]. SAA is an apolipoprotein that belongs to class 1 APP in which IL-1 and IL-6 are the main inflammatory mediators involved in its transcriptional induction [12]. Multiple *cis*-acting elements, including C/EBP and NF-κB, have been found to be important for SAA genes [13]. LPS injection in rabbit results in the activation of C/EBP and NF-κB, which may be responsible for LPS-induced SAA induction [14]. NF-κB and NF-IL6 are involved in the cytokine-induced SAA gene expression [15]. SAF-1 is a zinc finger transcription factor that is essential for cytokine-induced SAA induction [16]. It was demonstrated that MAPK signaling pathway regulated the DNA-binding activity and transactivation potential of SAF-1 and phosphorylation of SAF-1 in response to cytokines was markedly inhibited by MAPK inhibitors [17]. More recently, it was demonstrated that LPS-stimulated SAA protein induction was significantly reduced in TLR4-deificient mice, suggesting the critical role of TLR4 in SSA induction [18]. In the present study, to determine whether hepatocytes respond directly to LPS and produce SAA, we investigated the presence of TLR4, a ligand of LPS, in human hepatocytes.

2. Materials and methods

2.1. Cells

Human primary hepatocytes were purchased from Cell Systems (Kirkland, WA). The cells were cultured in a basal medium composed of Ham's F-12 and Leibovitz L-15 (1:1) medium (Invitrogen, Carlsbad, CA), 0.2% (v/v) bovine serum albumin, 5 mM glucose (Wako Chemical Co Inc., Osaka, Japan), 10^{-8} M dexamethasone (Wako), and 10^{-8} M bovine insulin (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco, Grand Island, NY). These hepatocytes prepara-

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Abbreviations: ERK, extracellular signal-related kinase; IκB-α, IkappaB-α; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; SAA, serum amyloid A protein; TLR, Toll-like receptor

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tions were less than 0.1% reactive with the CD68 monoclonal antibodies (eBioscience, San Diego, CA), indicating that these cells were free of monocyte/macrophage.

2.2. Flow cytometry for determination of TLR4 expression

Adherent human hepatocytes were detached using 1 mM EDTA. Cell surface staining was performed using anti-human TLR4 phycoerythrin (eBioscience, San Diego, CA). Isotype-matched non-bonding control antibodies were used for comparison. Cells were analyzed using a EPCS XL (Coulter, Fullerton, CA). A total of 10 000 immunofluorescent events were acquired for each sample.

2.3. Immunoblot analysis

Whole cell lysates were prepared from LPS-stimulated hepatocytes using Triton lysis buffer containing protease and phosphatase inhibitor (1% Nonidet-P 40, 50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 10 μg/mL aprotinin and 10 μg/mL leupeptin). One hundred micrograms of protein was electrophoresed on 10% SDS polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes and probed by anti-IkappaB-α (IκB-α), phospho-extracellular signal-related kinase (ERK)1/2, phospho-p38 and phospho-JNK1/2 antibodies (1:1000 dilution, Biosource, Camarillo, CA). Hepatocytes culture supernatants were also electrophoresed on 14% polyacrylamide gels. The fractionated proteins were transferred to a nitrocellulose membrane (pore-size: 0.2 μ, Bio-Rad, Hercules, CA) and probed with rabbit anti-SAA antibodies (1:2000 dilution) and developed using an enhanced chemiluminesence (ECL) system (Amersham, Arlington Heights, IL).

2.4. RNA preparation and RT-PCR assay

Total cellular RNA was extracted from hepatocytes using guanidium thiocyanate and phenol (RNAzol B, Cinna/Biotek Labs Int. Inc., Friendswood, TX). First-strand cDNA was synthesized by reverse transcription at 45 °C for 45 min in a 50 μl reaction mixture containing 1 μg of total RNA and MuLV reverse transcriptase (Invitrogen). After denaturing at 99 °C for 5 min followed by cooling at 5 °C, the cDNA was amplified using PCR. Two microliters of denatured cDNA was amplified in a 20 μl final volume containing 1 U *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD), 1 μM of each primer, *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin–Elmer–Cetus, Foster City, CA) using a program of 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final 10 min extension at 72 °C. The amplified products were subjected to electrophoresis on 2% agarose gel.

The specific primers used for TLR4 were
5′-TTGTATTCAAGGTCTGGCTGG-3′ (forward),
5′-GCAACCTTTGAAACTCAAGCC-3′ (reverse).
The predicted size of the fragment was 436 bp.
For *MyD88*:
5′-CCGCGCTGGCGGAGGAGATGGAC-3′ (forward),
5′-GCAGATGAAGGCATCGAAACGCTC-3′ (reverse).
The predicted size of the fragment was 356 bp.
For β-actin:
5′-GACGAGGCCCAGAGCAAGAGAG-3′ (forward),
5′-ACGTACATGGCTGGGGTGTTG-3′ (reverse).
The predicted size of the fragment was 236 bp.
For *SAA1*:
5′-CAGACAAATACTTCCATGCT-3′ (forward),
5′-ATTGTGTACCCTCTCCCCC-3′ (reverse).
The predicted size of the fragment was 303 bp.
For *SAA2*:
5′-CAGACAAATACTTCCATGCT-3′ (forward),
5′-ATTATATGCCATATCTCAGC-3′ (reverse).
The predicted size of the fragment was 328 bp.

3. Results

3.1. Expression of TLR4 in human hepatocytes

We first examined the mRNA expression of the LPS receptor molecule TLR4 and an intracellular adaptor protein for TLR4, *MyD88*, in human hepatocytes. Total RNA was extracted from hepatocytes and THP-1 cells, a monocyte cell line. The expression of TLR4 and *MyD88* genes was analyzed by reverse-transcription PCR. As shown in Fig. 1, TLR4 and *MyD88* mRNA were detected in human hepatocytes. THP-1 cells, which express TLR4 and *MyD88* mRNA, served as a positive control. To assess the protein expression of TLR4 in human hepatocytes, we undertook immunofluorescence staining for the presence of TLR4 in human hepatocytes using flow cytometry. As shown in Fig. 2, significant fractions of human hepatocytes constitutively expressed TLR4. To inves-

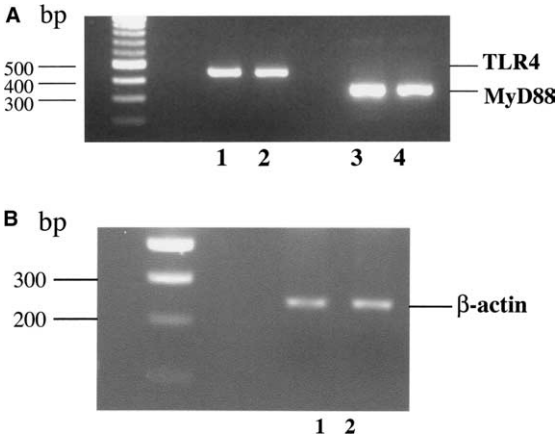

Fig. 1. Expression of TLR4 and *MyD88* mRNA in human hepatocytes. (A) Total RNA was obtained from THP-1 cells (lanes 1 and 3) and human hepatocytes and TLR4 and *MyD88* mRNA were analyzed by PCR following reverse transcription. (B) β-Actin expression in THP-1 cells (lane 1) and human hepatocytes (lane 2) were used as control.

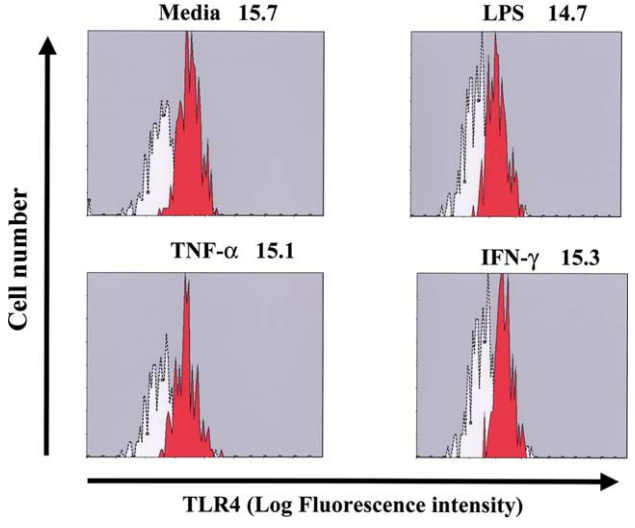

Fig. 2. Effects of cytokines on TLR4 expression on human hepatocytes. Human hepatocytes were treated with media, LPS (100 ng/ml), TNF-α (100 ng/ml) or IFN-γ(100 ng/ml) for 24 h. Expression of TLR4 on hepatocytes was measured by flowcytometer and represented as changes in fluorescence between TLR4 and the isotype control. Dotted line: isotype-matched control Ab. Solid line: TLR4 expression on hepatocytes. Values in upper right of graph represent mean fluorescence intensity of TRL4 expression. The data shown are representatives of at least three independent experiments.

tigate whether TLR4 expression could be regulated by inflammatory stimuli, we examined the effects of LPS, TNF-α and IFN-γ on TLR4 expression in hepatocytes. Human hepatocytes were stimulated with *E. coli* LPS (100 ng/ml), TNF-α (50 ng/ml) or IFN-γ (100 ng/ml) for 24 h, and the expression of TLR4 was analyzed by flow cytometry. TLR4 expression on hepatocytes was not modulated by these stimuli (Fig. 2).

3.2. LPS stimulates IκB-α degradation and MAPK activation

To determine whether LPS stimulation transmits a signal across the cell membrane in hepatocytes, we investigated the effects of LPS on the NF-κB and MAPK. Phosphorylation and subsequent degradation of IκB-α, an inhibitor of NF-κB, result in the activation of NF-κB [19]. To confirm LPS-induced NF-κB activation, we evaluated the IκB-α proteolysis. The protein levels of IκB-α in LPS-treated hepatocytes were measured by immunoblot analysis. LPS stimulation induced substantial IκB-α degradation in a time-dependent manner, suggesting the activation of NF-κB in hepatocytes (Fig. 3). LPS-mediated TLR4 signaling has also been found to trigger the activation of MAPKs. As shown in Fig. 4A, LPS had induced the phosphorylation of ERK1/2 at 15 min after stimulation. Similarly, LPS stimulation resulted in the phosphorylation of p38 (Fig. 4B) and JNK1/2 (Fig. 4C) in human hepatocytes. These results indicate that LPS activates MAPK signaling in human hepatocytes.

3.3. LPS induces SAA secretion from human hepatocytes

To investigate whether the TLR4 expressed on human hepatocytes is functional, the effects of LPS, a natural ligand for TLR4, on human hepatocytes were analyzed. We examined the secretion of SAA, a specific protein produced by hepatocytes. Human hepatocytes were incubated with varying doses of LPS in the presence or absence of 10% FCS for 24 h, and culture supernatants were removed and analyzed by anti-SAA immunoblot. Fig. 5A shows the secretion of SAA from LPS-stimulated human hepatocytes in a dose-dependent manner. LPS did not induce SAA production from human hepatocytes in the absence of serum (Fig. 5C). This result indicates that LPS-induced SAA production in hepatocytes was dependent on the presence of serum, a source of the LPS-binding protein (LBP) that is required for LPS to act through TLR4. Polymyxin B is an antibiotic that binds the lipid A motif of LPS and inactivates its biological function [20]. Preincubation with polymyxin B (100 IU/ml) before stimulation completely inhibited LPS-induced SAA production from human hepatocytes (Fig. 5B). To assess the functional role of TLR4, hepatocytes were incubated with anti-TLR4 monoclonal antibodies (HAT 125, eBioscience) for 6 h before stimulation with LPS. Hepatocytes preincubated with anti-TLR4 resulted in a reduced SAA synthesis, demonstrating that LPS utilizes TLR4 in human hepatocytes (Fig. 5D).

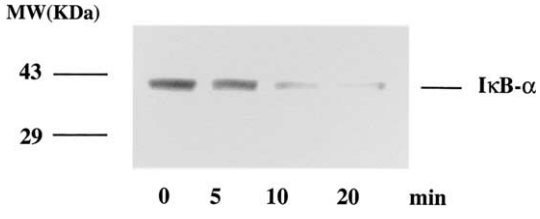

Fig. 3. LPS stimulated IκB-α degradation in hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Cells were lysed and cellular lysates were assessed by anti-IκB-α immunoblot analysis. The data shown are representatives of at least three independent experiments.

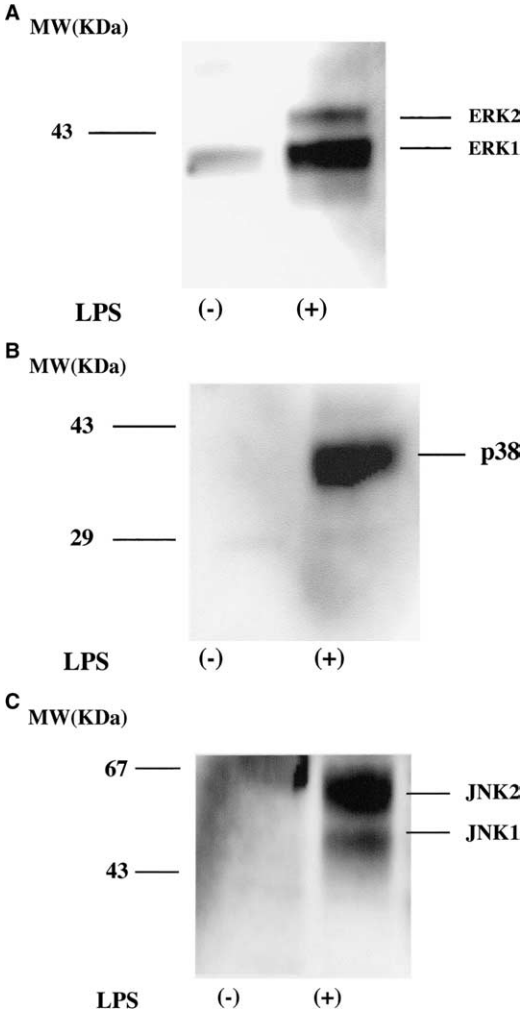

Fig. 4. LPS stimulation induces MAPK activation in hepatocytes. Quiescent human hepatocytes were stimulated with LPS (100 ng/ml) for 15 min. Cells were lysed and cellular lysates were analyzed by immunoblot using anti-phospho-specific ERK1/2 (A), p38 (B) and JNK1/2 (C) antibodies. The data shown are representatives of at least three independent experiments.

To confirm the LPS-stimulated induction of SAA at mRNA levels, we determined the levels of acute-phase SAA (*SAA1, SAA2*) in LPS-stimulated human hepatocytes using RT-PCR methods. Although the expression levels of *SAA1,2* mRNA in unstimulated hepatocytes were below the limit of detection, significant levels of *SAA1,2* mRNA expression were detected after 3 h of LPS stimulation (Fig. 6). In contrast, β-actin mRNA was constitutively expressed in hepatocytes and unchanged by LPS stimulation.

4. Discussion

Lipopolysaccharide (LPS) derived from gut bacteria has been implicated in this liver injury [21]. It has been suggested that LPS exerts an indirect effect on hepatocytes and that

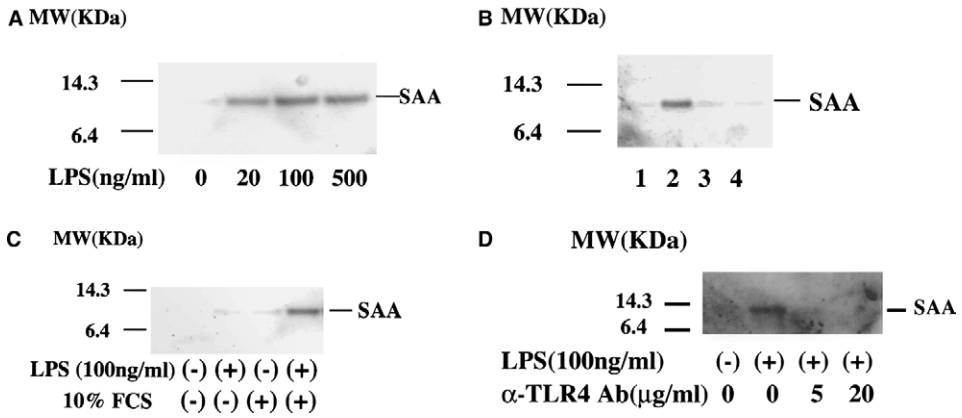

Fig. 5. (A) LPS induces SAA synthesis from human hepatocytes. Human hepatocytes were stimulated with the indicated concentrations of LPS for 24 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representatives of at least three independent experiments. (B) LPS-induced SAA production is inhibited by Polymyxin B. Human hepatocytes were stimulated by LPS (100 ng/ml) for 24 h with or without pretreatment of polymyxin B for 30 min. SAA production was measured by anti-SAA immunoblot using culture supernatants. Lane 1: untreated; lane 2: treated with LPS (100 ng/ml); lane 3: treated with Polymyxin B (2 IU/ml) plus LPS; lane 4: treated with Polymyxin B (10 IU/ml) plus LPS. The data shown are representative of at least two independent experiments. (C) LPS-induced SAA synthesis depends on the presence of serum. Human hepatocytes were stimulated with LPS (100 ng/ml) in the presence or absence of 10% FCS for 24 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representative of at least two independent experiments. (D) Effects of mAbs on TLR4 on the production of SAA by hepatocytes stimulated with LPS. Before the stimulation with LPS, human hepatocytes were pretreated with anti-TLR4 mAbs. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representatives of at least two independent experiments.

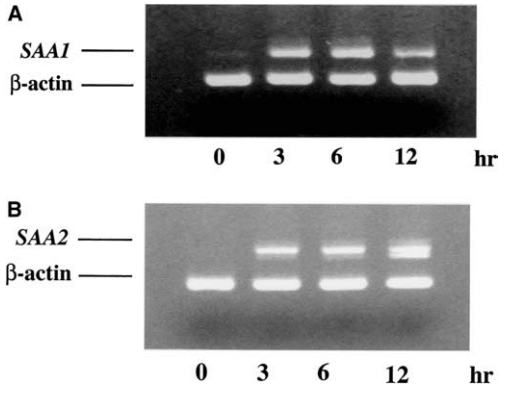

Fig. 6. LPS induces *SAA1,2* mRNA expression in human hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Expression on *SAA1* (A) and *SAA 2* (B) was analyzed by PCR following reverse transcription. β-Actin expression was used as control. The data shown are representatives of at least two independent experiments.

Kupffer cells are the major target of LPS [22]. However, several studies have raised the possibility that hepatocytes respond directly to LPS. Recently, it was demonstrated that murine hepatocytes express TLRs and respond to LPS through the TRL4 receptor pathway [23]. In this study, we demonstrated the mRNA expression of TLR4 and MyD88, a co-receptor for LPS, in human hepatocytes. Furthermore, hepatocytes expressed TLR4 on the cell surface at the protein level. These data indicate that human hepatocytes express LPS recognition molecules and suggest the possibility that hepatocytes respond to LPS directly.

Monocyte/macrophage exposed to LPS show the reduced surface TLR4 expression, which is thought to be one of the mechanisms of LPS tolerance [24]. In our data, TLR4 expression on hepatocytes was not modulated by LPS stimulation. Although the mechanism for the TLR4 downregulation in LPS-tolerant macrophages has not been clarified, these discrepancies in responses to LPS could be due to the differential cell types.

We then focused on the LPS signal transduction cascade in human hepatocytes. Upon binding of LPS to TLR4, MyD88, an adaptor protein, links TLR4 to IL-1R-associated protein kinase (IRAK) and IRAK is phosphorylated [25]. Phosphorylated IRAK dissociates from the receptor complex and activates tumor necrosis factor receptor-associated factor 6 (TRAF-6) [26]. Subsequently, TRAF-6 activates MAPKs and NF-κB to produce cytokines and chemokines [27]. To assess LPS signaling, we investigated whether LPS stimulation results in the activation of these kinases or transcription factors in human hepatocytes. MAPK are a group of serine/threonine protein kinases that participate in transmitting extracellular signals to the cell nucleus. LPS-induced MAPK activation was previously demonstrated in murine hepatocytes [23]. Consistent with these findings, our data indicate that LPS stimulation resulted in the activation of ERK1/2, p38 and c-Jun N-terminal kinase (JNK1/2). NF-κB is also shown to be activated by LPS stimulation. NF-κB is sequestered in an active form in the cytoplasm bound to inhibitor IκB-α. Extracellular stimuli induce IκB kinase (IKK), which degrades IκB [19]. After degradation of IκB, the NF-κB complex moves to the nucleus and activates NF-κB-dependent transcription [19]. The finding that LPS leads to the rapid degradation of IκB-α suggests that LPS stimulation induces IKK activation in human hepatocytes.

Hepatocytes support intra- as well as extrahepatic defense reactions by synthesis of APPs in inflammatory processes including endotoxemia. SAA, one of the APPs, is produced by hepatocytes in response to inflammatory cytokines such as TNF-α, IL-1β and IL-6 [11,28]. We evaluated the effects of LPS on SAA synthesis in hepatocytes. Our results clearly indicate that LPS directly induced SAA mRNA and protein expression in human hepatocytes. This LPS-induced SAA

synthesis was blocked by polymyxin B, a polypeptide antibiotic that inactivates the biological functions of LPS by removing FCS, a source of LPS-binding protein that is required for the interaction between LPS and TLR4 [29]. Recent reports indicated that NF-κB and SAA activating factor (SAF) are essential transcription factors for SAA gene expression [30] and that activation of SAF is mediated by MAPKs [17]. LPS-mediated NF-κB and MAPK activation coordinate the induction of SAA mRNA. SAA can induce chemotaxis of neutrophils [31] and matrix metalloproteinases (MMPs) induction [32], key processes involved in inflammation and tissue destructions. It is possible that human hepatocytes respond to LPS and participate in hepatic inflammation by producing SAA during LPS-induced liver injury.

In conclusion, our data suggest that human hepatocytes are capable of responding to a microbial product, LPS, directly without the mediation of Kupffer cells and macrophages. This LPS-mediated hepatocyte activation could be implicated in the pathogenesis of endotoxin-induced liver injury.

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