Regulation of Hepatic Drug-Metabolizing Enzyme Genes by Toll-Like Receptor 4 Signaling Is Independent of Toll-Interleukin 1 Receptor Domain-Containing Adaptor Protein

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ABSTRACT:

During inflammation, drug metabolism and clearance are altered due to suppression of hepatic drug-metabolizing enzyme (DME) genes and their regulatory nuclear receptors (NRs) [pregnan X receptor, constitutive androstane receptor, and retinoid X receptor (RXRα)]. The bacterial endotoxin, lipopolysaccharide (LPS), induces expression of proinflammatory cytokines in the liver, which contribute to altered DME expression. LPS binds to the cell-surface receptor, Toll-like receptor 4 (TLR4), which initiates a signal transduction cascade, including recruitment of the Toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP). However, the role of TLR4 and TIRAP in LPS-mediated regulation of hepatic DME expression is not known. Wild-type (C3H/HeJ), TLR4-mutant (C3H/HeJ), TIRAP+/+, and TIRAP−/− mice were injected i.p. with LPS. RNA levels of the major hepatic DME, Cyp3a11 and Ugt1a1, and the NRs were suppressed ~60 to 70% by LPS in wild-type but not in the TLR4-mutant mice. The nuclear protein levels of RXRα were reduced by LPS in wild-type but not in TLR4-mutant mice. Induction of hepatic cytokines (interleukin-1β, tumor necrosis factor-α, and interleukin-6), c-Jun N-terminal kinase, and nuclear factor-κB was blocked in TLR4-mutant mice. Surprisingly, LPS had the same effect on cytokines, kinases, NRs, and DME genes in livers of both TIRAP+/+ and TIRAP−/− mice, indicating that TIRAP is not essential for TLR4-mediating suppression of NRs and DMEs in liver. However, TIRAP−/− mice have reduced serum cytokine expression compared with TIRAP+/+ mice in response to LPS. This shows that although TIRAP mediates inflammatory responses induced by LPS, it is not essential in regulating LPS-mediated alterations of gene expression in liver.

Lipopolysaccharide-induced inflammatory responses in the liver lead to altered gene expression of DMEs due to reduced expression and function of the nuclear receptor (NRs), PXR, CAR, and RXRα (Beigneux et al., 2002). Regulation of hepatic DMEs by LPS has been attributed to the effects of the proinflammatory cytokines, interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNFα), and interferons (Renton, 2004; Aitken et al., 2006). However, the mechanism of suppression of hepatic DME genes in LPS-induced inflammation is not fully understood. We have reported that LPS treatment of mice suppresses PXR and CAR RNA levels and causes modification and nuclear export of the RXRα mutant (C3H/HeJ), TIRAP.

Some DME genes and their regulatory NRs are targeted by cell-signaling kinases, primarily by members of the mitogen-activated protein kinase family, which are activated by LPS (Li et al., 2002; Cheng et al., 2003; Rochette-Egly, 2003; Oesch-Bartlomowicz and Oesch, 2005). The transcription factors AP-1 and NF-κB are also activated during inflammation and can regulate expression and activity of some DME genes and NRs (Lee et al., 2000; Abdel-Razzak et al., 2004; Abdulla et al., 2006). The mitogen-activated protein kinases, c-Jun N-terminal kinase (JNK), and extracellular signal activated kinase are involved in regulation of some DMEs and NRs (Yu et al., 1999; Tan et al., 2004). Curcumin, a known inhibitor of JNK, can block LPS-mediated down-regulation of cytochrome P450 enzymes, although the underlying mechanism is not known (Cheng et al., 2003). Furthermore, we have shown that activation of JNK by LPS or IL-1β results in modification and nuclear export of RXRα, and this may contribute to suppression of RXRα-dependent hepatic genes (Li et al., 2002; Ghose et al., 2004). JNK inhibits glucocorticoid receptor activity, leading to suppression of CAR gene expression (Pascussi et al., 2003). Reduction in CAR gene expression in inflammation has also been attributed to the disruption of glucocorticoid receptor...
mediated transactivation of the CAR gene by NF-κB (Assenat et al., 2004, 2006). Recent work has demonstrated that NF-κB can interact with the PXR-RXR complex, leading to the suppression of Cyp3a4 gene expression by LPS (Gu et al., 2006).

Recent evidence indicates that inflammatory responses in the liver involve Toll-like receptor (TLR) signaling pathways (Akira and Takeda, 2004; Schwabe et al., 2006). Genetic evidence suggests that TLR4 is involved in signaling by LPS, as mutations of the gene Lps selectively inhibited LPS signal transduction in C3H/HeJ mice (Qureshi et al., 1999). TLR4-mediated signaling is initiated by the adaptor, TIRAP, which recruits MyD88 to the plasma membrane (Kagan and Medzhitov, 2006; O’Neill and Bowie, 2007). A dominant-negative form of TIRAP, in which Box 2 proline was mutated to histidine, resulted in inhibition of TLR4 signaling (Fitzgerald et al., 2001). TIRAP is associated with TLR4, and a TIRAP inhibitor peptide completely blocks TLR4 signaling (Horng et al., 2006). The specific role of TIRAP in the TLR4 signaling pathway was confirmed in TIRAP-deficient mice, which showed impairment of cytokine induction in response to LPS (Horng et al., 2002; Yamamoto et al., 2002a). Roles for TLR4 and TIRAP in regulating hepatic DME gene expression in inflammation are essentially unknown.

In this study we sought to determine the role of the TLR4 signaling pathway in regulating the expression of key hepatic phase I and phase II DME genes, Cyp3a11 and Ugt1a1, respectively. We find that activation of TLR4 by LPS leads to activation of JNK and NF-κB and to the suppression of NRs and DMEs. The effects of LPS on DME genes and NRs are completely abrogated in the TLR4-mutant mice. Surprisingly, the central TLR4 adaptor protein, TIRAP, was unnecessary for LPS-mediated suppression of DME genes and NRs in the liver. Induction of cell-signaling pathways and production of cytokines in TIRAP−/− mice were comparable with the levels in TIRAP+/− mice. However, serum IL-6 levels were significantly reduced in LPS-treated TIRAP−/− mice compared with wild-type mice, indicating that although TIRAP plays a role in eliciting immune responses, it may not be essential in mediating the effects of LPS in liver. This finding is surprising, because TIRAP is the first adaptor in the TLR4-signaling pathway, and previous studies had shown that TIRAP is essential for mediating the effects of LPS in macrophages and dendritic cells (Horng et al., 2002; Yamamoto et al., 2002b). Overall, these results suggest that the presence of multiple, intersecting, intracellular regulatory pathways allows the liver to adequately respond to inflammation.

Materials and Methods

Materials. Highly purified LPS (Escherichia coli serotype 0111:B4) was purchased from InvivoGen (San Diego, CA) and was freshly diluted to the desired concentration in pyrogen-free 0.9% saline before injection. Anti-JNK and phospho-JNK (nos. 9252 and 9251; Cell Signaling Technology Inc., Beverly, MA) and anti-RXRα (D-20) (no. sc-553; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used according to the manufacturer’s instructions. [γ-32P]ATP was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Oligonucleotides were obtained from Sigma Genosys (Houston, TX). All reagents for real-time PCR were purchased from Applied Biosystems (Foster City, CA).

Animals. TLR4-mutant (C3H/HeJ) mice with defects in LPS-mediated signaling as a result of a missense mutation (proline → histidine) at codon 712 and the genetically similar strain of TLR4-wt (C3H/HeFeJ) mice with intact TLR4-mediated signaling were obtained from The Jackson Laboratory (Bar Harbor, Maine). TIRAP+/− and TIRAP−/− mice (C57BL/6×SV129; F3) were obtained from Dr. Ruslan Medzhitov (Yale University School of Medicine, New Haven, CT). The animals were maintained in a temperature- and humidity-controlled environment and were provided with water and rodent chow ad libitum. Adult male (8–10 weeks) mice (20–25 g) were given an i.p. injection of 2 mg/kg b.w. LPS in saline or saline alone. LPS in this dose range has been shown previously to induce cytokines and reduce expression of hepatic genes without inducing hepatic damage (Ghose et al., 2004, 2007). Livers were removed at the times indicated in the figure legends (1–16 h) after treatment. All animal protocols were approved by the Institutional Animal Care and Use Committee. Experiments were performed in triplicate and repeated three to four times.

Preparation and Analysis of Nuclear and Cytoplasmic and Whole Cell Extracts. Nuclear, cytoplasmic, and whole cell extracts were prepared as described previously (Ghose et al., 2004, 2007). The protein concentration was determined by BCA assay according to the manufacturer’s protocol (Pierce Chemical, Rockford, IL). These fractions were analyzed by immunoblotting.

Electrophoretic Gel Mobility Shift Assays. Nuclear extracts were prepared according to published protocols (Ghose et al., 2004, 2007). Double-stranded oligonucleotide probes were end-labeled and purified, and 10 μg of nuclear extracts were incubated on ice for 30 min with 32P end-labeled oligonucleotide as described previously (Ghose et al., 2004, 2007). After binding, the samples were electrophoresed through a nondenaturing 6% polyacrylamide gel, dried, and exposed to X-ray film. In addition, gels were exposed to a PhosphorImager screen and quantified using a PhosphorImager and ImageQuant software.

Real-Time Quantitative PCR Analysis. Total RNA was isolated from mouse liver tissues using the RNasy kit from Qiagen (Valencia, CA). cDNA was synthesized from 7.5 μg of total RNA using the ProSTAR first-strand RT-PCR kit (Stratagene, La Jolla, CA). Real-time quantitative PCR was performed using an ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems). Briefly, each amplification reaction (25 μl) contained 50 to 100 ng of cDNA, 300 nM concentrations of forward and reverse primer, a 200 nM concentration fluorogenic probe, and 15 μl of TaqMan Universal PCR Master Mix. PCR thermocycling parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Quantitative expression values were extrapolated from standard curves and were normalized to cyclophilin. RNA levels of the LPS-injected samples were determined relative to the saline-injected samples in the wild-type and mutant mice. The sequences of the primers and probes were obtained from the literature or purchased from Applied Biosystems, as reported previously (Ghose et al., 2004, 2007).

Serum Cytokine Analysis. Serum IL-6 levels were determined by an enzyme-linked immunosorbent assay (BD OptEIA Mouse IL-6 ELISA kit; BD Biosciences, San Diego, CA) according to the manufacturer’s directions.

Results

Regulation of DME Gene Expression by TLR4 and TIRAP. Hepatic expression of the major phase I DME gene, Cyp3a11, and the phase II DME gene, Ugt1a1, are reduced during inflammation; however, the mechanism by which this suppression occurs is not fully understood (Renton, 2004; Abdulla et al., 2006; Aitken et al., 2006). To investigate the role of Toll-like receptor signaling in mediating regulation of DMEs, the TLR4 pathway was activated by i.p. injection of 2 mg/kg b.w. of LPS to TLR4-wt (C3H/HeFeJ) and TLR4-mutant (C3H/HeJ) mice (Fig. 1A). RNA was isolated from the livers harvested at 16 h after LPS injection and was analyzed by real-time PCR. There was no significant difference in RNA levels of the DME between the saline-injected TLR4-wt and TLR4-mutant mice. However, 16 h after LPS injection, RNA levels of Cyp3a11 and Ugt1a1 were suppressed 60 to 70% in the TLR4-wt mice, whereas this suppression was completely blocked in the TLR4-mutant mice.

The adaptor protein, TIRAP, is considered the primary adapter involved in mediating the intracellular signaling events initiated by the activation of TLR2 and TLR4 on the cell surface (Horng et al., 2001, 2002; Akira and Takeda, 2004). The primary function of TIRAP is to recruit the adaptor, MyD88, to TLR4, and this leads to the activation of downstream kinases, which are involved in expanding and targeting the responses to TLR4 signaling (Kagan and Medzhitov, 2006).
Because TIRAP is the first adaptor in the TLR4 signaling pathway, we hypothesized that TLR4-mediated effects on DME genes would be significantly attenuated in the absence of TIRAP. Interestingly, LPS treatment resulted in suppression of Cyp3a11 and Ugt1a1 RNA levels in both the TIRAP$^{+/+}$ and TIRAP$^{-/-}$ mice. This indicates that suppression of hepatic DME genes during inflammation is mediated by the LPS receptor TLR4, but the downstream adaptor, TIRAP, is not essential (Fig. 1B).

Regulation of NR Expression by TLR4 and TIRAP. It is well established that the xenobiotic NRs, PXR and CAR, regulate the expression of DME genes. PXR and CAR heterodimerize with the central NR, RXR, to bind to conserved sequences in the promoter regions of DME genes including Cyp3a11 and Ugt1a1, resulting in activation of these DME genes (Karpen, 2002; Chen et al., 2003). RNA levels of CAR were suppressed ~60% by LPS treatment of TLR4-wt mice, and this down-regulation was blocked in the TIRAP$^{+/+}$ and TIRAP$^{-/-}$ mice. This indicates that suppression of NR RNA levels is partially mediated by TIRAP, whereas no such reduction was detected in the TLR4-mutant mice. This indicates that suppression of NR gene expression and function by LPS is independent of the first adaptor of the TLR4 signaling pathway, TIRAP.

Regulation of Cytokine Gene Expression by TLR4 and TIRAP. LPS treatment results in the induction of proinflammatory cytokines in several cell types in liver, including the Kupffer cells, the resident liver macrophages, which are the primary sites of hepatic cytokine production. These cytokines then act on hepatocytes to suppress DME gene expression in the liver (Aitken et al., 2006). As expected, nuclear RXRα protein levels were reduced in TIRAP$^{+/+}$ mice upon LPS treatment (Fig. 2C); however, this suppression was not blocked in TIRAP$^{-/-}$ mice. This indicates that suppression of NR gene expression and function by LPS is independent of the first adaptor of the TLR4 signaling pathway, TIRAP.

Role of TLR4 and TIRAP in Regulation of Cell-Signaling Pathways. The NR-mediated regulation of DME gene expression in inflammation involves cross-talk with cell-signaling components (Li et al., 2002; Cheng et al., 2003; Pascussi et al., 2003; Oesch-Bartlomowicz and Oesch, 2005). The mitogen-activated protein kinase, JNK, has been shown to be involved in phosphorylation and nuclear export of the central...
NR, RXRα (Ghose et al., 2004; Zimmerman et al., 2006), and in the regulation of expression and activity of some phase I and phase II DMEs (Yu et al., 1999; Pascussi et al., 2003; Tan et al., 2004). JNK activation by LPS was markedly attenuated in TLR4-mutant mice as demonstrated by a reduction in P-JNK levels compared with the TLR4-wt mice (Fig. 4A). It has recently been shown that there is a cross-talk between NRs, DMEs, and NF-κB, a key regulator of inflammation and immune response (Ke et al., 2001; Zhou et al., 2006). LPS treatment of TLR4-wt mice led to NF-κB activation as measured by degradation of IκBα, whereas no such activation was observed in TLR4-mutant mice (Fig. 4A). These results were confirmed by electrophoretic mobility shift assay, in which nuclear binding activity of NF-κB and AP-1 was activated by LPS treatment of TLR4-wt mice, whereas there was essentially minimal activation of NF-κB and AP-1 in livers of TLR4-mutant mice (Fig. 4A). The cell-signaling components, JNK and NF-κB, were activated by LPS treatment of TIRAP+/+ mice, and this effect was not attenuated in the TIRAP−/− mice, as determined by immunoblotting and electrophoretic mobility shift assay (Fig. 4, C and D). These results support earlier observations that in the liver, cell-signaling components are activated by TLR4, without the involvement of TIRAP. Interestingly, there was a direct correlation between LPS-mediated activation of P-JNK and suppression of nuclear RXRα protein levels, supporting a link between P-JNK and modification of nuclear RXRα function and activity.

**Induction of Serum IL-6 Expression Is Regulated by TIRAP.** The results show that the LPS receptor, TLR4, down-regulates hepatic DME genes and NRs without the involvement of TIRAP. Furthermore, TIRAP is not involved in induction of cytokine RNA levels and cell-signaling pathways by TLR4 in liver of LPS-treated mice. TIRAP has been shown to be present in the liver (Nishimura and Naito, 2005), which was confirmed by our results (data not shown). So, the next goal was to investigate whether LPS treatment affected secretion of cytokines in mouse serum in the TIRAP+/+ mice compared with the corresponding wild-type. Expression of the proinflammatory cytokine, IL-6, was induced in the serum of LPS-treated TIRAP+/+ mice as expected (Fig. 5), whereas IL-6 levels in the serum were significantly reduced in TIRAP−/− mice. This indicates that TIRAP is involved in regulating the expression levels of cytokines in the serum after LPS treatment, and TIRAP may be nonfunctional in the liver tissue.

**Discussion**

The suppression of key phase I and phase II DMEs during inflammation is highly relevant to the care and treatment of critically ill...
patients, and the underlying molecular mechanisms remain to be fully elucidated. Inflammatory responses involve TLR-signaling pathways that can be activated by microbial components and endogenous ligands from damaged or stressed cells (Beg, 2002; Akira and Takeda, 2004). This study demonstrates that LPS-mediated suppression of the representative phase I and II DME genes, Cyp3a11 and Ugt1a1,
respectively, involve the LPS receptor, TLR4, but, surprisingly, was independent of the main TLR4 adaptor protein, TIRAP. The NRs, PXR, CAR, and RXRα, which regulate expression of DME genes are also suppressed by TLR4-mediated mechanism, also without the involvement of TIRAP.

In many cell types and models, TLR4-mediated signaling appears to require TIRAP, which is a specific adaptor for both TLR2 and TLR4 (Horng et al., 2002; Yamamoto et al., 2002a). Recently it has been shown that TIRAP initiates TLR4-mediated cell signaling by facilitating the delivery of the adaptor, MyD88, to activated TLR4 (Kagan and Medzhitov, 2006). Surprisingly, this study shows that expression of DMEs are suppressed by LPS treatment in both TIRAP+/+ and TIRAP−/− mice, indicating that TLR4-mediated suppression of DMEs in the liver does not involve TIRAP-dependent mechanism.

Initial studies with TIRAP−/− mice had shown that TIRAP is essential the induction of cytokines by LPS in bone marrow-derived dendritic cells (Horng et al., 2002) and peritoneal macrophages (Yamamoto et al., 2002a). Recently, it has been shown that in vivo TIRAP plays a critical role in mediating early immune responses to LPS in the lung (Jeyaseelan et al., 2005). In our studies, TIRAP has no role in induction of cytokine RNA levels in the liver, indicating that cytokine production, probably mainly by the resident liver macrophages (Kupffer cells), is not dependent on TIRAP. Furthermore, the activation of cell-signaling components such as JNK and NF-κB by LPS is mediated by TLR4 without the involvement of TIRAP in the liver.

The overall results indicate that alteration of metabolic processes in the liver during LPS-mediated inflammation requires TLR4 but is independent of TIRAP. The involvement of TLR4 in mediating the effects of LPS on hepatic genes was expected, because TLR4 is the specific receptor for LPS. TIRAP has been found to be expressed in the liver (Nishimura and Naito, 2005); however, it is not known whether it is functional in the complex environment of the liver tissue in vivo. This raises the likely possibility that TLR4-mediated effects in the liver may involve a TIRAP-independent/TRIF-dependent pathway. TRIF is an adaptor involved in TLR4-mediated induction of interferon-β, which leads to the activation of NF-κB with delayed kinetics (Akira and Takeda, 2004; O’Neill and Bowie, 2007). It has been shown that i.p. or i.v. administration of live or heat-killed E. coli in mice results in apoptosis of dendritic cells, and this process is mediated by TLR4 via a TRIF-dependent pathway and is independent of MyD88 (De Trez et al., 2005). Recently, a TRIF-specific and MyD88-independent pathway for TLR4 has been identified that provides a molecular mechanism relating how LPS induces major histocompatibility class II expression during dendritic cell maturation (Kamon et al., 2006). It remains to be determined whether LPS-mediated suppression of NRs and DMEs in the liver is controlled by a TRIF-dependent mechanism. However, it is also possible that TLR4 and MyD88, but not TIRAP, regulate LPS-mediated effects on hepatic metabolic processes. It has been shown that activation of NF-κB in endothelial cells by LPS is mediated by TLR4 and MyD88, without the involvement of TIRAP (Li et al., 2003).

The overall results indicate that activation of TLR4 leads to induction of NF-κB, which results in induction of proinflammatory cytokines (IL-1β, TNFα, and IL-6) in the keratinocytes, by TIRAP-independent pathways. Cytokines can bind to their respective receptors on hepatocytes to initiate signaling events. It has been shown previously that TIRAP is not involved in the cytokine signaling pathway (Horng et al., 2002), so in TIRAP−/− mice, cytokine signaling is probably mediated by MyD88 to activate JNK and NF-κB, which alter NR function, leading to suppression of DME gene expression in the hepatocytes. However, hepatocytes also express TLRs, and LPS can directly activate TLR4 signaling in the hepatocytes and alter gene expression. The role of TIRAP, MyD88, and TRIF in mediating TLR4 signaling in hepatocytes remains to be investigated.

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Fig. 5. Induction of serum IL-6 levels is regulated by TIRAP. TIRAP+/+ and TIRAP−/− mice were injected i.p. with 2 mg/kg LPS or saline, mice were sacrificed after 4 h, and blood was collected. Serum IL-6 levels were determined by enzyme-linked immunosorbent assay (n = 6/group). Error bars denote S.D.


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