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Role of TLR-4 in Liver Macrophage and Endothelial Cell Responsiveness During Acute Endotoxemia

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Abstract

Liver macrophages and endothelial cells have been implicated in hepatotoxicity induced by endotoxin (ETX). In these studies, we analyzed the role of toll-like receptor 4 (TLR-4) in the response of these cells to acute endotoxemia. Treatment of control C3H/OuJ mice with ETX (3 mg/kg, i.p.) resulted in increased numbers of activated macrophages in the liver. This was associated with morphological changes in the cells and a rapid (within 3 hr) induction of nitric oxide synthase-2, cyclooxygenase-2, microsomal PGE synthase-1, interleukin-1 beta and tumor necrosis factor alpha gene expression. In endothelial cells, acute endotoxemia led to increased expression of these genes, as well as 5-lipoxygenase. In contrast, liver sinusoidal cells from C3H/HeJ TLR-4 mutant mice were relatively unresponsive to ETX. Treatment of C3H/OuJ, but not C3H/HeJ mice with ETX, resulted in activation of transcription factors AP-1 and NF-κB in liver sinusoidal cells, which was evident within 3 hr. Whereas in macrophages, transcription factor activation was transient, in endothelial cells, it persisted for 24 hr. In C3H/OuJ mice treated with ETX, activation of p38 MAP kinase was also evident in macrophages and endothelial cells, and JNK kinase in macrophages. In contrast, reduced protein kinase B (AKT) was noted in macrophages. In C3H/HeJ mice, ETX administration also led to activation of p38 MAP kinase in macrophages with no effects on JNK, p44/42 MAP kinase or AKT. These studies demonstrate that liver macrophages and endothelial cells are highly responsive to acute endotoxemia. Moreover, this activity is largely dependent on TLR-4.

Keywords

inflammatory mediators; eicosanoids; MAP kinases; leukotrienes; prostaglandins

Introduction

Lipopolysaccharide (LPS) is a constituent of the cell wall of gram-negative bacteria (Raetz and Whitfield, 2002). It is a complex glycolipid composed of a hydrophilic polysaccharide portion and a toxic hydrophobic domain known as lipid A or endotoxin (ETX). The liver is continuously exposed to ETX via the portal circulation. Liver sinusoidal cells in particular,

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macrophages and endothelial cells, play an important role in clearance of ETX from the blood (Knolle and Gerken, 2000). Excessive levels of ETX can readily overcome this clearance mechanism leading to liver damage (Fujihara et al., 2003). This is thought to be due, in part, to oxidants, eicosanoids and cytotoxic proinflammatory cytokines released by LPS-activated macrophages and endothelial cells (Iredale, 2003; Oda et al., 2000; Su, 2002).

LPS exerts its biological activity by complexing with CD14 and toll-like receptor 4 (TLR-4) (Dobrovolskaia and Vogel, 2002; Fujihara et al., 2003; Raetz and Whitfield, 2002). These receptors belong to a family of pattern recognition receptors important in elimination of microbial pathogens (Aderem and Ulevitch, 2000). Activation of TLR-4 initiates biochemical signaling leading to increased activity of nuclear factor-kappa B (NF- κ B) and activating protein-1 (AP-1) transcription factors and the release of cytokines such as tumor necrosis factor alpha (TNF α) and interleukin-1 β (IL-1 β), as well as nitric oxide, which have been implicated in tissue injury and septic shock (Raetz and Whitfield, 2002). In the absence of functional TLR-4, mice are hyporesponsive to ETX (Hoshino et al., 1999; Qureshi et al., 1999). This is associated with impaired production of inflammatory mediators and protection against ETX-induced injury (Hoshino et al., 1999; Manthey et al., 1994; Vogel et al., 1999).

In the present studies, we analyzed the role of TLR-4 in the response of liver macrophages and endothelial cells to acute endotoxemia. For these experiments, we compared the response of these cells from C3H/HeJ mice, which possess a mutated nonfunctional TLR-4, with control C3H/OuJ mice (Hoshino et al., 1999). Our results demonstrate that murine hepatic nonparenchymal cells contribute to hepatic responsiveness to ETX. Moreover, cellular activities are regulated in large part via TLR-4.

Materials and Methods

Reagents

Collagenase type IV, *Escherichia coli* LPS (serotype 0128:B12) and protein A beads (Sepharose from *Staphylococcus aureus*) were purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-labeled rat anti-mouse F4/80 and anti-mouse CD68 antibodies, FITC-labeled rat IgG control, FITC-labeled goat anti-rat IgG and Leucoperm were from Serotec (Raleigh, NC). Rat anti-mouse MECA32 was from BD Biosciences (San Diego, CA). Polyclonal rabbit anti-mouse p44/42, p38 and JNK MAP kinase and AKT antibodies were obtained from Upstate Cell Signaling (Charlottesville, VA). Horse radish peroxidase (HRP)-conjugated goat anti-rabbit antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Repurification of LPS

LPS was repurified as previously described (Manthey et al., 1994). Briefly, LPS (2.5 mg) was reconstituted in endotoxin-free water (500 μ l) containing 0.2% triethylamine (TEA). Deoxycholate (0.5%, DOC) was added, followed by water-saturated phenol (500 μ l). The sample was then vortexed intermittently for 5 min and allowed to separate at room temperature for 5 min. After an additional 5 min on ice, the sample was centrifuged at 4°C for 2 min at 10,000 $\times$ g. The aqueous layer was transferred to a new tube and the phenol phase re-extracted with 500 μ l of 0.2% TEA/0.5% DOC. The first and second aqueous phases were pooled and re-extracted with one ml of water-saturated phenol. The resulting aqueous phase was adjusted to 75% ethanol with 30 mM sodium acetate, allowed to precipitate at -20°C for one hr and then centrifuged for 10 min, 4°C, at 10,000 $\times$ g. The precipitated pellets were washed with one ml of cold 100% ethanol to remove remaining phenol. Nitrogen gas was used to evaporate the ethanol. LPS powder was resuspended (5 mg/ml) in 0.2% TEA and diluted to 0.25 mg/ml in PBS for animal treatments.

Animals

Male TLR-4-mutant C3H/HeJ mice and control C3H/OuJ mice (8–12 weeks) were obtained from Jackson Laboratory (Bar Harbor, ME). All animals were housed under specific-pathogen-free conditions and allowed free access to sterile water and food. The animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences. To induce acute endotoxemia, mice were administered a single intraperitoneal dose of repurified LPS (3 mg/kg) or PBS control.

Hepatic macrophage and endothelial cell isolation

Mice were euthanized with Nembutal (200 mg/kg). Liver sinusoidal cell isolation was performed as previously described with some modifications (Ahmad et al., 1999). Briefly, the liver was perfused through the portal vein with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks' balanced salt solution (HBSS, pH 7.3) containing 0.5 mM EGTA and 25 mM HEPES, followed by Leibovitz L-15 medium containing 25 mM HEPES and 100 U/ml collagenase type IV for 2 min. All buffers were maintained at 37°C during the perfusion. The liver was then extracted, disaggregated and the resulting cell suspension filtered through 220 μm nylon mesh. Hepatocytes were separated from nonparenchymal cells by three successive washes ($50 \times g$) for 5 min. Nonparenchymal cells were recovered by centrifugation of the supernatant at $300 \times g$ for 7 min. Macrophages and endothelial cells were then purified according to their size and density on a Beckman J-6 elutriator (Beckman Instruments Inc., Fullerton, CA) equipped with a centrifugal elutriation rotor set at 2500 rpm. The pump speed was set at 12 ml/min to load the cells. Endothelial cells were collected at 17 ml/min and macrophages at 33 ml/min. The purity for macrophages and endothelial cells were about 85% as determined by flow cytometry and differential staining.

Flow Cytometry and Immunofluorescence

Macrophages (1×10^6) and endothelial cells (2×10^6) were fixed (15 min, room temperature) in 0.5% paraformaldehyde and washed in PBS containing 1% BSA. For indirect immunofluorescence assays, cells were incubated (30 min, 4°C) with a 1:10 dilution of rat anti-mouse MECA32 antibody followed by incubation (30 min, 4°C) with FITC-labeled goat anti-rat IgG. For direct immunofluorescence assays, FITC-labeled primary antibodies (F4/80 or CD68) or FITC-labeled rat IgG control were used (30 min, 4°C). For analysis of CD68 expression, cells were resuspended in Leucoperm B together with the labeled antibody or control. Cells were then washed in PBS with 1% BSA and fixed in 0.5% paraformaldehyde overnight. Forward-angle light scatter (FLS), side scatter (SS) and cell-associated fluorescence were analyzed on a Coulter Cytomics FC500 (Beckman Coulter). For each analysis, at least 15,000 events were collected and analyzed using CXP software. The percentage positive cells were calculated using Overton cumulative subtraction method of Coulter Cytometrics Software.

Electron Microscopy

Liver tissue (16 mg), macrophages (2×10^6) and endothelial cells (5×10^6) were fixed with 3% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4. The specimens were then treated with 1% osmium tetroxide for one hr, followed by ethanol dehydration in graded steps through propylene oxide, and then embedded in Embed 812 (Electron Microscopy Sciences, Hatfield, PA). One micrometer sections were stained with methylene blue and azure II for light microscopic examination. Ultra-thin sections cut from representative areas were stained with uranyl acetate and lead citrate, and then examined using a Hitachi H7000 transmission electron microscope.

Electrophoretic mobility gel shift assay

Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). Binding reactions were conducted at room temperature for 20 min in a total volume of 20 μ l containing 10 μ g of nuclear extracts, 4 μ l of 5X gel shift binding buffer [20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5], 2 μ g poly dI-dC, and 3×10^4 counts/min/ml [γ -³²P]ATP (3,000 Ci/mmol at 10 mCi/ml)-labeled probe containing NF- κ B (AGT TGA GGG GAC TTT CCC AGG C) or AP-1 (CGC TTG ATG ACT CAG CCG GAA) consensus oligonucleotides (Santa Cruz Biotechnologies, Santa Cruz, CA). Protein-DNA complexes were separated on 7% non-denaturing polyacrylamide gels run at 250 V in $0.5 \times$ TBE (45 mM Trisborate and 1 mM EDTA, pH 8.0), and visualized after the gels were dried and autoradiographed. For supershift assays, the reaction mixtures were pre-incubated at room temperature for 30 min with one μ g of antibody to NF- κ B (p65 or p50) or AP-1 (cJun or cFos) subunits prior to the addition of labeled oligonucleotide. For competition assays, the reaction mixtures were incubated with a 40-fold excess of the respective unlabeled oligonucleotide for 30 min prior to analysis.

cDNA synthesis and real time PCR analysis

Cells were stored in RNA LATER solution (Ambion) at -20°C until RNA isolation. DNase I-treated total RNA was extracted using a RNeasy Miniprep kit (Qiagen Inc, Valencia, CA) following the manufacturer's instructions. RNA was quantified using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). For cDNA synthesis, RNA (0.2 μ g) in 9 μ l RNase-free water was denatured at 65°C for 4 min, rapidly cooled on ice, and then resuspended in a 20 μ l final volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM of each dNTP, 20 mM random hexamers and 200 U Superscript II RNase H⁻ RT (Invitrogen, Carlsbad, CA). After one hr incubation at 37°C , RNase H⁻ (2 U) was added and the samples incubated for an additional 20 min. The samples were then denatured at 95°C for 5 min and chilled on ice.

Real time quantitative PCR was performed using the ABI Prism 7000 Sequence Detection System. Each reaction contained 0.01 μ g cDNA template, forward and reverse primers and SYBR Green PCR master mix in a 25 μ l final volume. The thermal cycling parameters were set for the following conditions: one 2-min cycle at 50°C , one 10-min cycle at 95°C (for AmpliTaq gold enzyme activation), and forty 15 s at 95°C with 1-min at 60°C cycles.

Normalization for the relative quantity of mRNA was accomplished by comparison to 18S rRNA. The primers used were: COX-2, GTCTGGTGCCTGGTCTGATGA and CACTCTGTTGTGCTCCCGAAG; NOS-2, GGCAGCCTGTGAGACCTTTG and GCATTGGAAGTGAAGCGTTTC; TNF α , AAATTCGAGTGACAAGCCGTA and CCCTTGAAGAGAACCTGGGAGTAG; IL-1 β , CCAAAGATGAAGGGCTGCT and TCATCTGGACAGCCCAGGTC; mPGE-1, GGCCTTCTGCTCTGCAGC and GCCACCGCGTACATCTTGAT; mPGE-2, AGCCCCTGGAAGAGGTCATC and CATTCATGGCCTTCATGGGT; 5-LOX, CAGGGAGAAGCTGTCCGAGT and GCAGAGGCCGTGAAGATCAC; 12-LOX, ACCAGCAAGGACGACGTGAC and ATCAGGTAGCGACCCCATCA; and 15-LOX, TCGGAGGCAGAATTCAAGGT and CAGCAGTGGCCCAAGGTATT.

Western Blotting

Cells were lysed in buffer containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 1% protease inhibitor and 1% phosphatase inhibitors in PBS. Lysates were clarified by centrifugation at 16,000 g for 10 min at 4°C . Protein concentrations were measured using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Samples were fractioned on

10% SDS-polyacrylamide gels and transferred to nitrocellulose (Amersham Biosciences, Piscataway, NJ). Nonspecific binding was blocked using 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-Tween 20). The membrane was then incubated overnight (4°C) with primary antibody in 1% milk (in TBS-Tween 20); washed for one hr using TBS-Tween 20, and then incubated for one hr with secondary antibody (1:5000) in 2.5% milk (in TBS-Tween 20). Antibody binding was visualized by autoradiography using enhanced chemiluminescence ECL detection reagents (Amersham Life Science, Arlington Heights, IL). The dilutions of primary antibodies were as followed: p38, p44/42 and JNK MAP kinases and AKT (1:1300); and phospho-p38, phospho-p44/42 and phospho-JNK MAP kinases and phospho-AKT (1:1000). Ten micrograms of protein were loaded onto each lane of the gel.

Statistics

All experiments were repeated at least three times using four mice per treatment group. Data were analyzed by one-way analysis of variance using SigmaStat 3.5. A *P* value of ≤ 0.05 was considered statistically significant.

Results

Effects of acute endotoxemia on the physical and antigenic properties of liver macrophages and endothelial cells

Initially, we analyzed the effects of acute endotoxemia on the morphology of liver macrophages and endothelial cells *in situ*. Examination of liver sections from untreated C3H/OuJ mice by electron microscopy revealed a relatively large nuclear region and numerous cytoplasmic vacuoles in both macrophages and endothelial cells (Fig. 1). Endothelial cells also displayed long thin attenuated cytoplasmic processes. The greatest morphologic effects of ETX administration were observed 3 hr post treatment (Fig. 1, panels C and D). At this time increased numbers of vacuoles were observed in macrophages and endothelial cells. In endothelial cells, lipid droplets were also evident, suggesting cellular damage (Van Bossuyt and Wisse, 1988). Macrophages and neutrophils containing lysosomal granules were also apparent in the sinusoids. By 24 hr post treatment (Fig. 1, panels E and F), the morphology of the cells resembled control.

We next analyzed the morphology of macrophages and endothelial cells isolated from livers of control and ETX treated mice. Light microscopic analysis indicated that macrophages from both control and ETX treated mice were in general, larger than endothelial cells and possessed a greater cytoplasmic to nuclear ratio (Fig. 2, panels A–D). After 6 hr incubation (Fig. 2, panels E–H), both macrophages and endothelial cells flattened and spread on the culture dishes, and exhibited stellate morphology. Following ETX treatment of the mice, both freshly isolated and cultured macrophages appeared more irregularly shaped. In contrast to macrophages, no major structural changes were noted in endothelial cells after ETX administration. Electron microscopic evaluation confirmed that freshly isolated macrophages were relatively round with projecting microvilli and pseudopodia, while endothelial cells appeared as clusters of folded cell processes around the cell body (Fig. 3). Macrophages and endothelial cells isolated from ETX treated mice were significantly more vacuolated than cells from control animals and displayed increased lysosomal density. The folded processes of the endothelial cells were also less attenuated and contracted into the cell body.

In further studies, we analyzed the effects of acute endotoxemia on the physical properties of liver macrophages and endothelial cells by flow cytometric assessment of their light-scattering properties. In general, light scattered in the forward-angle direction is proportional to the size of the cells, and in the right-angle direction (side scatter), to the density or granularity of the cells. Flow cytometric analysis revealed one relatively homogeneous population of endothelial

cells (Fig. 4, left panel). In contrast, two subpopulations of macrophages were identified (Fig. 4, left panel); one population ($58 \pm 0.02\%$) that was relatively small in size and density (subpopulation 1) and a second population ($42 \pm 0.02\%$) that was larger and more dense (subpopulation 2). Whereas ETX treatment of mice had no major effects on the light-scattering properties of endothelial cells (Fig. 4), marked changes were noted in the macrophages (Fig. 4). Thus, following ETX administration, the percentage of macrophages in subpopulation 1 increased, while the percentage of cells in subpopulation 2 decreased. This became evident with ETX treatment. At this time, $70 \pm 0.9\%$ of the macrophages were contained within subpopulation 1, and $30 \pm 0.9\%$ in subpopulation 2.

To confirm their identity, cells were stained with antibodies to the macrophage markers, F4/80 and CD68 (Gordon, 1999; Rabinowitz and Gordon, 1991), or the endothelial cell marker, MECA32 (Penn et al., 1993), and then analyzed by flow cytometry. Approximately 85% of macrophages from control animals stained positively for F4/80, and 80% for CD68 (Fig. 5). Binding of F4/80 to the cells was homogeneous, indicating that both macrophage subpopulations expressed similar levels of this antigen. In contrast, two populations of macrophages expressing relatively low and high levels of CD68 were identified. Whereas the low CD68⁺ cells were distributed equally in subpopulations 1 and 2, the majority of cells (70%) expressing high levels of CD68 were from the smaller and less dense macrophage population (subpopulation 1). Interestingly, approximately 40% of liver macrophages were also found to express low levels of the endothelial cell marker, MECA32. The majority of these cells were from subpopulation 2, the larger denser macrophage population. Treatment of mice with ETX had no major effects on expression of F4/80, CD68 or MECA32 by the macrophages (Fig. 5). We also analyzed expression of these antigens on liver endothelial cells. The majority of endothelial cells (85%) from both control and ETX treated mice were identified as positive for MECA32. Endothelial cells were also found to express F4/80 and CD68; however the percentage positive cells and the intensity of fluorescence were reduced when compared to macrophages. Thus, about 25% of the endothelial cells were positive for F4/80 and 65% for CD68. As observed in macrophages, ETX administration had no effect on expression of these antigens by endothelial cells.

Role of TLR-4 in liver macrophage and endothelial cell responsiveness to ETX

We have previously demonstrated that acute endotoxemia is associated with functional activation of liver macrophages and endothelial cells (Ahmad et al., 2002; Feder and Laskin, 1994; McCloskey et al., 1992; Pilaro and Laskin, 1986). To determine if TLR-4 plays a role in the response of these cells to ETX, we compared C3H/OuJ mice with C3H/HeJ mice, which possess a mutated nonfunctional TLR-4 (Hoshino et al., 1999). As observed in C3H/OuJ mice, two subpopulations of liver macrophages were identified in control C3H/HeJ mice that differ with respect to size and density (Fig. 4, right panel). Subpopulation 1 ($57 \pm 1.2\%$) was relatively small in size and density, while subpopulation 2 ($43 \pm 1.2\%$) was larger and more dense. In contrast to C3H/OuJ mice, ETX had no major effects on macrophages from C3H/HeJ mice (Fig. 4). Flow cytometric analysis also revealed one relatively homogeneous population of endothelial cells from control and ETX treated C3H/HeJ mice, which is similar to findings in C3H/OuJ mice.

We also analyzed the effects of ETX on the phenotype of macrophages and endothelial cells from TLR-4 mutant mice. As observed in cells from C3H/OuJ mice, in cells from both control and ETX treated C3H/HeJ mice; one homogenous population of F4/80⁺ macrophages was identified whereas two subpopulations of macrophages, binding low and high levels of CD68 were noted (Fig. 6). Moreover, approximately 45% of liver macrophages from the mutant mice were also found to express the endothelial cell marker, MECA32. Endothelial cells from C3H/HeJ mice, like C3H/OuJ mice, were highly positive (80%) for MECA32. These cells also

expressed F4/80 and CD68 although at reduced level when compared to macrophages. Thus, approximately 40% of endothelial cells were positive for F4/80 and 20% for CD68. ETX administration had no effect on expression of these markers in endothelial cells from C3H/HeJ mice.

The effects of acute endotoxemia on the number of macrophages and endothelial cells recovered from the liver were also assessed. ETX administration to C3H/OuJ resulted in a twofold increase in the number of macrophages recovered from the liver 48 hr post treatment when compared to control mice (Table 1). In contrast, although two- to three-fold greater numbers of endothelial cells were recovered from the liver relative to macrophages, ETX had no effect on numbers of these cells. In C3H/HeJ mice, ETX had no effect on number of macrophages or endothelial cells recovered from the livers (Table 1). Microscopic analysis of macrophages and endothelial cells isolated from C3H/HeJ mice treated with ETX also showed no major changes when compared to control mice (data not shown).

Effects of acute endotoxemia on inflammatory gene expression

In further studies, we analyzed the role of TLR-4 in macrophage and endothelial cell mRNA expression of nitric oxide synthase-2 (NOS-2), TNF α and IL-1 β , inflammatory proteins important in the liver response to ETX (Jirillo et al., 2002). Induction of acute endotoxemia was associated with a rapid and transient increase in expression of these genes in both macrophages and endothelial cells from control C3H/OuJ mice. This was evident within 3 hr and decreased toward control by 24 hr (Fig. 7). Whereas macrophages expressed greater levels of NOS-2 and TNF α , when compared to endothelial cells, levels of IL-1 β were similar in the two cell types. In C3H/HeJ mice, acute endotoxemia had minimal effects on expression of these inflammatory mediators.

Cyclooxygenase-2 (COX-2), microsomal PGE synthases (mPGES-1 and mPGES-2) and lipoxygenases (5-LOX, 12-LOX and 15-LOX) are enzymes mediating the biosynthesis of eicosanoids, which are important in liver inflammation and cellular proliferation during acute endotoxemia (Titos et al., 2005; Uematsu et al., 2002). Treatment of C3H/OuJ mice with ETX resulted in a marked induction of COX-2 and mPGES-1 mRNA expression within 3 hr in both macrophages and endothelial cells (Fig. 8). These effects were transient returning to control by 24 hr. 5-LOX mRNA was also found to increase after ETX administration but only in endothelial cells. In contrast, 15-LOX mRNA expression decreased (7-fold) in endothelial cells, which was evident within 3 hr post ETX administration. Acute endotoxemia had no significant effects on mPGES-2 or 12-LOX mRNA expression in either cell type. In general, in C3H/HeJ mice, no effects were observed in the expression of eicosanoid metabolism genes, with exception to mPGES-1, which increased 12-fold in macrophages and 3-fold in endothelial cells after ETX administration (Figs. 7 and 8).

Effects of acute endotoxemia on NF- κ B and AP-1 nuclear binding activity

The transcription factors NF- κ B and AP-1 are known to regulate expression of variety of genes involved in inflammation and immune responses, including IL-1 β , TNF α , NOS-2 and COX-2 (Blackwell and Christman, 1997; Karin et al., 1997). In further studies, we determined if acute endotoxemia was associated with altered nuclear binding activity of these transcription factors in macrophages and endothelial cells, and if this was dependent on TLR-4. Constitutive NF- κ B and AP-1 nuclear binding activity was detectable in macrophages and endothelial cells from control C3H/OuJ mice (Fig. 9). In both cell types, ETX administration resulted in a rapid increase in nuclear binding of the transcription factors, which was noted within 3 hr. Whereas in macrophages, transcription factor activity subsequently returned to control levels, in endothelial cells, NF- κ B and AP-1 binding activity persisted for at least 24 hr. Note that in both macrophages and endothelial cells, ETX altered the migration of AP-1 in the gels. This

is likely due to ETX-induced alterations in the composition of proteins in the AP-1 complex (Karin et al., 1997). This might contribute to different functional activities in these cells. NF- κ B and AP-1 nuclear binding activity was blocked by incubating the samples with 40-fold excess of the respective unlabeled probes, demonstrating the specificity of the probes. Moreover, supershift assays using antibodies to p50 and p65 blocked NF- κ B binding activity. Similarly, c-Jun antibody partially blocked AP-1 binding activity. As observed in C3H/OuJ mice, in C3H/HeJ TLR-4 mutant mice, low constitutive NF- κ B and AP-1 nuclear binding activity was detectable in both macrophages and endothelial cells (Fig. 9). However, acute endotoxemia had no effect on this activity.

Effects of acute endotoxemia on mitogen activity protein (MAP) kinase and protein kinase B (AKT) expression in macrophages and endothelial cells

MAP kinases and AKT are signaling molecules important in regulating expression of inflammatory genes (Guha and Mackman, 2002; Saklatvala, 2004). In further studies, we analyzed the effects of acute endotoxemia on expression of c-Jun NH₂-terminal kinase (JNK), extracellular signal-regulated kinase 1 and 2 (p44/42) and p38 MAP kinase, and AKT in liver sinusoidal cells. Freshly isolated liver macrophages and endothelial cells from both C3H/OuJ and C3H/HeJ mice were found to constitutively express each of the MAP kinase proteins, as well as AKT (Fig. 10). ETX administration had no major effects on expression of these proteins in either cell type or mouse strain. In macrophages from C3H/OuJ mice, ETX administration caused an increase in phospho-JNK and phospho-p38 MAP kinase. In these cells, phospho-p44/42 MAP kinase and phospho-AKT were constitutively elevated in both control and endotoxin-treated mice. In contrast, in endothelial cells from C3H/OuJ mice, all three MAP kinases and AKT were constitutively activated. Moreover, while ETX transiently increased activation of p38 MAP kinase in endothelial cells at 3 hr, p44/42 MAP kinase and AKT transiently decreased. The MAP kinases and AKT were also constitutively activated in the macrophages and endothelial cells from C3H/HeJ mice. ETX administration resulted in an increase in phospho-p38 MAP kinase in macrophages, which was evident at 24 hr. No further changes in activation of the MAP kinases or AKT were observed in endothelial cells from C3H/HeJ mice following endotoxin administration.

Discussion

Liver macrophages and endothelial cells have been implicated in the pathophysiology of ETX-induced hepatic injury (Oda et al., 2000; Su, 2002). This is due in part to excessive production of inflammatory mediators by these cells. In the present studies, we analyzed the role of TLR-4 in the response of these liver sinusoidal cells to acute endotoxemia. Both macrophages and endothelial cells were found to undergo a process of cellular activation after *in vivo* exposure to ETX. This was characterized by physical and functional alterations, including increased expression of proinflammatory and cytotoxic mediators and regulatory signaling molecules. However, these responses were not observed or were greatly reduced in C3H/HeJ mice, which possess a mutated nonfunctional TLR-4. These findings demonstrate that TLR-4 signaling plays an important role in ETX-induced activation of liver nonparenchymal cells.

Light and electron microscopy revealed that macrophages from control mice were larger than endothelial cells, and exhibited greater cytoplasmic to nuclear ratio. Moreover, whereas macrophages possessed short microvilli, long thin fenestrated cytoplasmic extensions were noted in endothelial cells. These morphologic differences are consistent with previous reports on macrophages and endothelial cells from rat and human liver (Carr, 1973; Gendault JL 1988). After incubation for 6 hr, both macrophages and endothelial cells flattened and spread on the culture dishes and displayed stellate morphology. Similar findings have been described in rat liver macrophages and endothelial cells (McCloskey et al., 1992). Like rat macrophages

(Ahmad et al., 1999), mouse macrophages were heterogeneous with respect to size and density. These results are in accord with reports of macrophage heterogeneity within the liver lobules (Kono et al., 2002; Laskin et al., 2001a). Macrophage heterogeneity was also observed in antigen expression. Thus, subpopulations of macrophages that expressed relatively high and low levels of CD68 were identified. Flow cytometric analysis of these cells demonstrated that the high CD68 expressing cells consisted mainly of small macrophages, which are thought to reside in centrilobular regions of the liver (Bouwens et al., 1986; Sleyster and Knook, 1982). It has been suggested that CD68 is important in scavenging oxidized low-density lipoprotein (oxLDL) (Kurushima et al., 2000). Higher expression of CD68 on macrophages in the centrilobular regions of the liver may facilitate the removal of oxLDL from the portal circulation. F4/80 is highly expressed on mature macrophages with little subpopulation heterogeneity (Hirsch et al., 1981). Similarly we found that F4/80 expression on resident liver macrophages was homogeneous. Interestingly, endothelial cells were found to express CD68 and F4/80 antigens, although at reduced levels when compared to macrophages. These findings, together with our observation that macrophages express low levels of the endothelial cell protein MECA32, provide support for the idea that liver macrophages and endothelial cells share some similar phenotypic characteristics (Knolle et al., 1999; McCloskey et al., 1992).

Although treatment of mice with ETX had no major effects on CD68, F4/80 or MECA32 expression by macrophages or endothelial cells, morphological evidence of macrophage activation was noted, which is consistent with previous studies in rats (McCloskey et al., 1992; Van Bossuyt and Wisse, 1988). Thus, macrophages isolated from ETX treated mice were significantly more vacuolated than cells from control animals and displayed increased lysosomal density. We also noted increases in the percentage of a small dense macrophage population (subpopulation 1) after ETX administration. In previous studies with rats, the smaller dense macrophages were shown to be activated to produce increased amounts of cytokines and oxidants when compared to the larger macrophages and it is likely that these cells function similarly in mice (Ahmad et al., 1999; Itoh et al., 1992). Consistent with previous studies in rats, ETX administration to C3H/OuJ mice was found to be associated with an increase in the number of macrophages, but not endothelial cells, recovered from the liver (Ahmad et al., 1999; McCloskey et al., 1992; Van Bossuyt and Wisse, 1988). This is most likely due to infiltration of macrophages into the liver from blood and bone marrow precursors.

To assess the role of TLR-4 in the liver nonparenchymal cell response to ETX, we used C3H/HeJ TLR-4 mutant mice. In contrast to C3H/OuJ mice, ETX had no major effects on the light scattering properties of macrophages from C3H/HeJ mice or the number of macrophages recovered from liver. These data suggest that TLR-4 plays an important role in leukocyte trafficking into the liver during acute endotoxemia. This is supported by studies demonstrating that the lack of functional TLR-4 impairs processes required for recruitment of leukocytes into the liver, lung and muscle (Andonegui et al., 2002; Kerfoot and Kubes, 2005).

NF- κ B and AP-1 are important transcriptional regulators of inflammatory genes, a number of which have been implicated in ETX-induced hepatotoxicity (Cohen, 2002; Fujihara et al., 2003; Su, 2002). The present studies demonstrate that both NF- κ B and AP-1 nuclear binding activity are upregulated in liver macrophages and endothelial cells within 3 hr of ETX administration to C3H/OuJ mice. A similar rapid activation of NF- κ B and AP-1 in response to ETX has been previously described in intact rat liver (Li et al., 2006). Interestingly, lower constitutive levels of NF- κ B and AP-1 activity were detected in both liver macrophages and endothelial cells from control mice. This is most likely due to continuous exposure of these cells to gut-derived ETX through the portal circulation.

In C3H/OuJ mice, increased NF- κ B and AP-1 nuclear binding activity after ETX administration was correlated with increased expression of NOS-2, IL-1 β , TNF α , COX-2 and

mPGES-1 mRNA in both macrophages and endothelial cells. Interestingly, whereas ETX-induced inflammatory gene expression and transcription factor activity returned to control levels in macrophages after 3 hr, in endothelial cells they persisted for at least 24 hr. These findings are consistent with the idea that endothelial cells play a more prominent role late in the inflammatory response to ETX (Knolle and Gerken, 2000). Recent studies have shown that inhibition of NF- κ B prevents ETX-induced inflammatory mediator production, including TNF α and IL-6, in intact liver (Li et al., 2006). These data suggest that inflammatory gene expression in liver sinusoidal cells is dependent on activation of NF- κ B. Proteins encoded by inflammatory genes, including NOS-2, COX-2, TNF α and IL-1 β aid in host defense against microbial infection and may also contribute to tissue injury (Cohen, 2002; Laskin et al., 2001b; Lin and Yeh, 2005). Prostaglandins generated via COX-2 and mPGES-1 are also involved in vasodilation, chemotaxis and the generation of inflammatory cytokines (Fahmi, 2004; Park and Christman, 2006). Findings that these genes are upregulated in liver macrophages and endothelial cells provide support for the idea that these cells are important effectors of ETX-induced hepatic inflammation. Of note is our observation that hepatic endothelial cells expressed similar levels of TNF α as macrophages. TNF α has been reported to be primarily expressed by activated monocyte/macrophages (Xiong and Hales, 1993). These data support the idea that endothelial cells and macrophages in the liver exhibit similar phenotypic and functional reactivity during acute endotoxemia. Our findings that COX-2 and mPGES-1, but not mPGES-2, are upregulated by ETX are in accord with studies in isolated rat liver macrophages treated with LPS (Bezugla et al., 2006).

The present studies also showed that 5-LOX mRNA expression is increased after ETX administration, but only in endothelial cells. A similar lack of effect of LPS on 5-LOX has been described in cultured alveolar and peritoneal macrophages (Brock et al., 2003; Coffey et al., 2000). 5-LOX catalyzes the production of leukotrienes, which are known to be important in leukocyte activation, promoting adhesion of these cells to vascular endothelium, and stimulating chemotaxis and the production of proinflammatory cytokines (Goetzl et al., 1995; Peters-Golden et al., 2005). The observation that 5-LOX is expressed in liver endothelial cells provides additional support for the idea that these cells play a role in recruitment and activation of leukocytes into liver tissue during acute endotoxemia (Kerfoot and Kubes, 2005; Oda et al., 2000). Of interest is our finding that 15-LOX decreases in endothelial cells after ETX administration. Leukotrienes produced via 15-LOX have been reported to inhibit neutrophil migration across cytokine-activated endothelium, and to block the generation of superoxide anion (Kuhn et al., 2002). These data suggest that 15-LOX is important in limiting the inflammatory response and it may play a similar role in the liver.

In contrast to C3H/OuJ mice, ETX administration to C3H/HeJ mice had minimal effects on NF- κ B and AP-1 nuclear binding activity, or on NOS-2, IL-1 β , TNF α and COX-2 mRNA expression in macrophages and endothelial cells. Similar defects in LPS-induced inflammatory genes (mPGES-1, TNF α , COX-2) and nuclear transcription factor binding activity (NF- κ B) have been described in peritoneal macrophages from C3H/HeJ mice, confirming the importance of TLR-4 in ETX responsiveness (Uematsu et al., 2002; Vogel and Fenton, 2003). Interestingly, mPGES-1 was induced to a relatively small extent in both cell types from these mice. This suggests that multiple signaling pathways regulate expression of this gene in hepatic nonparenchymal cells. Of note, low levels of constitutive NF- κ B and AP-1 binding activity were detected in C3H/HeJ mice indicating that this activity may not require TLR-4 signaling during homeostasis.

MAP kinases and AKT are signaling molecules important in regulating expression of inflammatory genes, including NOS-2 and COX-2 (Chen et al., 1999; Saklatvala, 2004; Schabbauer et al., 2004). Interestingly, these kinases were constitutively active in macrophages and endothelial cells from C3H/HeJ mice and, in general, not altered by ETX administration.

These data suggest that TLR-4 is only partially involved in expression and activation of these kinases. Lack of functional TLR-4 may be compensated by the presence of IL-1R. These receptors contain homologous cytoplasmic domain and thus, may induce similar intracellular pathways culminating in constitutive MAP kinases and AKT activation (Aderem and Ulevitch, 2000; Martin and Wesche, 2002; Qureshi et al., 1999). In macrophages and endothelial cells from C3H/OuJ mice, p44/42 MAP kinase and AKT were also constitutively activated whereas JNK was activated only in endothelial cells. Administration of ETX was found to activate JNK and p38 MAP kinases in macrophages, and of p38 MAP kinase in endothelial cells. A decrease was also noted in activated p44/42 MAP kinase and AKT in endothelial cells. These data indicate that only JNK in macrophages and p38 in macrophages and endothelial cells are modulated by ETX in C3H/OuJ mice and that these enzymes may be required for functional activation of the cells. PI3-kinase-AKT pathway has been shown to negatively regulate LPS-induced expression of TNF α and NOS-2. Decreased activated AKT in endothelial cells may mediate the increases in inflammatory gene expression observed in these cells in our studies (Park et al., 1997; Schabbauer et al., 2004). Taken together, it appears that in macrophages ETX-induced TLR-4 signaling pathways involves JNK MAP kinase, while in endothelial cells p38 MAP kinase is important.

TLR-4 participates in host defense against invading pathogens (Aderem and Ulevitch, 2000). Our data provides evidence that ETX utilizes TLR-4 for activation of intracellular signals, including MAP kinases and nuclear binding factors (NF- κ B and/or AP-1) and upregulation of inflammatory genes in liver nonparenchymal cells. Moreover, TLR-4 appears to be required for recruitment of macrophages into the liver during acute endotoxemia. These findings suggest that TLR-4 may be a useful target for developing therapeutic interventions aimed at controlling ETX-induced liver injury.

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Figure 1.

Transmission electron microscopy of liver sinusoidal cells. Livers, collected 3 hr and 24 hr after treatment of C3H/OuJ mice with endotoxin (ETX) (panels C–F) or control (CTL) (panels A–B) were processed for electron microscopy as described in the Materials and Methods. Hepatocyte, H; macrophage, MP; endothelial cell, EC; vacuole, V; neutrophil, N.

Figure 2.

Effects of ETX on liver macrophage and endothelial cell morphology. Cells, isolated from livers 24 hr after treatment of C3H/OuJ mice with endotoxin (ETX) or control (CTL), were stained with Geimsa and photographed (40x magnification). *Panels A–D:* Cytospin preparations of cells were analyzed immediately after isolation. *Panels E–H:* Cells were cultured (2.5×10^5 /well, 8-well slide chambers) in DMEM medium containing 10% FBS for 6 hr prior to analysis.

Figure 3.

Transmission electron microscopy of liver macrophages and endothelial cells. Cells, isolated 24 hr after treatment of C3H/OuJ mice with ETX or CTL, were fixed with glutaraldehyde and processed for electron microscopy. Inset, representative enlarged macrophage or endothelial cell.

Figure 4.

Characterization of liver endothelial cells by flow cytometry. Cells, isolated from livers of C3H/OuJ or C3H/HeJ mice 48 hr after administration of ETX or control (CTL), were analyzed by flow cytometry according to their light-scattering properties. One representative histogram for each time point from 4 separate experiments is shown. FLS, forward-angle light scatter; SS, side scatter.

Figure 5.

Antigen expression by liver macrophages and endothelial cells from C3H/OuJ mice. Cells, isolated from livers of C3H/OuJ mice 24 hr or 48 hr after administration of ETX or control (CTL), were stained with antibodies to F4/80, CD68 or MECA32 or IgG control and then analyzed by flow cytometry. One representative from 2–3 separate experiments is shown.

Figure 6.

Antigen expression by liver macrophages and endothelial cells from C3H/HeJ mice. Cells, isolated from livers of C3H/HeJ mice 48 hr after administration of ETX or control (CTL), were stained with antibodies to F4/80, CD68 or MECA32 or IgG control and then analyzed by flow cytometry. One representative from 2–3 separate experiments is shown.

Figure 7.

Effects of acute endotoxemia on inflammatory gene expression. Macrophages and endothelial cells were isolated from livers of C3H/OuJ (white bars) or C3H/HeJ (black bars) mice 3 or 24 hr after administration of ETX or control (CTL). NOS-2, IL-1 β and TNF α mRNA expression were quantified by real-time PCR. Data are presented as fold increase over CTL. Each bar is the mean $\pm$ SE (n=8–12) from 3 separate experiments. *Significantly different (p<0.05) from CTL.

Figure 8.

Effects of acute endotoxemia on expression of genes involved in eicosanoid metabolism. Macrophages and endothelial cells were isolated from livers of C3H/OuJ (white bars) or C3H/HeJ (black bars) mice 3 or 24 hr after administration of ETX or control (CTL). mRNA expression for the indicated genes was quantified by real-time PCR. Data are presented as fold increase over CTL. Each bar is the mean $\pm$ SE (n=12) from 3 separate experiments.

*Significantly different ($p < 0.05$) from CTL.

Figure 9.

Effects of acute endotoxemia on NF- κ B and AP-1 nuclear binding activity. Macrophages (MP) and endothelial cells (EC) were isolated from livers of C3H/OuJ or C3H/HeJ mice 3–48 hr after administration of ETX or control (CTL). *Upper panel*, nuclear extracts were analyzed for NF- κ B binding activity by EMSA. Endothelial cell extracts from C3H/OuJ mice isolated 48 hr after ETX administration were incubated at room temperature for 30 min with antibodies to NF- κ B p50, NF- κ B p65, or a 40-fold excess of unlabeled cold competitor (CC) prior to the labeled probe. One representative gel from three separate experiments is shown. *Lower panel*, nuclear extracts were analyzed for AP-1 binding activity by EMSA. Macrophage extracts from C3H/OuJ mice isolated 3 hr after ETX administration were incubated at room temperature for 30 min with antibodies to cFos, cJun, or a 40-fold excess of unlabeled cold competitor (CC) prior to the labeled probe. One representative gel from two separate experiments is shown.

Figure 10.

Effects of acute endotoxemia on expression of cell signaling molecules. Macrophages and endothelial cells were isolated from livers of C3H/OuJ or C3H/HeJ mice 3–48 hr after administration of ETX or control (CTL). Cell lysates were prepared and analyzed by western blotting using antibodies to the total or phospho-JNK, p38 and p44/42 MAP kinase, and AKT. One representative gel from 3–4 separate experiments is shown. 10 μ g of cellular protein was loaded onto each lane of the gels.

Table 1

Effects of acute endotoxemia on the number of macrophages and endothelial cells recovered from the liver

		Cells $\times 10^6$ /mouse		
		CTL	ETX (24 hr)	ETX (48 hr)
Macrophages	OuJ	1.6 $\pm$ 0.1	2.0 $\pm$ 0.1	2.9 $\pm$ 0.4 *
	HeJ	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	1.3 $\pm$ 0.2
Endothelial Cells	OuJ	4.5 $\pm$ 0.4	4.8 $\pm$ 0.4	5.3 $\pm$ 0.9
	HeJ	4.6 $\pm$ 0.9	4.5 $\pm$ 0.3	5.0 $\pm$ 0.9

Cells were isolated 24 hr or 48 hr after treatment of mice with endotoxin (ETX) or control (CTL). Each value represents the mean $\pm$ SEM from 3–18 separate experiments (n=11–72).

* Significantly ($p < 0.05$) different from OuJ mice (ANOVA and t-test).