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Toll-like receptor-4 regulation of hepatic *Cyp3a11* metabolism in a mouse model of LPS-induced CNS inflammation

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Goralski, Kerry B., Dalya Abdulla, Christopher J. Sinal, Andre Arsenault, and Kenneth W. Renton. Toll-like receptor-4 regulation of hepatic *Cyp3a11* metabolism in a mouse model of LPS-induced CNS inflammation. *Am J Physiol Gastrointest Liver Physiol* 289: G434–G443, 2005. First published May 5, 2005; doi:10.1152/ajpgi.00562.2004.—Central nervous system (CNS) infection and inflammation severely reduce the capacity of cytochrome *P*-450 metabolism in the liver. We developed a mouse model to examine the effects of CNS inflammation on hepatic cytochrome *P*-450 metabolism. FVB, C57BL/6, and C3H/HeouJ mice were given *Escherichia coli* LPS (2.5 μg) by intracerebroventricular (ICV) injection. The CNS inflammatory response was confirmed by the elevation of TNF-α and/or IL-1β proteins in the brain. In all mouse strains, LPS produced a 60–70% loss in hepatic *Cyp3a11* expression and activity compared with saline-injected controls. Adrenalectomy did not prevent the loss in *Cyp3a11* expression or activity, thereby precluding the involvement of the hypothalamic-adrenal-pituitary axis. Endotoxin was detectable (1–10 ng/ml) in serum between 15 and 120 min after ICV dosing of 2.5 μg LPS. Peripheral administration of 2.5 μg LPS by intraperitoneal injection produced similar serum endotoxin levels and a similar loss (60%) in *Cyp3a11* expression and activity in the liver. The loss of *Cyp3a11* in response to centrally or peripherally administered LPS could not be evoked in Toll-like receptor-4 (TLR4)-mutant (C3H/HeJ) mice, indicating that TLR4 signaling pathways are directly involved in the enzyme loss. In summary, we conclude that LPS is transferred from the brain to the circulation in significant quantities in a model of CNS infection or inflammation. Subsequently, LPS that has reached the circulation stimulates a TLR4-dependent mechanism in the periphery, evoking a reduction in *Cyp3a11* expression and metabolism in the liver.

lipopolysaccharide; cytochrome *P*-450; drug metabolism

THE CYTOCHROMES *P*-450 are a gene superfamily of heme-containing enzymes that contribute to the metabolism and elimination of exogenous and endogenous substances (1, 5). The highest amounts of drug-metabolizing cytochromes *P*-450 are found in the liver and intestine, with lower amounts found in other organs, including the kidney, lung, and brain. In rodents and humans, enzymes of the CYP3A subfamily comprise the majority of cytochrome *P*-450 in the liver (30–50%) and metabolize 50–60% of clinically used drugs (1).

It is well known that cytochromes *P*-450 are modulated during inflammatory responses and/or infectious diseases (28, 39). In general, acute systemic inflammatory responses trigger reductions in hepatic cytochrome *P*-450 enzyme expression and activity, with the consequence of reduced drug metabolism during episodes of inflammation. Bacterial LPS is the major stimulus for inflammatory responses in the central nervous system (CNS) caused by gram-negative meningeal pathogens (42). Inflammatory responses in the brain caused by the administration of *Escherichia coli* LPS into the lateral ventricle of rats reduce hepatic CYP1A1/2, CYP2B1/2, CYP2D1, CYP2E1, and CYP3A1/2 enzyme activities and/or protein levels (14, 27, 31, 40, 41, 45). The signaling mechanisms that produce peripheral responses during CNS infection have been proposed to include peripheral inflammatory cytokines, the hypothalamic-adrenal-pituitary (HPA) axis, the sympathetic nervous system, and reactive oxygen species (8, 15, 48). Because of the complex nature of these signaling mechanisms, it has been difficult to determine which pathways are most important for the loss in hepatic drug metabolism in vivo. Mice with disruptions of genes contributing to inflammatory signaling pathways (TNF-α receptor, IL-6, signal transducer and activator of transcription-1, and nitric oxide synthase-2-null mice) have been used as a tool to delineate the factors that are important for decreasing hepatic drug metabolism during peripheral inflammatory responses (32, 43, 46, 50). The availability of mice with genetic alterations in immune signaling pathways will allow a more precise mechanistic examination of the biochemical links between CNS infection and inflammation and drug metabolism and disposition in vivo.

Toll-like receptor-4 (TLR4) has been identified as the transmembrane receptor that specifically recognizes LPS and initiates intracellular signal transduction and an immune response to that bacterial cell wall component (6, 36). LPS signaling during conditions of systemic inflammation involves the binding of LPS to the serum protein LPS binding protein, followed by association with CD14, a receptor found either in the membrane or in soluble form. The complex is then thought to translocate to TLR4 to initiate intracellular signal transduction pathways, including NF-κB activation and inflammatory cytokine production (4, 6). A similar process occurs in the regions of the brain (choroid plexus, leptomeninges, and circumventricular organs) that coexpress the CD14 and TLR4 components of the LPS signal transduction pathway (20, 30). Despite the importance of TLR4 in LPS signaling, it has received minimal attention with regard to regulation of drug metabolism by inflammatory processes. C3H/HeJ mice contain a single point mutation at amino acid 712 that renders TLR4 ineffective and the mice unresponsive to stimulation by LPS (36, 37). Thus the C3H/HeJ mouse strain represents a useful model to examine whether changes in hepatic cytochrome *P*-450 expression during LPS exposure can be specifically attributed to activation of TLR4 signaling pathways.

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The aim of this study was to examine the signaling mechanisms responsible for the loss in hepatic cytochrome *P*-450 3A in a model of gram-negative bacterial infection or inflammation of the mouse brain. Our novel findings indicated that, in this experimental model, the flux of LPS from brain to blood and the activation of a peripheral TLR4-mediated immune response lead to a reduction *Cyp3a11* drug metabolism in the liver.

MATERIALS AND METHODS

Mice and production of CNS inflammation. FVB and C57BL/6 mouse strains were used initially in the study to develop methodology and to characterize the effects of CNS infection or inflammation on mouse cytochrome *P*-450 metabolism. Subsequently, the C3H/HeJ and closely related C3H/HeouJ strains were used to specifically address the role of TLR4 in mediating changes in hepatic cytochrome *P*-450 expression and activity. Adult male mice (FVB strain) and adrenalectomized FVB mice were obtained from Taconic Laboratories (Germantown, NY). Male mice (6–8 wk old) containing either a spontaneous mutation in *TLR4* gene (C3H/HeJ) and age-matched LPS-responsive (C3H/HeouJ) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were obtained from our in-house breeding colony. The mice were housed in cages lined with corncob bedding and had free access to water and Purina mouse chow. Drinking water for adrenalectomized mice was supplemented with 5% glucose. Mice were kept on a 12:12-h day-night cycle and were allowed to acclimatize in the animal holding facilities for a period of 1 wk before experimentation. The Dalhousie University Committee on Laboratory Animals approved all experimental procedures involving mice according to guidelines of the Canadian Council on Animal Care.

On the day of the experiments, mice were anesthetized with enflurane (3.0–4.0%) and were given an infusion of either endotoxin-free saline (2.5 μl) or gel-purified *E. coli* LPS (serotype 0127:B8; 2.5 μg in 2.5 μl) into the lateral cerebral ventricle [intracerebroventricular (ICV)] using a Harvard Apparatus syringe pump. The solutions were injected at a rate of 2.5 μl/min. The coordinates for the sterotaxic injections were −2.5 mm dorsal/ventral, −1.5 mm lateral, and −0.2 mm anterior/posterior from the bregma (11).

For the intraperitoneal experiments, mice were injected intraperitoneally with either a low (2.5 μg) or high (125 μg) dose of purified *E. coli* LPS suspended in 100 μl of 0.9% endotoxin-free saline. Control mice were injected with 100 μl of the saline vehicle.

Phenol-extracted LPS, but not the gel-purified LPS, contains micromolar levels of biologically active contaminants, including glutamate and adenosine (16), which could precipitate nonspecific responses in our model. For those reasons, chromatographically purified LPS is the preferred preparation for in vivo studies of this nature. To assess blood-brain barrier permeability changes, a tracer amount (4 nmol) of D-[1-^{14}C]mannitol was administered peripherally to control mice and to mice 0, 1, 4, and 24 h after 2.5 μg LPS was administered into the lateral ventricle. Brain levels of total radioactivity were measured by scintillation counting 1 h after the [^{14}C]mannitol injection. For the TNF-α experiment, 50 ng of recombinant mouse TNF-α (R&D systems) or vehicle containing sterile filtered PBS and 0.1% BSA were administered into the lateral ventricle in a total volume of 2.5 μl.

Tissue and microsome preparation. Mice were killed 4 or 24 h after the ICV administration of either LPS or saline, at which point liver and brain samples were obtained. The whole brain was homogenized in 1.5 ml of PBS (pH 7.4) and spun at 13,000 rpm for 10 min. Brain supernatants were stored at −80°C until utilized for cytokine measurements. Samples (100 mg) were obtained from the liver and frozen in liquid nitrogen for total RNA isolations. The remaining liver was removed and used to prepare microsomes according to previously published methods (10). We homogenized liver tissue in ice-cold 1.15% KCl for 15–20 s using a Polytron homogenizer. The homogenates were centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatants were recentrifuged at 110,000 *g* for 40 min. The resulting microsomal pellets were resuspended in 0.8 ml of glycerol buffer (pH 7.5) that contained 50 mM KH_2PO_4, 20% glycerol, and 0.40% KCl. Samples were stored at −80°C until enzymatic assays were performed. Microsomal protein content was determined by the method of Lowry et al. (23) using BSA as a standard.

Cyp3a11 enzyme assays. The formation of α-hydroxytriazolam from triazolam was used as a specific indicator for mouse *Cyp3a11* activity (34). Incubation mixtures (performed in duplicate) contained 500 μg of total microsomal protein, 20 μM triazolam, and 1 mM NADPH in 100 mM PBS (pH 7.4). The reaction mixtures (1 ml final volume) were incubated in a 37°C water bath with a shaker set at 100 oscillations/min. After 15 min, the reactions were stopped by the addition of 5 ml of dichloromethane; 2,000 ng of phenacetin were added to each sample as an internal standard. The samples were then centrifuged at 2,700 rpm for a period of 10 min, and the lower organic phase containing the metabolites and internal standard was transferred into a clean glass tube and evaporated to dryness using a nitrogen evaporator followed by reconstitution in 200 μl of HPLC mobile phase. The HPLC mobile phase consisted of acetonitrile [20% (vol/vol)] and methanol [35% (vol/vol)] in 0.01 M potassium phosphate buffer (45% vol/vol) buffered to pH 7.4 with NaOH. Reconstituted samples (50 μl) were separated on a reverse-phase C-8 (25 cm × 4.5 mm inner diameter) analytical column (Beckman Coulter, Fullerton, CA) attached to a Waters 2690 separation module. The mobile phase flow rate for separations was set at 1.0 ml/min. The approximate retention times were 5.0 min for α-hydroxytriazolam, 8.0 min for phenacetin, and 12.0 min for triazolam; these were detected by UV absorption at 220 nm (Waters 2487 Dual λ absorption unit).

RNA isolation and Northern analyses. Total liver RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quality of the RNA was determined spectrally by measuring the 260-to-280-nm absorbance ratio and was typically between 1.8 and 2.0. For Northern blot analyses, 10 μg of total RNA were resolved on a 1.1% agarose-formaldehyde denaturing gel. RNA was transferred overnight to a nylon membrane (Immobilon NY$^+$; Millipore, Bedford, MA) and was fixed to the membrane by UV cross-linking and heating for 1 h at 65°C. [^{32}P]CTP probes for mouse *Cyp3a11* and *Gapdh* mRNA were generated from the corresponding cDNAs using the RmT Random Primer labeling kit according to manufacturer's instructions (Stratagene). Blots were prehybridized for 1 h at 65°C in 10 ml of Sigma Perfecthyb Plus (Sigma, St. Louis, MO) followed by overnight hybridization with [^{32}P]CTP probes. Blots were exposed to storage phosphor screen (Amersham Biosciences, Piscataway, NJ) for 16–24 h and scanned with a phosphor imager (Amersham Biosciences). Quantification was performed using ImageQuant 5.2 software (Amersham Biosciences).

Quantitative real-time PCR. Total RNA (5 μg) was reverse transcribed using Stratascript reverse transcriptase (Stratagene, Cedar Creek, TX) with 12.5 ng/μl random hexamers pd(N)$_6$ and 1.0 mM dNTPs according to the supplier's instructions. We amplified cDNA product (1 μl) by quantitative PCR using 125 nM gene-specific primers in a total volume of 20 μl with brilliant SYBR green QPCR Master Mix using the Stratagene MX3000p thermocycler. For TNF-α, a 61-bp product was generated with the forward 5′-CCC TCA CAC TCA GAT CAT CTT CT-3′ and reverse 5′-GCT ACG ACG TGG GCT ACA G-3′ primers (GenBank accession no. NM_013693). For GAPDH, a 245-bp amplicon was generated with the forward 5′-AAG GTC GGT GTG AAC GGA TTT GG-3′ and reverse 5′-TTG ATG TTA GTG GGG TCT CGC TCC-3′ primers (GenBank accession no. NM_008084). The amplification protocol consisted of a 10-min hot start at 94°C, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 18 s, and elongation at 72°C for 30 s. Melting curves followed by separation of PCR products on a 2.5% agarose gel were used to ensure the formation of a single product at the appropriate size. The threshold cycle values for TNF-α and control gene

Gapdh were obtained with a threshold of 3 SD above background. Relative TNF-α expression normalized to *Gapdh* was calculated using the $\Delta\Delta C_T$ (where C_T is threshold cycle) method (see Ref. 22).

Cytokine measurements. We measured the levels of TNF-α and IL-1β in brain homogenates and serum 2 or 4 h after LPS administration ICV using a murine sandwich ELISA prepared as previously described (26). The results are expressed as nanograms of cytokine per gram of brain wet weight or nanograms of cytokine per milliliter of serum.

Serum LPS measurements. LPS (2.5 μg) was administered by the ICV or intraperitoneal route to endotoxin-responsive C3H/HeouJ mice. Mice were killed 15 min, 30 min, 2 h, or 24 h later, and trunk blood was collected. All groups were compared with noninjected control mice. We measured serum LPS levels using the kinetic chromogenic *Limulus amebocyte* lysate assay as per the manufacturer's instructions (Associates of Cape Cod, Cape Cod, MA). The assay was linear from 0.395 pg/ml to 100 ng/ml LPS. We calculated the area under the curve ($AUC_{0.25-2\ h}$) from the mean serum LPS concentrations using the trapezoidal method.

Data analyses. Data are expressed as means ± SE of three or four separate animals. An unpaired *t*-test was used to compare means between two groups. ANOVA was used for multiple-comparison procedures. A Tukey's test was used for post hoc analysis of the significant ANOVA. A difference in mean values with a value of $P \leq 0.05$ was considered to be significant.

Chemicals. Purified *E. coli* LPS (serotype 0127:B8), phenacetin, α-hydroxytriazolam, ethoxyresorufin, resorufin, NADPH, and dichloromethane were obtained from Sigma. [^{32}P]dCTP was obtained from Perkin-Elmer (Boston, MA). Triazolam was obtained from Roche Pharmaceuticals. All other reagents were of the finest grade available and were purchased from commercial suppliers.

RESULTS

Administration of LPS into the lateral ventricle reduces hepatic Cyp3a11 expression and activity. The initial experiments carried out in the two mouse strains (FVB and C57BL/6) were required to develop a mouse model of CNS infection and inflammation and to determine whether this model exhibited reductions in *Cyp3a11* expression and activity. The dose of LPS was based on previous studies that demonstrated that 0.1 μg/kg LPS administered ICV was required for significant downregulation of hepatic cytochrome *P*-450 enzymes in rats (14, 31, 41). The expression of *Cyp3a11* and *Gapdh* mRNA was measured in the liver by Northern blot analyses 4 and 24 h after the administration of *E. coli* LPS into the left lateral cerebral ventricle (Fig. 1*A*). *Cyp3a11* mRNA was unchanged at 4 h and was decreased by 60% 24 h after LPS treatment compared with the respective saline controls (Fig. 1, *B* and *D*). Liver *Gapdh* expression was not altered by LPS treatment. Consistent with mRNA expression, the rate of α-hydroxylation of triazolam (*Cyp3a11* activity) was unchanged at 4 h but was decreased by 60% at 24 h by LPS treatment compared with the respective saline control (Fig. 1, *C* and *E*). LPS (2.5 μg) administered ICV to C57BL/6 mice produced a similar reduction in *Cyp3a11* mRNA (80%) and α-hydroxylation of triazolam (60%) and indicated that the effect of LPS was not restricted to the FVB strain of mice (Fig. 2).

Administration of LPS into the lateral cerebral ventricle produces CNS inflammation. Two hours after LPS was administered into the lateral ventricle of FVB mice, TNF-α and Il-1β levels were significantly increased (2-fold) compared with the saline controls (Fig. 3, *A* and *B*). The increased level of TNF-α and Il-1β are consistent with the production of CNS inflammation in response to LPS. A parallel increase (2-fold) in TNF-α and IL-1β in serum indicated a systemic response to ICV administered LPS (Fig. 3, *C* and *D*).

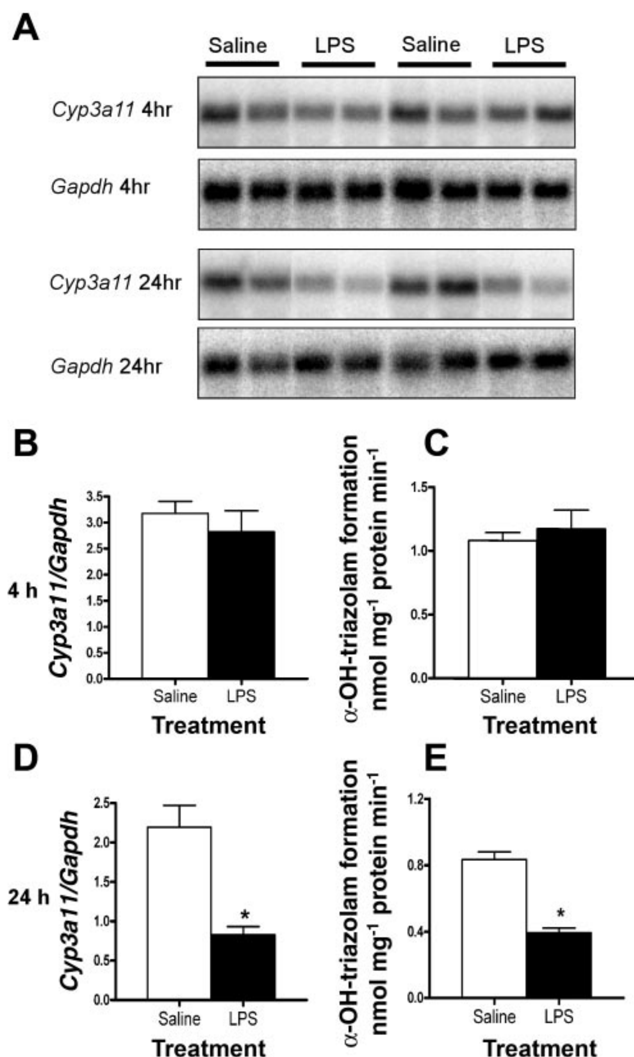

Fig. 1. Hepatic *Cyp3a11* mRNA and activity were downregulated 24 h after intracerebroventricular (ICV) administration of LPS (2.5 μg) to male FVB mice. *A*: representative Northern blots of *Cyp3a11* and *Gapdh* at 4 and 24 h after ICV administration of LPS or saline. Band intensities were determined by densitometry, and hepatic *Cyp3a11* (*B* and *D*) mRNA was normalized to *Gapdh* mRNA. *Cyp3a11* activity was measured 4 h (*C*) and 24 h (*E*) after LPS or saline administration by monitoring the formation of α-hydroxytriazolam (α-OH-triazolam) from triazolam in liver microsomal fractions. Each bar represents the mean ± SE of 4 mice. **Cyp3a11* mRNA and activity were lower compared with the respective saline-treated mice ($P < 0.05$, unpaired *t*-test).

HPA axis does not mediate hepatic Cyp3a11 downregulation following ICV administration of LPS. The release of inflammatory cytokines after LPS injection into the brain is known to activate the HPA axis and increase circulating corticosteroids, which can affect the acute phase response and gene transcription in the liver (2, 9, 48). Thus it was important to determine the relevance of the HPA axis in contributing to the downregulation of *Cyp3a11* mRNA and enzyme activity in our model (Fig. 4). Adrenalectomized and control FVB mice were left untreated or were injected with 2.5 μg or LPS or saline ICV. LPS reduced *Cyp3a11* expression and activity to the same degree (70%) in both control and adrenalectomized

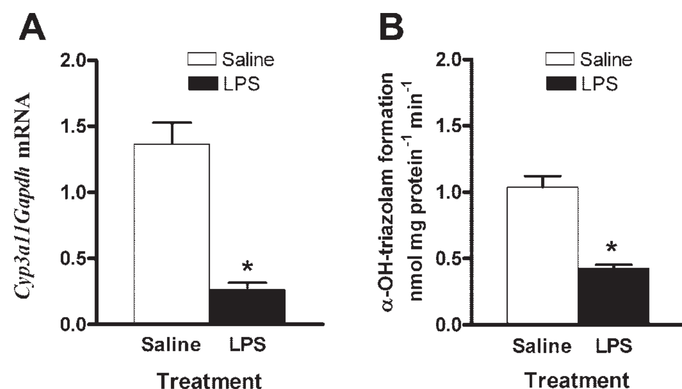

Fig. 2. Hepatic *Cyp3a11* mRNA and activity were downregulated 24 h after ICV administration of LPS (2.5 μg) to male C57BL/6 mice. *A*: hepatic *Cyp3a11* mRNA expression normalized to *Gapdh* expression as determined by Northern blot analysis. Band intensities were determined by densitometry. *Cyp3a11* activity was measured by monitoring the formation of α-OH-triazolam from triazolam in liver microsomal fractions (*B*). Each bar represents the mean ± SE of 4 mice. *$P < 0.05$ compared with the respective saline-treated mice (unpaired *t*-test).

mice (Fig. 4, *A–C*). In control mice and adrenalectomized mice, saline administration had no effect on hepatic *Cyp3a11* mRNA or triazolam metabolism compared with the respective untreated animals. Similarly, basal *Cyp3a11* expression and triazolam hydroxylation in adrenalectomized mice was not significantly different from controls. ICV LPS in control mice significantly increased serum corticosterone compared with saline-treated or untreated control mice, indicating activation of the HPA axis (Fig. 4*D*). Adrenalectomy completely prevented the elevation in serum corticosterone after ICV administration of LPS.

TLR4 signaling regulates hepatic Cyp3a11 expression. TLR4-expressing (C3H/HeouJ) and TLR4 mutant (C3H/HeJ) mice were utilized to determine whether the downregulation of hepatic *Cyp3a11* by LPS was mediated through stimulation of the TLR4. Twenty-four hours after LPS (2.5 μg) administration to C3H/HeouJ mice, *Cyp3a11* mRNA was decreased by 70% compared with the saline control (Fig. 5, *A* and *B*). In sharp contrast, no loss in *Cyp3a11* mRNA was evoked 24 h after LPS was administered directly into the lateral cerebral ventricle of TLR4 mutant (C3H/HeJ) mice. Liver *Gapdh* expression was similar in both mouse strains and was not altered by LPS treatment. Correspondingly, triazolam metabolism to α-hydroxytriazolam was reduced by 60% in the C3H/HeouJ (wild-type) mice but was unchanged in the C3H/HeJ (TLR4 mutant) mice compared with the respective saline controls (Fig. 5*C*). Basal *Cyp3a11* mRNA and triazolam hydroxylation were 40% lower in saline-treated C3H/HeJ vs. C3H/HeouJ mice. After 4 h, LPS increased brain TNF-α protein in the C3H/HeouJ mice but not in the C3H/HeJ mice compared with the respective saline controls (Fig. 5*D*). Liver TNF-α mRNA expression was increased 60-fold in the C3H/HeouJ mice but only 5-fold in the C3H/HeJ mice 4 h after LPS treatment compared with the respective saline controls (Fig. 5*E*). These results demonstrate an inhibition of the brain and liver inflammatory responses to LPS in the TLR4-mutant C3H/HeJ mice and indicate that the reduction in hepatic *Cyp3a11* expression and activity are linked to stimulation of TLR4 by LPS. Recombinant TNF-α (50 ng) administered ICV to the C3H/HeouJ and C3H/HeJ mice did not produce a loss in *Cyp3a11* expression in either mouse strain 24 h after they were dosed (Fig. 5*F*). The dose of TNF-α was chosen to approximate the TNF-α level detected in the brain 2–4 h after the LPS dose.

LPS is rapidly transferred from the CNS to the periphery after administration into the lateral cerebral ventricle. During CNS infection, the leakage of endotoxin into the periphery could play a role in reducing hepatic cytochrome *P*-450 metabolism; however, the systemic distribution of LPS after its administration ICV has not been previously characterized. LPS (2.5 μg) administered by the ICV route was detected in the serum (1,000–10,000 pg/ml range) between 15 min and 2 h after mice were dosed. LPS was not detectable in the serum 24 h after ICV administration or in untreated control mice (Fig. 6*A*). When 2.5 μg of LPS were administered via the intraperitoneal route, the levels of endotoxin detected were very similar to those obtained after administration of LPS by the ICV route (Fig. 6*B*). The intraperitoneal administration of 2.5 μg LPS also produced a 60% reduction in hepatic *Cyp3a11* mRNA and triazolam metabolism (Fig. 6, *C–E*). The $AUC_{0.25–2\ h}$ results calculated for the serum LPS data were 11,817 and 8,847 $pg \cdot ml^{-1} \cdot h^{-1}$ after ICV and intraperitoneal injection of LPS, respectively. This result directly supports the idea that a peripheral inflammatory response produced by LPS reaching the circulation after ICV injection is sufficient to produce reductions in hepatic cytochrome *P*-450 metabolism. The uptake of peripherally administered [^{14}C]mannitol was used as a marker of changes in blood-brain permeability induced by ICV administration of LPS (Fig. 6*F*). Increased blood-brain permeability was suggested by the significant increase in total brain [^{14}C]mannitol 5 and 24 h after LPS administration compared with control mice.

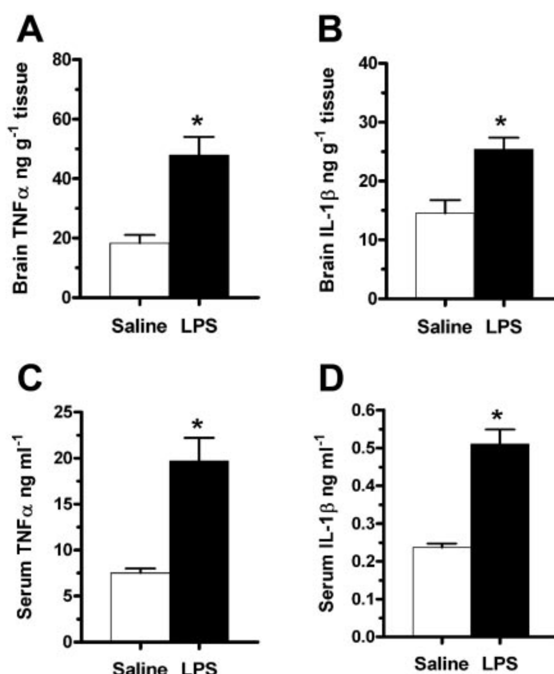

Fig. 3. Brain and serum inflammatory cytokines were elevated after ICV administration of LPS. Male FVB mice were injected with 2.5 μg of LPS or 2.5 μl of saline into the lateral cerebral ventricle. Levels of TNF-α and IL-1β proteins in brain (*A* and *B*) and serum (*C* and *D*) were measured 2 h after ICV administration of LPS or saline. Each bar represents the mean ± SE of 4 mice. *Mean cytokine levels were higher compared with the respective saline-treated control ($P < 0.05$, unpaired *t*-test).

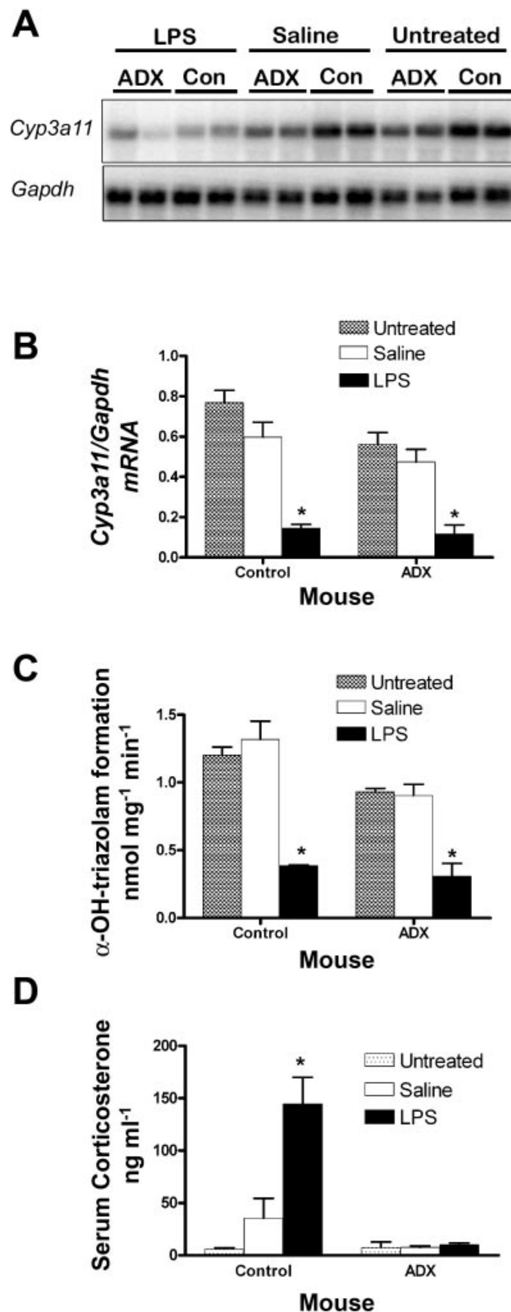

Fig. 4. Hypothalamic-adrenal-pituitary (HPA) axis does not contribute to hepatic *Cyp3a11* downregulation after ICV administration of LPS. Experiment was performed 2 wk after adrenalectomy. Control (Con) mice and adrenalectomized (ADX) mice were injected with 2.5 μg of LPS or 2.5 μl of saline into the lateral cerebral ventricle. Analyses were performed 24 h after the ICV injections. *A*: representative Northern blots for hepatic *Cyp3a11* and *Gapdh*. *B*: hepatic *Cyp3a11*/*Gapdh* expression as determined by densitometry. *C*: hepatic *Cyp3a11* activity (α-OH-triazolam formation). *D*: serum corticosterone level. For the corticosterone measurement, the LPS-ADX (adrenalectomy) bar represents the average of 2 mice. All other bars represent means ± SE of 3 or 4 mice. *Different compared with the respective saline-treated control or the untreated control ($P < 0.05$, ANOVA followed by Tukey's post hoc test). Statistical comparison of serum corticosterone in the ADX mice was not performed because of insufficient sample size ($n = 2$) in the LPS-ADX group.

Systemic administration of LPS mediates a reduction in hepatic Cyp3a11 expression and activity through TLR4 signaling. The importance of TLR4 in mediating hepatic *Cyp3a11* downregulation after the administration of LPS by the intra-

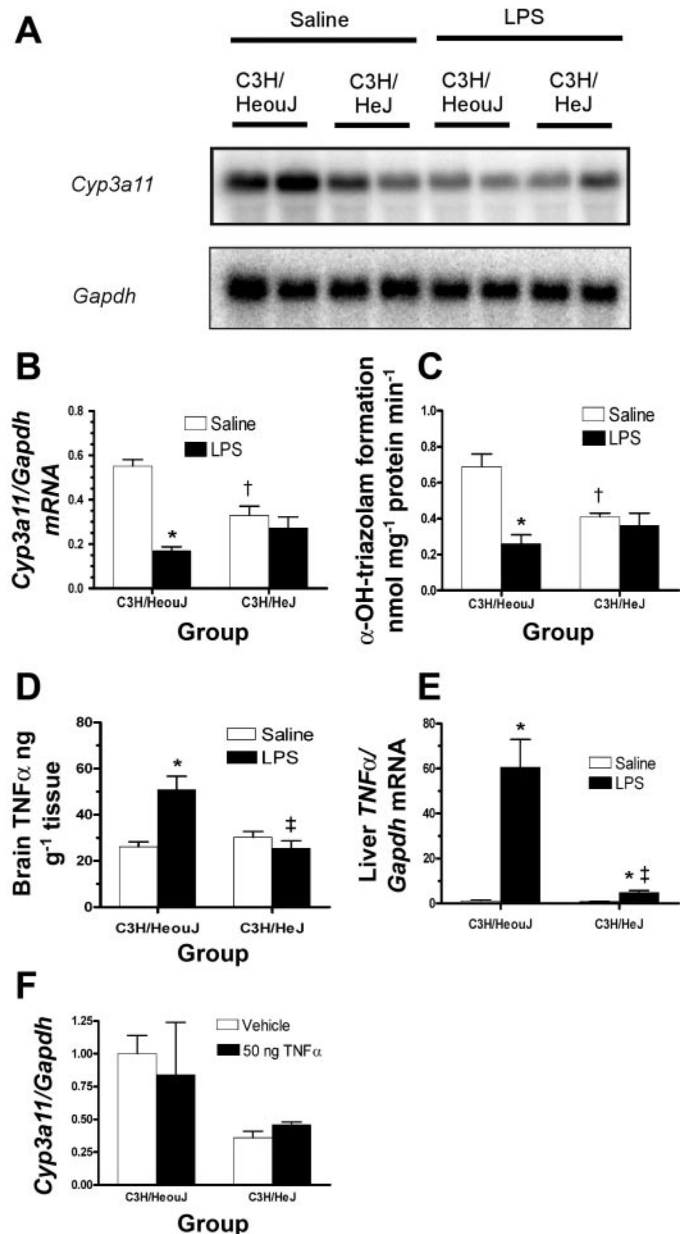

Fig. 5. Toll-like receptor-4 (TLR4) mutant mice (C3H/HeJ) are resistant to downregulation of *Cyp3a11* mRNA and activity after ICV administration of LPS. TLR4 mutant (C3H/HeJ) and wild-type (C3H/HeouJ) mice were injected with 2.5 μg of LPS or 2.5 μl of saline into the lateral cerebral ventricle. *A*: representative Northern blots of *Cyp3a11* and *Gapdh*. *B*: hepatic *Cyp3a11/Gapdh* mRNA expression as determined by densitometry. *C*: *Cyp3a11* enzyme activity (α-OH-triazolam formation) 24 h after treatment. *D*: TNF-α protein was measured by ELISA in brain homogenates 4 h after ICV injection of LPS as a marker of the central nervous system (CNS) inflammatory response. *E*: TNF-α mRNA was measured in liver 4 h after ICV injection of LPS as a marker of an inflammatory response in that organ. *F*: effect of ICV administration of 50 ng of TNF-α on hepatic *Cyp3a11* expression. Each bar represents the mean ± SE of 4 mice. *Different in LPS-treated compared with respective saline-treated mice ($P < 0.05$). †*Cyp3a11* mRNA and activity were lower in saline-treated C3H/HeJ mice compared with saline-treated C3H/HeouJ mice, and ‡TNF-α was lower compared with LPS-treated C3H/HeouJ mice ($P < 0.05$, ANOVA followed by Tukey's post hoc test).

peritoneal route was examined with TLR4-expressing and TLR4 mutant mice. In C3H/HeouJ mice, a high dose (5 mg/kg) of LPS produced a 90% reduction in *Cyp3a11* mRNA and a corresponding 60% loss of α-hydroxytriazolam formation (Fig. 7). In contrast, TLR4 mutant (C3H/HeJ) mice were completely resistant to the loss in *Cyp3a11* mRNA and activity produced by the high dose of LPS administered intraperitoneally. These data identify that peripheral TLR4 stimulation is a requirement for the downregulation of the major drug-metabolizing hepatic cytochrome *P*-450 enzyme by systemic *E. coli* LPS.

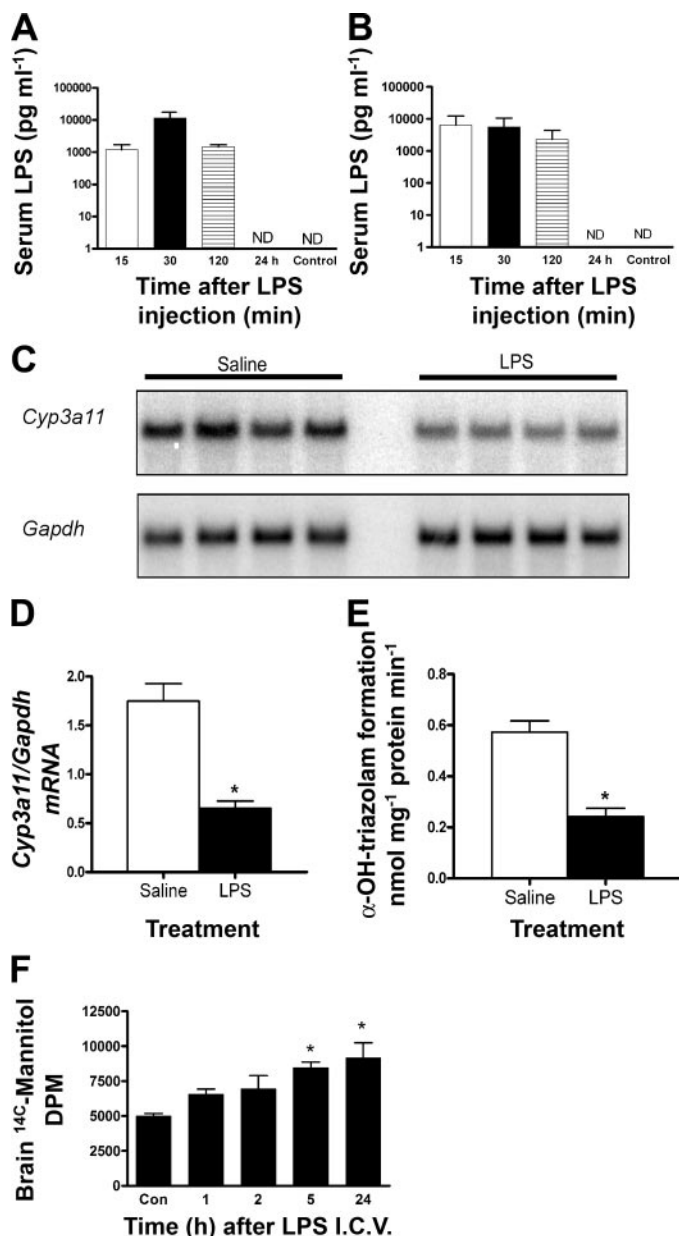

Fig. 6. After ICV administration, LPS is transferred from the brain to the periphery. Serum endotoxin levels were measured by the kinetic chromogenic *Limulus* ameboctye lysate assay in noninjected mice or at various times after 2.5 μg of LPS were administered ICV (*A*) or intraperitoneally (IP) (*B*) to C3H/HeouJ mice. ND, not determined. *C*: Northern blots for hepatic *Cyp3a11* and *Gapdh* mRNA. *D*: hepatic *Cyp3a11*/*Gapdh* mRNA expression determined by densitometry. *E*: hepatic microsomal *Cyp3a11* activity (α-OH-triazolam formation) 24 h after IP administration of 2.5 μg of LPS or saline to C3H/HeouJ mice. *F*: brain accumulation of peripherally administered [^{14}C]mannitol (4 nmol) was used as marker of blood-brain solute permeability changes at various times after ICV injection of 2.5 μg of LPS. Each bar represents the mean ± SE of 3 or 4 mice. *D* and *E*: **Cyp3a11* mRNA or enzyme activity was lower compared with the saline-treated control ($P < 0.05$, unpaired *t*-test). *F*: *$P < 0.05$ compared with control (ANOVA followed by Tukey's honestly significant difference test).

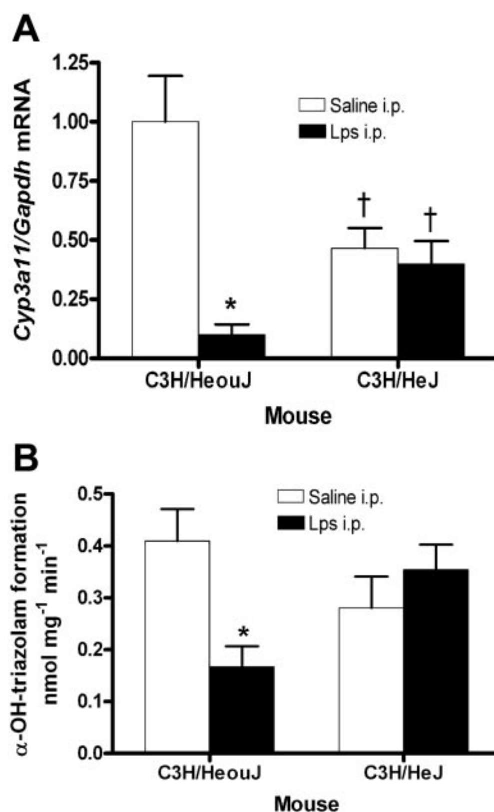

Fig. 7. Regulation of hepatic *Cyp3a11* metabolism by systemic LPS is mediated via TLR4 signaling pathways. TLR4 mutant (C3H/HeJ) and wild-type (C3H/HeouJ) mice were injected with 125 μg (5 mg/kg) of LPS or saline into the peritoneal cavity. *A*: hepatic *Cyp3a11/Gapdh* mRNA expression as measured by densitometry. *B*: *Cyp3a11* enzyme activity 24 h after IP injection of LPS. Each bar represents the mean ± SE of 4 mice. **Cyp3a11* expression or activity was lower compared with the saline-treated C3H/HeouJ mice ($P < 0.05$). †*Cyp3a11* expression in the C3H/HeJ mice was different compared with the respective saline-treated or LPS-treated C3H/HeouJ mice ($P < 0.05$, ANOVA followed by Tukey's post hoc test).

DISCUSSION

In experimental models of CNS infection and/or inflammation, the central administration of LPS produces an inflammatory response in the brain characterized by the activation of microglia, astrocytes, and proinflammatory cytokine pathways; the release of inflammatory cytokines (TNF-α, IL-1β, and IL-6); and leukocyte infiltration (30, 35). The CNS inflammatory response in our mouse model was confirmed by the presence of elevated inflammatory cytokines (TNF-α and IL-1β) in the brains of LPS-treated FVB or C3H/HeouJ mice. We have observed a significant downregulation of expression and metabolic activity of hepatic *Cyp3a11* in FVB, C57BL/6, and C3H/HeouJ mice but not in C3H/HeJ mice. A large reduction in hepatic CYP3A metabolism produced by an inflammatory response would have implications for drug toxicity because the cytochrome *P*-450 subfamily metabolizes a large percentage of

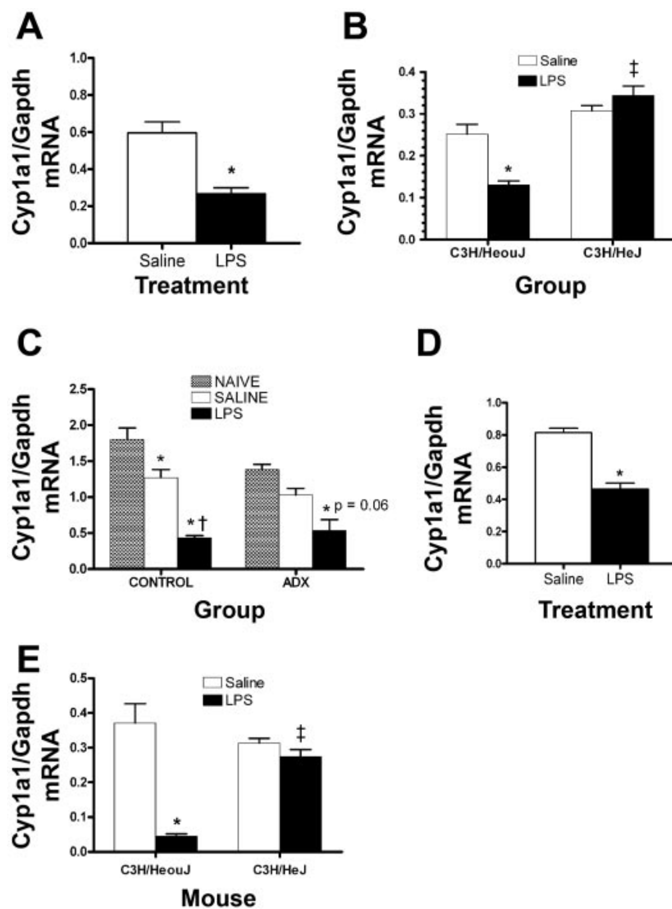

Fig. 8. Regulation of hepatic *Cyp1a1* mRNA expression by *E. coli* LPS. In *A–C*, mice received ICV injection of LPS (2.5 μg) or saline (2.5 μl). Hepatic *Cyp1a1* levels were measured 24 h after treatment. ICV treatment with LPS decreased hepatic *Cyp1a1* mRNA in male FVB mice (*A*) and in TLR4-expressing (C3H/HeouJ) mice but in not TLR4 mutant (C3H/HeJ) mice (*B*) and in control and adrenalectomized FVB mice (*C*) compared with relative saline controls and/or naive mice. *D*: hepatic *Cyp1a1* mRNA was decreased (45%) 24 h after IP administration of LPS (2.5 μg) to C3H/HeouJ mice compared with that in saline-injected control mice. *E*: hepatic *Cyp1a1* mRNA was decreased (85%) 24 h after IP administration of 125 μg of LPS to TLR4-expressing (C3H/HeouJ) but not TLR4 mutant (C3H/HeJ) mice compared with that in the respective saline controls. Each bar represents the mean ± SE of 3 or 4 samples. An unpaired *t*-test was used for statistical comparison in *A* and *D*. ANOVA was used for statistical comparison in *B*, *C*, and *E*. **P* < 0.05 compared with the respective saline-treated mice. †*P* < 0.05 compared with the respective naive mice (*C*). ‡*P* < 0.05 compared with LPS-treated C3H/HeouJ mice (*B* and *E*). Mean *Cyp1a1* mRNA level in LPS-treated adrenalectomized mice approached statistical significance compared with the saline control (*P* = 0.06).

clinically used drugs in humans (1). The present results were not restricted to *Cyp3a11* expression as similar losses in hepatic *Cyp1a1* expression were generated by the imposed experimental conditions (Fig. 8). The extent (50–70%) of hepatic cytochrome *P*-450 downregulation in the mouse was similar to the loss in hepatic cytochromes *P*-450 that occurred in our laboratory's previously established rat model of CNS infection/inflammation (14, 31, 41).

The existence of a pathway to activate a peripheral acute phase response during infections of the CNS was supported by the previous observations that inflammatory cytokine levels in the periphery were elevated and the hepatic acute phase response was stimulated (8, 15, 48, 51). This undetermined signaling pathway has been proposed to regulate drug metabolism in the liver during CNS infections or inflammation (41, 45). The HPA axis is activated by cytokines during the acute phase response and may have a suppressive role against the reduction of ethoxyresorufin *O*-deethylase (*Cyp1a*), pentoxyresorufin *O*-depentylase (*Cyp2b*), imipramine *N*-demethylase (*Cyp2d*), and erythromycin *N*-demethylase (*Cyp3a*) metabolism after ICV injection of LPS to rats (9, 45, 48). In the present study, adrenalectomy prevented the elevation of plasma corticosterone but did not protect or enhance the LPS-evoked depression in hepatic *Cyp3a11* expression and activity after LPS administration. This confirms that the HPA axis plays no role in hepatic *Cyp3a11* downregulation in the mouse model of CNS infection or inflammation. Others have examined the stimulation of the sympathetic nervous system or transduction of TNF-α, IL-1β, and IFN-γ from the brain to the periphery, but neither of these pathways could explain the link between CNS infection/inflammation and the loss in hepatic cytochrome *P*-450 enzyme activity in rats (31, 39, 45). Consistent with the findings of Nicholson et al. (31), in the present study, centrally administered TNF-α did not reduce *Cyp3a11* expression in C3H/HeouJ or C3H/HeJ mice, suggesting that CNS-derived TNF-α is not the major factor involved in downregulation of hepatic *Cyp3a11* after the ICV injection of LPS. IL-6 could also contribute to *Cyp3a11* downregulation. However, LPS decreased hepatic *Cyp3a11* expression by 80% in both IL-6-expressing and IL-6-null mice and suggested against a major role for that cytokine for the downregulation of *Cyp3a11* expression by LPS (46).

LPS has been identified as a specific ligand of TLR4 (6, 36). Signaling through the LPS receptor (TLR4) is an additional pathway that could initiate the reduction in hepatic drug metabolism in the model of CNS inflammation. We observed that the TLR4 mutants (C3H/HeJ mice) were totally resistant to the LPS-mediated loss in hepatic *Cyp3a11* expression and activity that was observed in mice that express functional TLR4. Basal levels of *Cyp3a11* mRNA expression and triazolam hydroxylation were reduced in the C3H/HeJ mice. On the basis of those data, C3H/HeJ mice behaved phenotypically as slow metabolizers of triazolam, whereas C3H/HeouJ mice were fast metabolizers of the drug. The LPS effect in C3H/HeouJ mice was functionally significant because it was able to change the metabolic phenotype of the C3H/HeouJ mice from that of a fast metabolizer to that of a slow metabolizer of triazolam. Several of our findings also support that C3H/HeJ mice are resistant to LPS-mediated reductions in *Cyp3a11* expression and activity because of inhibition of signal transduction through the TLR4 and not because of reduced basal expression of that cytochrome *P*-450 gene. First, it was clear from the peripheral inflammation study that LPS could reduce *Cyp3a11* mRNA and triazolam hydroxylation in C3H/HeouJ mice below basal levels in the C3H/HeJ mice. Second, basal *Cyp1a1* expression was similar in C3H/HeouJ and C3H/HeJ (Fig. 8) mice but was only depressed by LPS in the C3H/HeouJ mice. Finally, hyporesponsiveness to LPS in the TLR4-mutant mice was confirmed by the lack of a TNF-α response in the brain and liver.

The lack of the TNF-α response in C3H/HeJ mice after the ICV administration of LPS indicated the absence of a CNS inflammatory response. However, we could not conclude that

blockade of the CNS inflammatory response was the only factor that protected the TLR4 mutant mice from the loss in hepatic *Cyp3a11* because TLR4 are also present on peripheral macrophages and hepatocytes and could still elicit an immune response to *E. coli* LPS (4, 21). This idea of a peripheral inflammatory response is supported by the observation that liver TNF-α mRNA and serum levels of TNF-α and IL-1β were elevated by ICV administered LPS. If LPS was secreted into the blood from the cerebral spinal fluid, then a resulting peripheral inflammatory response could explain the decrease in hepatic *Cyp3a11* metabolism.

Several studies have suggested that LPS was restricted to the CNS when it was injected into the lateral ventricle of the brain (41, 45). In contrast, the present observations provide convincing evidence that LPS is not restricted to the CNS after central administration and is transferred to the blood in significant quantities. Similar quantities of LPS were detected in serum, and a similar loss in hepatic *Cyp3a11* expression and activity was observed after the intraperitoneal administration of LPS (2.5 μg). The systemic bioavailability, as approximated by AUC, was similar after both routes of administration. These results indicated that, after ICV administration, the amount of LPS reaching the systemic circulation is large enough to alter hepatic cytochrome *P*-450 solely by actions in peripheral tissues. Thus a transfer of LPS from the CNS may have lead to the previous observation of reduced hepatic cytochrome *P*-450 after ICV administration of LPS to rats (41, 45).

E. coli LPS (0127:B8, 200 μg) injected ICV to rats increased blood-brain barrier permeability between 4 and 16 h after the injection (18). Changes in blood-brain barrier permeability could result in LPS leakage into the peripheral circulation. However, our experimental results suggested that peak LPS transfer (between 1 and 2 h) from the ventricles occurred on a time scale that preceded the opening of the blood-brain barrier. The majority of cerebral spinal fluid is resorbed by bulk flow into the systemic circulation via transfer from the subarchnoid villi into the superior sagittal venous sinus (7). The bulk flow mechanism begins to return other blood-brain barrier-impermeable substances (albumin and leptin) to the systemic circulation within minutes of their injection by the ICV route (7, 24). Thus the bulk flow reabsorption of cerebral spinal fluid is likely the more important mechanism for LPS transfer to the peripheral circulation as opposed to passage across the blood-brain barrier.

The decrease in hepatic *Cyp3a11* expression and activity in response to circulating levels of LPS also appears to involve TLR4, as the intraperitoneal injection of LPS had no effect in TLR4-mutant mice. Others have shown that C3H/HeJ mice were resistant to LPS downregulation of hepatic ethoxycoumarin dealkylase (*Cyp2a*) and ethoxyresorufin dealkylase (*Cyp1a/2*) enzyme activities 24 h after LPS was administered intraperitoneally, but this work was conducted before the identification of TLR4 as a critical component of the LPS signal transduction pathway (13, 44). Also, in a model of indomethacin-induced intestinal injury, TLR4 activation by LPS released from intestinal flora may contribute to downregulation of hepatic *Cyp3a11*, *Cyp1a1/2*, and *Cyp2d9* enzyme activities (25). Together, these results directly support the idea that peripheral TLR4 are involved in the regulation of hepatic cytochrome *P*-450 enzymes by peripherally administered LPS. TLR4-mediated signaling occurs through two intracellular pathways (47). The MyD88-dependent pathway results in the direct induction of inflammatory cytokines (e.g., TNF, IL-1β, and IL-6). The MyD88-independent pathway activates NF-κB with delayed kinetics as well as the interferon response factor-3, which upregulates the expression of interferon-inducible genes. Although not determined in the present study, experiments in MyD88-deficient and interferon response factor-3-deficient mice should reveal which of these two pathways is most important for *Cyp3a11* downregulation by LPS.

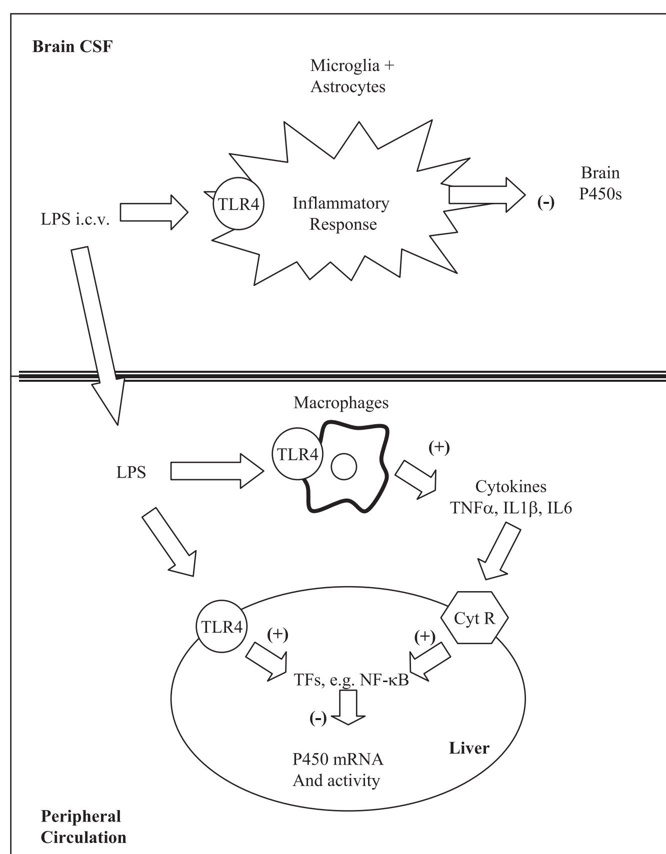

Fig. 9. Downregulating hepatic cytochrome *P*-450 metabolism in a model of gram-negative bacterial infection of the CNS. LPS injected within the lateral ventricle produces TLR4-dependent CNS inflammation, which may be important for decreased cytochrome *P*-450 activity in the brain but is not likely the major factor that reduces hepatic cytochrome *P*-450 metabolism. We demonstrate an alternative mechanism in which centrally administered LPS is rapidly transferred from the cerebral spinal fluid (CSF) to the blood. Peripheral LPS activates TLR4-dependent inflammatory pathways in peripheral immune cells (e.g., macrophages), leading to an elevation in circulating inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6). It is well known that those inflammatory cytokines activate signal transduction pathways within the hepatocyte and the expression/activation hepatic transcription factors (e.g., NF-κB), which inhibit expression of cytochrome *P*-450. Additionally, direct LPS stimulation of TLR4 signaling pathways in the hepatocyte represents a potential pathway that could trigger intracellular events that lead to *Cyp3a11* downregulation. TF, transcription factor; Cyt R, cytokine receptor; +, activation; −, inhibition or downregulation.

Our findings have led to a putative model of how CNS infection could lead to the loss of cytochrome *P*-450 expression and activity in the liver, as shown in Fig. 9. LPS injected within the lateral ventricle produces TLR4-dependent CNS inflammation. The localized inflammation is likely important for the loss in cytochrome *P*-450 that occurs in the brain (41) but is not the major factor that depresses cytochrome *P*-450

metabolism in the liver. We propose that centrally administered LPS is rapidly transferred from the cerebral spinal fluid to the blood. Through a TLR4-dependent mechanism, circulating LPS then reduces hepatic *Cyp3a11* mRNA expression and enzyme activity. The loss of hepatic cytochrome *P*-450 produced by systemic LPS could occur indirectly through the action of circulating cytokines such as TNF-α, IL-1β, and IL-6 that are released from a peripheral source (e.g., macrophages or Kupffer cells) (12, 13, 28, 31, 39, 44). Those cytokines activate nuclear transcription factors within the hepatocyte, which regulate cytochrome *P*-450 expression in the liver (2, 3, 17, 19, 29, 33, 38). Despite the direct link between cytokines and cytochrome *P*-450 expression, large doses of LPS (1–5 mg/kg) were able to mediate hepatic *Cyp3a11* downregulation in vivo in mice with targeted disruptions in IL-6 and the TNF-α receptor (46, 50). This could be explained by the observations that LPS stimulates NF-κB signaling in isolated hepatocytes through TLR4 present on those cells (21, 49). Thus the direct action of LPS on the hepatocyte represents a potential mechanism for *Cyp3a11* downregulation in the liver.

In summary, in the presence of a CNS infection, the transfer of endotoxin from the brain to the periphery could stimulate a peripheral immune response that produces a loss in cytochrome *P*-450-mediated drug metabolism in the liver. We have also identified that stimulation of TLR4 is responsible for linking the immune response to *E. coli* endotoxin with a major drug metabolizing cytochrome *P*-450 in the mouse liver. The reduction in cytochrome *P*-450 enzymes during inflammatory responses or infections of the CNS may reduce the metabolism and elimination of several clinically used drugs and increase the potential for adverse drug interactions.

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GRANTS

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