DMD Fast Forward. Published on July 8, 2005 as doi:10.1124/dmd.105.004564

The signal transduction pathways involved in hepatic cytochrome P450 regulation in the rat during an LPS-induced model of CNS inflammation

Dalya Abdulla, Kerry B. Goralski, Elena Garcia Del Busto Cano, and Kenneth W. Renton

Department of Pharmacology (D.A., K.B.G., E.G.D.B.C., K.W.R), Department of Pediatrics, IWK Health Center (K.B.G.), Dalhousie University, Halifax, Nova Scotia, B3H 4H7, Canada

Running Title: CYP regulation during CNS inflammation

Corresponding Author:

Kenneth W. Renton

Dept. of Pharmacology

Sir Charles Tupper Medical Bldg.

Dalhousie University

Halifax, N.S.

B3H 4H7

Telephone: (902)494-2562

Fax: (902)494-1388

Email: Ken.Renton@dal.ca

Number of Text Pages: 39

Number of Figures: 10

Number of Tables: 2

Number of References: 38

Number of words in Abstract: 200

Number of words in Introduction: 486

Number of words in Discussion: 1432

Abbreviations: LPS, lipopolysaccharide; CNS, central nervous system; CYP, cytochrome P450; NO, nitric oxide; TNFα, tumor necrosis factor alpha; IL-1β, interleukin-1β; IFNγ; interferon gamma; NF-κB, nuclear factor-kappa B; AP-1, activator-protein-1; CREB, cAMP response element binding protein; C/EBP, CAAT enhancer binding protein; EROD, 7-ethoxyresorufin O-dealkylase; PROD, pentoxyresorufin O-dealkylase; ICE-1, IL-1 converting enzyme; TLR, Toll-Like receptor.

Abstract

It is well known that inflammatory and infectious conditions of the central nervous system (CNS) differentially regulate hepatic drug metabolism through changes in cytochrome P450 (CYP), however the pathways leading to this regulation remain unknown. We provide evidence delineating a signal transduction pathway for hepatic CYP gene expression downregulation in an established rat model of CNS inflammation using lipopolysaccharide (LPS) injected directly into the lateral cerebral ventricle (i.c.v.). Brain cytokine levels were elevated and the expression of TNFα and IκBα were increased in the liver following the i.c.v. administration of LPS, indicating the presence of an inflammatory response in the brain and liver. The expression of CYP2D1/5, CYP2B1/2, and CYP1A1 was downregulated following CNS inflammation. The binding of several transcription factors (NF-κB, AP-1, CREB, C/EBP) to responsive elements on CYP promoter regions was examined using electromobility shift assays. Binding of both NF-κB and C/EBP to the promoter regions of CYP2D5 and CYP2B1, respectively, was increased, indicating that they play an important role in the regulation of these two isoforms during inflammatory responses. Evidence is also provided suggesting that the rapid transfer of LPS from the CNS into the periphery likely accounts for the downregulation of CYPs in the liver.

Introduction

Cytochrome P450 constitutes a superfamily of heme containing enzymes that are well known for their role in the metabolism and elimination of various exogenous and endogenous substances (Chang and Kam, 1999; Renton, 2001). In addition to metabolism, CYP isoforms play a major role in many biochemical and physiological pathways such as the biosynthesis and/or degradation of steroid hormones and fatty acids (Chang and Kam, 1999). Changes in the levels of CYP isoforms may contribute to the development of cancer, Parkinson's disease, and adrenal hyperplasia (Chang and Kam, 1999). The majority of the CYP isoforms are found in the liver, however other extra-hepatic sites of CYP localization include the CNS, gastrointestinal tract, kidney, lungs, and adrenal glands (Anzenbacher and Anzenbacherova, 2001).

The effects of host defence and immune stimulation on CYP isoforms have been well documented (Renton, 2001; Morgan et al., 2002). Both viral and bacterial inflammatory conditions can lead to differential regulation of hepatic CYP isoforms (Renton and Nicholson, 2000; Nicholson and Renton, 2001). Cytokines are known to play a dominant role in this regulation; IFNγ, IL-1β, IL-6, and TNFα have been shown to downregulate hepatic CYP1A1, CYP1A2, CYP2B1/2, and CYP3A1/2 in rat models of systemic inflammation when given alone or in combination (Barker et al., 1992; Barker et al., 1994; Pan et al., 2000; Morgan, 2001; Renton, 2001; Morgan et al., 2002; Nicholson and Renton, 2002). The injection of immunostimulants directly into the CNS produces a highly regulated inflammatory response characterized by the production of cytokines, immune cell infiltration, and tissue damage (Nicholson and Renton, 1999; Renton, 2001; Gavrilyuk et al., 2002; Nicholson and Renton, 2002; Rivest, 2003). We have previously demonstrated that the catalytic activity of both hepatic and CNS CYP isoforms are downregulated in rat models of CNS inflammation/infection (Renton

and Nicholson, 2000; Nicholson and Renton, 2001; Garcia Del Busto Cano and Renton, 2003). A reduction in brain CYP metabolism may exacerbate susceptibility to neurotoxic agents such as 1-methyl-4-phenylpyridinium (Goralski and Renton, 2004). A loss or reduction in hepatic CYP levels has direct consequences for reduced metabolism of therapeutic agents (Chang and Kam, 1999; Renton, 2001). The pathways directly causing the downregulation of CYP isoforms following conditions of CNS inflammation are not completely understood, however multiple mechanisms are implicated.

Our objective was to determine the signal transduction mechanisms that contribute to hepatic CYP regulation in a rat model of LPS-induced CNS inflammation. To perform that, we examined the regulation of hepatic CYP gene expression following the intracerebroventricular (i.c.v.) administration of LPS, a well known and utilized model of CNS inflammation/infection (Shimamoto et al., 1999; Renton and Nicholson, 2000; Nadeau and Rivest, 2002). Our major findings are that several hepatic transcription factors play a vital role in CYP gene regulation during conditions of CNS inflammation and that LPS is rapidly transferred from the CNS into the periphery where it likely contributes to the effects observed in this rodent model of CNS inflammation.

DMD Fast Forward. Published on July 8, 2005 as DOI: 10.1124/dmd.105.004564
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Reagents

All laboratory reagents were purchased from Sigma (St. Louis, MO) with the exceptions noted in the text. Gel purified *Escherichia coli* lipopolysaccharide (LPS) of sereotype 0127:B8 (Sigma, St Lois, USA) was utilized in the experiments as outlined below.

Animals and treatments

Male Sprague-Dawley rats (125 – 150 grams) were obtained from Charles River Laboratories (Quebec, Canada) and were housed on corncob bedding for a period of 5 days on a 12-hour light/dark cycle. All animal procedures were performed according to the Dalhousie University Committee on Laboratory Animals following the guidelines established by the Canadian Council on Animal Care. Rats were allowed *ad-libitum* access to food and water prior to and following the experimental procedure. On the day of the experiment, rats were anaesthetized using enflurane and maintained on a 4% level of the anaesthetic during the surgery. Intracerebroventricular (i.c.v.) injections into the lateral ventricle were performed using a KOPF sterotaxic instrument. The coordinates utilized relative to bregma were 1.7 mm lateral and 4.7 mm below the skull surface. A dose of 25 μg LPS was dissolved in pyrogen-free saline and injected in a volume of 5 μL. In a separate set of experiments, rats were injected intravenously (i.v.) through the tail vein with 25 μg LPS dissolved in 100 μL of saline and tissue samples obtained at either 3, 6, or 24 hours following injection. Some experiments required intraperitoneal injection of rats with 25 μg LPS dissolved in 100 μL of saline. All experiments utilized 4 – 6 male rats per treatment. For the cytokine experiments, animals received a cytokine cocktail containing 100 ng TNFα, 50 ng IL-1β, 45 ng IL-1α, 50 ng IL-6, and 50 ng IFNγ (all cytokines obtained from CedarLane Laboratories Ltd, Ontario, Canada) made in 0.1 % sterile

filtered BSA. For the cytokine inhibitor experiments, rats received 25 μg of LPS i.c.v. in combination with one of the following treatments: the TNFα soluble antibody etanercept (Enbrel™, Immunex, Wyeth-Ayerst, Canada) administered either i.p. (25 mg kg^{-1}) or i.c.v. (40 μg) or the IL-1 inhibitor YVAD (Alexis Biochemicals, California, USA) administered i.c.v. (0.63 μg). Doses for the cytokine cocktail and the cytokine inhibitor experiments were selected based on results from dose response curves (data not shown).

Tissue isolation, microsomal fraction preparation, and microsomal metabolism assays

At 2, 4, 6, and 9 hours following the i.c.v. injection of either saline or LPS, rats were anaesthetized, decapitated, and liver (~100 mg) for RNA isolation was obtained, serum collected, and whole brain homogenates for cytokine measurement was obtained. For the i.v. experiments, total liver RNA was isolated at 3 and 6 hours following treatment. Liver microsomal fractions were obtained at 24 hours following either the i.v. or i.c.v. injection of LPS as described previously (Renton and Nicholson, 2000) and were suspended in a glycerol-phosphate buffer (50 mM KH_2PO_4 buffer, pH 7.4, containing 20% glycerol and 0.4% KCl). Liver microsomal fractions were stored at –80^{o}C until usage. Total protein concentrations were determined according to a modified Lowry protocol (Lowry et al., 1951). The activity of CYP1A1/2 and CYP2B1/2 were determined using the 7-Ethoxyresorufin O-dealkylase (EROD) assay and the Pentoxyresorufin O-dealkylase (PROD) assay, respectively, as described by Burke *et al* (Burke et al., 1985). Total P450 values were determined according to the method of Omura and Sato (Omura and Sato, 1964).

RNA extraction and northern blot analysis

Total liver RNA was extracted using the TriZol® method according to manufacturer's instructions and quality was determined using 260/280 nm ratios. 10 μg of total RNA was

DMD Fast Forward. Published on July 8, 2005 as DOI: 10.1124/dmd.105.004564
This article has not been copyedited and formatted. The final version may differ from this version.

electrophoresed on a 1.1% formaldehyde gel and transferred onto an immobolin–NY+ membrane (Millipore Corporation, MA, USA) overnight and fixed to the membrane by UV cross linking and heating for 1 hour at 65^{o}C. Blots were prehybridized for 1 hour in 10 mL of Sigma Perfecthyb™ Plus (Sigma, St Lois, MS) after which the [^{32}P]dCTP (Perkin and Elmer, Canada) labelled probes (RmT Random Primer Labelling kit, Stratagene, USA) were added to a specific activity of 1×10^{7} cpm. Blots were exposed to a storage phosphor screen (Amersham Biosciences, NJ, USA) for 16 – 24 hours and scanned using a phospher imager (Amersham Biosciences, NJ, USA). Bands were quantified using ImageQuant 5.2™ software (Amersham Biosciences, NJ, USA). CYP2D1/5 (Chow et al., 1999), TNFα (Cearley et al., 2003), and IκBα (Gavrilyuk et al., 2002) probes were constructed from forward and reverse primers (CYP2D1/5 FWD 5′ ATC GCT GGA CTT CTC GCT AC 3′, CYP2D1/5 REV 5′ GTC TTC TGA CCT TGG AAG AC 3′, TNFα FWD 5′ TAC TGA ACT TCG GGG TGA TTG GTC C 3′, TNFα REV 5′ CAG CCT TGT CCC TTG AAG AGA ACC 3′, IκBα FWD 5′ CAT GAA GAG AAG ACA CTG ACC ATG GAA 3′, IκBα REV 5′ TGG ATA GAG GCT AAG TGT AGA CAC G 3′) using a TOPO TA Cloning® kit (Invitrogen, Ontario, Canada) according to manufacturer's instructions. CYP2B1/2, MAPKK, and GAPDH probes were a generous gift from Dr. C. J. Sinal (Dalhousie University, Canada).

Real-time quantitative PCR

A total of 5 μg of liver RNA was reverse transcribed in a 25 μL reaction containing 62.5 nM random primers, 20 Units of RNaseOUT (Invitrogen, Ontario, Canada), 1× StrataScript® RT-buffer and 12.5 Units of StrataScript® Reverse Transcriptase (both Stratagene, California, USA) according to the instructions provided with the Reverse Transcriptase enzyme. Real-time quantitative PCR was performed using an MX3000P™ instrument (Stratagene, California, USA)

in a total volume of 20 μL. Reactions contained 10 μL of 2× Brilliant® SYBR® Green QPCR mix (Stratagene, California, USA), 62.5 ng of both forward and reverse primers, and 25 nM of reference dye. Cycle parameters consisted of an initial 10 minute denaturation step at 95°C followed by either 35 cycles for GAPDH or 45 cycles for CYP1A1 as follows: 30 second denaturation at 95°C, 18 seconds annealing at 60°C, and 30 seconds extension at 72°C. Dissociation curves were also performed to verify the amplicon being amplified. Primers specific for CYP1A1 and GAPDH were specifically designed using the published sequence for rat CYP1A1 (Accession number X00469) and rat GAPDH (Accession number X02231) as follows: CYP1A1 FWD 5′ GGA GCT GGG TTT GAC ACA AT 3′, CYP1A1 REV 5′ GAT AGG GCA GCT GAG GTC TG 3′ (amplicon size 157 bp), GAPDH FWD 5′ AGA CAG CCG CAT CTT CTT GT 3′, GAPDH REV 5′ CTT GCC GTG GGT AGA GTC AT 3′ (amplicon size 207 bp). Data was analysed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001), where the cycle threshold (C_T) values for CYP1A1 and GAPDH were ~ 32 and ~19, respectively.

Cytokine measurements

Whole brain was obtained following the i.c.v. injection of LPS at 2, 4, 6, and 9 hours and homogenized in 2 mL of phosphate buffer saline, pH 7.4. The homogenates were spun at 13,000 rpm for 10 minutes at 4°C and the supernatant was stored at –80°C until used for cytokine measurements. Protein levels in the brain homogenates were determined using a modified Lowry method (Lowry et al., 1951). Levels of TNFα and IL-1β in the brain were measured using a sandwich ELISA (R&D systems, Minneapolis, USA) and results are reported as pg of cytokine per mg of protein present in the brain homogenate. The limits of detection for both TNFα and IL-1β cytokine ELISA assays was 5 pg mL^{-1}.

Nitrite measurement

The total amount of NO in plasma was indirectly determined by measuring total nitrites and nitrates (end products of NO oxidation) using a NO assay kit (Cayman Chemical, Ann Arbour, Michigan, USA) according to manufacturer's instructions. The kit involved converting nitrates into nitrites and then measuring the converted products using Griess Reagent. Nitrite accumulation was determined in a 96-well plate format with the absorbances read at 540nm. The limit of detection for this assay was 2.5 μM.

Determination of endotoxin levels

LPS concentrations were determined in serum at 15 minutes, 30 minutes, 2 hours, 4 hours, 6 hours, 15 hours, and 24 hours following the administration of LPS by either the i.c.v. or i.p. route. Rats were injected with 25 μg of LPS either i.c.v (in 5 μL of pyrogen-free saline) or i.p. (in 100 μL of pyrogen-free saline). Following decapitation, trunk blood was collected and allowed to clot for 1 hour at room temperature and 1 hour on ice, and serum was obtained following a 10-minute spin at 3000 rpm, and stored at –80^{o}C until usage. All groups were compared to non-injected rats. LPS levels were determined per manufacturer's instructions from a commercially available kinetic assay kit based on ChromoLAL as a substrate (Associates of Cape Cod, MA, USA). The assay was linear from 0.0395 pg mL^{-1} to 100 ng mL^{-1} of LPS. The levels of endotoxin in the saline used to resuspend LPS were below the limit of detection of the assay, indicating that samples preparation was sufficiently aseptic for endotoxin detection.

Liver nuclear fraction isolation

Liver nuclear fractions were isolated from rats 1 and 3 hours following the i.c.v. injection of LPS according to a previously described method (Gorski et al., 1986). Briefly, animals were decapitated and livers were homogenized in 20 mL of Homogenization Buffer (100 mM HEPES,

pH 7.4, containing 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 5 μg mL^{-1} pepstatin A, and 5 μg mL^{-1} leupeptin) and centrifuged at 17,000 rpm for a period of 20 minutes at 4^{o}C. The nuclear pellet was resuspended in 10 mL of Nuclear Lysis Buffer (100 mM HEPES, pH 7.4, containing 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, 5 μg mL^{-1} pepstatin A, and 5 μg mL^{-1} leupeptin) and homogenized using a Dounce homogenizer. The extraction was initialized by the addition of $(NH_4)_2SO_4$ to the nuclear lysate in drop-wise fashion to a final concentration of 0.4 M. The viscous lysates were incubated for period of 30 minutes on ice with constant shaking, after which they were ultra-centrifuged at 35,000 rpm for a period of 60 minutes at 4^{o}C. Solid $(NH_4)_2SO_4$ was added to the supernatants at a concentration of 0.3 g mL^{-1}. The solutions were inverted several times and incubated on ice for a period of 20 minutes until all the $(NH_4)_2SO_4$ had dissolved. The solutions were then further centrifuged at 35,000 rpm for a period of 25 minutes at 4^{o}C and the pellets were re-suspended in Suspension Buffer (25 mM HEPES, pH 7.6, containing 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 5 μg mL^{-1} pepstatin A, and 5 μg mL^{-1} leupeptin) and stored at –80^{o}C until usage. Total protein concentrations were determined according to a modified Lowry protocol (Lowry et al., 1951).

Electromobility shift assays

Reactions were carried out in a total volume of 20 μL and contained 5 μg of protein, 50000 cpm of [^{32}P]-labeled probes, Binding Buffer (50 mM Tris-HcL, pH 7.9 containing 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 20% glycerol), and 2 μg of polydIdC. Reactions were pre-incubated with the polydIdC for a period of 15 minutes after which the radiolabeled probe was added to initiate the reaction. In cases of specific competitions, a 20× excess amount of non-radioactive self oligonucleotide was utilized and was included in the reaction mixture.

Reactions were incubated at room temperature for a period of 30 minutes and run on a 5% TBE-acrylamide gel at a voltage of 170 V. In the case of non-specific competitions, a 20× excess amount of non-radioactive non-self oligonucleotide was utilized in the reaction mixture. For supershift reactions, the reaction mixture was incubated with either a p65 antibody (Santa Cruz, California, USA) or a C/EBPα antibody (generous gift from Dr. M. W. Nachtigal) for a 30-minute period following the addition of the radioactive specific probe. The gels were then dried using a Bio-Rad gel dryer (Bio-Rad, Ontario, Canada) operating under vacuum and set at 80^{o}C for 105 minutes. Gels were then exposed to a phospher-storage screen for a period of 16 – 24 hours and scanned using a phospher imager (Amersham Biosciences, NJ, USA). Bands were quantified using ImageQuant 5.2™ software (Amersham Biosciences, NJ, USA). The probes (described in Table 1) were either obtained commercially from Santa Cruz or as single-stranded oligonucleotides from Sigma Genosys (Sigma-Aldrich, Ontario, Canada) and annealed according to a standard protocol. Briefly, 300 pmoles of each oligonucleotide were incubated in Annealing Buffer (100 mM Tris, pH 7.9 and 50 mM $MgCl_2$) for 10 minutes at 95^{o}C and allowed to gradually cool down to 25^{o}C.

Statistical analysis

All data are reported as the mean ± the standard error of the mean. Each time point constituted a separate experiment in which LPS treatment was compared to saline and was carried out on a different day, and therefore an unpaired t-test was utilized to compare saline versus LPS groups, where $p < 0.05$ determined statistical significance. Data is presented in one graph for all time points for convenience in illustrating the data.

DMD Fast Forward. Published on July 8, 2005 as DOI: 10.1124/dmd.105.004564
This article has not been copyedited and formatted. The final version may differ from this version.

Results

The intracerebroventricular injection of LPS initiates an inflammatory response in the brain and liver

Levels of TNFα and IL-1β proteins were increased in the brains of animals at 2, 4, and 6 hours following the i.c.v. administration of LPS as compared to saline control rats (Figure 1A and B). These data indicate that a CNS inflammatory response occurs in response to the i.c.v administration of LPS.

The liver is considered as the major target organ of the acute phase response (Akiyama and Gonzalez, 2003) and therefore we measured the expression of various important acute phase proteins in that organ. IκBα expression levels in the liver were increased at 2, 4, 6, and 9 hours following the induction of CNS inflammation (Figure 2A). MAPKK expression levels in livers of rats treated with LPS i.c.v. were significantly increased by 3.4 fold and 3.7 fold at 4 and 6 hours, respectively, following the i.c.v. administration of LPS (Figure 2B). The expression of TNFα in the liver was significantly increased following LPS i.c.v. at 2, 4, and 6 hours compared to saline i.c.v. administration (Figure 2C). These results indicate the activation of hepatic acute phase signalling proteins in this rat model of LPS-induced CNS inflammation.

We have previously reported increased amounts of cytokines in the serum of rats treated with LPS i.c.v. compared to saline-injected controls (Nicholson and Renton, 2001). Since the elevation of nitrites/nitrates is also associated with inflammation, we examined nitric oxide levels in the plasma of animals treated with LPS i.c.v. compared to saline. The levels of total nitrates and nitrites increased at 4, 6, and 9 hours and were 19 fold higher than saline treated rats 15 hours following LPS administration (Figure 3).

The effects of CNS inflammation on mRNA expression of CYP isoforms

Based on the observed changes in TNFα, IκBα, and MAPKK in the liver following the i.c.v. administration of LPS, we examined the expression of CYPs in the liver at these time points. The expression level of CYP2D1/5 was unchanged at 2 and 4 hours but was significantly reduced by 45% and 58% at 6 and 9 hours, respectively, following the i.c.v. administration of LPS (Figure 4A). The expression levels of CYP2B1/2 were significantly downregulated (by 45%) only at 6 hours following the induction of CNS inflammation, and were unchanged at 2, 4, and 9 hours following LPS administration (Figure 4B). The levels of hepatic CYP1A1 expression began to decline between 2 and 4 hours following CNS inflammation and were significantly reduced by 90 % at 6 hours following the i.c.v. administration of LPS (Figure 4C). At 9 hours following the i.c.v. administration of LPS, CYP1A1 expression levels in the liver had returned to normal in the LPS treated rats compared to saline. These results indicate that the changes in the expression of these CYP isoforms correlates with the increased expression of hepatic acute phase signalling molecules.

The involvement of hepatic transcription factors in the regulation of CYPs during LPS-induced CNS inflammation

The importance of several transcription factors in the regulation of CYP expression during CNS inflammation was observed using EMSA assays. NF-κB, activator protein-1 (AP-1), CCAAT-enhancer binding protein (C/EBP), and cAMP response element binding protein (CREB) are important down-stream transcription factors that are activated by the acute phase response and are responsible for gene regulation in the liver during conditions of inflammation (Della Fazia et al., 1997; Akiyama and Gonzalez, 2003). At 3 hours following the administration of LPS i.c.v., a significant upregulation in the binding of P^{32}-labeled NF-κB, AP-1, and CREB oligonucleotides

to response elements in liver nuclear fractions isolated from LPS treated animals compared to saline treated animals was observed (Figure 5A, B, and C). One hour following the induction of CNS inflammation, the binding of AP-1 in liver nuclear fractions obtained from LPS treated animals was also significantly upregulated (data not shown). These data indicate that NF-κB, AP-1, and CREB proteins are elevated in hepatic nuclear fractions prior to the loss of CYP mRNA expression.

The binding of transcription factors to the promoter regions of specific CYP isoforms is illustrated in Figures 6 and 7. An upregulation in the binding of an NF-κB response element (identified using MacVector®) on the CYP2D5 promoter occurred in liver nuclear fractions isolated 3 hours following the treatment of rats with LPS i.c.v. (Figure 6A). The binding of NF-κB to this response element on CYP2D5 was confirmed using specific and non-specific competitions and was supershifted using a p65 antibody. NF-κB and C/EBP response elements have been identified on the CYP2B1 promoter (Park and Kemper, 1996; Shaw et al., 1996). An increased binding of NF-κB and C/EBP response elements occurred in hepatic nuclear fractions 3 hours following the administration of LPS i.c.v. (Figure 6B and Figure 7A). The binding of both proteins was confirmed using specific and non-specific competitions and was supershifted using a p65 and a C/EBPα antibody. No binding was observed to the CREB response element on the promoter region of CYP1A1 (identified using MacVector®) (data not shown). Finally, an upregulation in the binding of an NF-κB response element (identified using MacVector®) on the CYP1A1 promoter was observed in liver nuclear fractions isolated 3 hours following the treatment of rats with LPS i.c.v. (Figure 7B). The binding was confirmed using specific and non-specific competitions.

DMD Fast Forward. Published on July 8, 2005 as DOI: 10.1124/dmd.105.004564
This article has not been copyedited and formatted. The final version may differ from this version.

The peripheral effects on hepatic CYP expression observed following the i.c.v. administration of LPS can not be completely accounted for by cytokines

The above results indicate that several important intra-hepatic biochemical changes are occurring during this rodent model of LPS-induced CNS inflammation. We examined several pathways to explain the mechanisms by which the i.c.v. administration of LPS is causing the peripheral effects on hepatic CYPs and inflammatory mediator expression. It is known that total cytochrome P450 levels are downregulated in response to the i.c.v. administration of LPS (Renton and Nicholson, 2000). Thus we chose to examine the effects of cytokines administered centrally and cytokine inhibitors on total cytochrome P450 levels 24 hours following the i.c.v. administration of LPS. When rats were administered a cytokine cocktail i.c.v., we observed no change in total cytochrome P450 levels (Figure 8A). Co-administration of Enbrel™ (TNFα soluble antibody) either i.c.v. or i.p. with LPS i.c.v. could not prevent the LPS-induced downregulation in total cytochrome P450 levels (Figure 8B and C). YVAD, the IL-1 inhibitor, was also not able to prevent the LPS-induced downregulation in total cytochrome P450 levels when both agents were administered i.c.v. (Figure 8D).

LPS levels are detected in the serum of animals given 25 μg of LPS by i.c.v. or i.p. injection

Since the distribution of LPS following its administration i.c.v. to rats has not been previously characterized, we determined whether LPS transfer to the periphery might contribute to the observed changes in hepatic CYPs. We measured the amounts of LPS (in pg/mL) in the serum of animals administered 25 μg of LPS either intracerebroventricularly or intraperitoneally. Following the i.c.v. administration of LPS, the serum endotoxin levels were a 100 fold greater than the amounts detected in the serum following the i.p. administration of the same dose of LPS (Figure 9). We have previously shown that the administration of 25 μg LPS i.p. does not affect

liver CYP activity (Renton and Nicholson, 2000). Minimal LPS was detected in control rats that did not receive LPS.

We then examined the effects on hepatic CYP levels if the entire amount of 25 μg of LPS we normally inject i.c.v. was present in the bloodstream. We chose to examine the expression of CYP2D1/5, CYP2B1/2, CYP1A1, TNFα, and IκBα at 3 and 6 hours following the intravenous (i.v.) administration of LPS since these genes were differentially regulated at these time points following the i.c.v. administration of LPS. We observed a significant reduction in CYP2D1/5, CYP2B1/2, and CYP1A1 expression at 6 hours following the i.v. administration of LPS (22 %, 54 %, and 93 % less than the respective saline groups, respectively) (Table 2). In addition, we observed a significant increase in TNFα expression at 3 hours following the i.v. administration of LPS (328 % increase compared to respective saline group) and a significant increase in IκBα expression at 3 and 6 hours following the i.v. administration of LPS (10 fold and 3.3 fold increase compared to respective saline group, respectively) (Table 2). These results indicate that the effects of administering LPS i.v. on the expression of CYPs and inflammatory mediators is similar to the effects observed following the i.c.v. administration of LPS. We have previously shown that at 24 hours following the administration of 25 μg of LPS i.c.v., a downregulation in total cytochrome P450 levels, CYP1A catalyzed EROD activity, and CYP2B catalyzed PROD activity occurs (Renton and Nicholson, 2000). Examination of these endpoints revealed that the administration of 25 μg LPS i.v. was able to cause a significant decrease in total cytochrome P450 levels (30% less than saline treated rats), CYP1A catalyzed EROD activity (42% less than saline treated rats), and CYP2B catalyzed PROD activity (48 % less compared to saline treated rats) following the i.v. administration of LPS (Table 2).

Discussion

Inflammatory mediators such as cytokines, prostaglandins, and reactive oxygen species are known to be released from activated microglia, the resident macrophages of the brain, during conditions of CNS inflammation (Rivest, 2003). Our current results indicate that an inflammatory response occurs in the brain and in the peripheral tissues following the administration of LPS i.c.v. manifested by increases in the levels of pro-inflammatory cytokines in rat brain and blood (Renton and Nicholson, 2000) and increases in the mRNA expression of the acute-phase response genes TNFα, IκBα, and MAPKK in rat liver, as outlined in Figure 10. Changes in CYP gene regulation are well documented during inflammation responses occuring *in vitro* (Ke et al., 2001; Kelicen and Tindberg, 2004). It is likely that the regulation of hepatic CYPs observed *in vivo* (Renton and Nicholson, 2000; Nicholson and Renton, 2001) also occurs as a result of changes in gene expression. We tested this idea by examining the regulation of hepatic CYP2D1/5, CYP2B1/2, and CYP1A1 using a well utilized model of CNS inflammation induced by the i.c.v. injection of LPS. These particular isoforms were selected since they are known to play a significant role in the metabolism of many clinically relevant drugs (Anzenbacher and Anzenbacherova, 2001). Our results indicate a significant reduction in the mRNA expression of CYP2D1/5, CYP2B1/2, and CYP1A1 in the liver following the i.c.v. administration of LPS, which is in agreement with the loss in enzymatic activity and/or protein levels previously reported for these isoforms (Renton and Nicholson, 2000).

NF-κB, AP-1, and CREB are important transcription factors that regulate a number of genes in the liver during the acute phase response (Della Fazia et al., 1997; Ruminy et al., 2001; Akiyama and Gonzalez, 2003). Our current results indicate an increase in the binding of NF-κB, AP-1, and CREB in hepatic nuclear fractions obtained 3 hours following the i.c.v. administration

of LPS, indicating a potential role for these acute phase proteins in the downregulation of hepatic CYPs during LPS-induced CNS inflammation. In support of this, we have observed an increased production of both TNFα and IκBα in the liver, known target genes upregulated through the NF-κB pathway. The acute phase response in the liver characterized by cytokine production and transcription factor upregulation could be an intrahepatic mechanism responsible for the repression in CYP2D1/5, CYP2B1/2, and CYP1A1 expression in the liver following the i.c.v. administration of LPS. To test the hypothesis, we examined the effects of the i.c.v. administration of LPS to the binding of NF-κB to the promoter regions of specific CYP isoforms. Our results indicate that hepatic NF-κB is likely to play an important role in the regulation of CYP2B1, CYP1A1, and CYP2D5 during CNS inflammation through binding to NF-κB response elements on the promoters of these genes. In support of this finding, Ke *et al* has also demonstrated a role for this transcription factor in the regulation of CYP1A1 in an in vitro model of CNS inflammation. Furthermore, Morgan and co-workers have shown that NF-κB is responsible for the LPS-mediated downregulation in CYP2C11 (Iber et al., 2000; Morgan et al., 2002). Figure 10 outlines the role of these acute phase proteins in the regulation of hepatic CYPs following the i.c.v. administration of LPS. We also observed using rat liver nuclear fractions that the i.c.v. administration of LPS caused an upregulation in the binding to a C/EBP region on the CYP2B1 promoter (Park and Kemper, 1996). Although the importance of this C/EBP region in the regulation of CYP2B1 in the liver is not completely understood (Akiyama and Gonzalez, 2003), these results are consistent with it playing some role in the regulation of CYP2B1 during inflammatory conditions. It has been shown CYP2B1/2 isoforms can be regulated at the post-translational level (Agrawal and Shapiro, 1996), and therefore it is possible that in our model of LPS-induced CNS inflammation, the downregulation in CYP2B1/2 activity

observed at 24 hours following the induction of CNS inflammation (Renton and Nicholson, 2000) may be occurring post-translationally or through enhanced proteolytic degradation of CYP2B proteins (Han et al., 2005; Lee and Lee, 2005).

Previous studies have suggested that a signalling pathway must exist between the brain and liver to account for the loss in hepatic CYPs during conditions of LPS-induced CNS inflammation (Terrazzino et al., 1997; Shimamoto et al., 1998; Shimamoto et al., 1999; Renton and Nicholson, 2000). TNFα, IL-1β, and IL-6 have all been shown to regulate several CYP isoforms at the level of the enzyme and mRNA in both peripheral and CNS models of inflammation (Barker et al., 1992; Ke et al., 2001; Morgan, 2001; Nicholson and Renton, 2002). We observed no effects on total cytochrome P450 levels following the i.c.v. administration of a cytokine cocktail (TNFα, IL-1α, IL-1β, IFNγ, and IL-6). In addition the central and peripheral blockade of the TNFα pathway (using the TNFα soluble antibody etanercept or Enbrel^{TM}) and the IL-1 signal transduction pathway (using the IL-1 inhibitor YVAD) could not prevent the downregulation in total cytochrome P450 levels induced by the i.c.v. administration of LPS. Similar results were observed when we examined the effects of the cytokine cocktail and cytokine inhibitors on CYP1A EROD catalyzed activity (data not shown). Shimamoto *et al* demonstrated that adrenalectomy did not block the loss in hepatic CYPs in response to the i.c.v. administration of LPS (Shimamoto et al., 1998; Shimamoto et al., 1999). In support of these results, we observed that hypophesectimized rats maintained the response to the i.c.v. administration of LPS (data not shown), indicating the lack of involvement of the hypothalamic-pituitary axis in the downregulation of hepatic CYPs during CNS inflammation. All these results support the idea that hepatic CYP regulation during the i.c.v. administration of LPS is occurring

at an intrahepatic level with various acute phase proteins such as NF-κB playing a dominant role in this regulation.

The injection of LPS i.c.v. is a commonly used model to produce CNS inflammation, however it has not been determined whether endotoxin leakage into the periphery plays a role in the effects on hepatic CYPs observed by us and others (Terrazzino et al., 1997; Shimamoto et al., 1998; Shimamoto et al., 1999; Renton and Nicholson, 2000). To test whether endotoxin leakage from the CNS might account for the effects observed on hepatic CYPs in LPS treated rats, we measured endotoxin levels in serum obtained from animals administered LPS directly into the lateral cerebral ventricle. We were able to detect significant amounts of LPS in the serum of rats as early as 15 minutes and for up to 2 hours following the i.c.v. administration of LPS. An energy mediated transport mechanism or bulk re-absorption of the cerebrospinal fluid likely accounts for the extremely rapid transfer of LPS from the CNS into the periphery. When animals were treated with the same dose of LPS (25 μg) given by the i.p. route, only small amounts of LPS were detected in the serum following LPS administration. Based on these results, the bioavailability of LPS from its i.p. administration is relatively small compared to the bioavailability which occurs from i.c.v. administration. This difference in bioavailability could explain previous results by our laboratory and Shimamoto *et al* where the administration of LPS by the i.p. route did not cause the downregulation in hepatic CYPs that is observed by administering the same dose directly into the lateral cerebral ventricle (Shimamoto et al., 1998; Shimamoto et al., 1999; Renton and Nicholson, 2000). It is likely that the rapid transfer of LPS from the CNS into the periphery in significant amounts is what accounts for the observed effects of i.c.v. LPS on hepatic CYPs (as shown in Figure 10). In support of this idea, we have observed that the administration of 25 μg of LPS by the intravenous route also causes a significant

downregulation in CYP2B1/2, CYP2D1/5, and CYP1A1 expression and a significant upregulation in the expression of TNFα and IκBα at 3 – 6 hours.

In summary, we suggest a signal transduction mechanism to explain the differential regulation of cytochrome P450 intrahepatically following a well-utilized LPS-induced model of CNS inflammation and provide insight into some of the molecular mechanisms by which rapid regulation of cytochrome P450 occurs in this model. Our results indicate that CYP regulation following the i.c.v. administration of LPS occurs at an intrahepatic level, with proteins such as NF-κB and C/EBP playing a dominant role in this regulation. We also show that the regulation of hepatic CYPs during LPS-mediated CNS inflammation results by a novel mechanism through which rapid transfer of LPS from the CNS into the periphery occurs following its i.c.v. administration.

Acknowledgements:

The authors would like to thank Sandra Dibb for her technical assistance. We would also like to thank Dr. C. J. Sinal for helpful discussions and help with the electromobility shift assays.

References

Agrawal AK and Shapiro BH (1996) Phenobarbital induction of hepatic CYP2B1 and CYP2B2: pretranscriptional and post-transcriptional effects of gender, adult age, and phenobarbital dose. *Mol Pharmacol* **49:**523-531.

Akiyama TE and Gonzalez FJ (2003) Regulation of P450 genes by liver-enriched transcription factors and nuclear receptors. *Biochim Biophys Acta* **1619:**223-234.

Anzenbacher P and Anzenbacherova E (2001) Cytochromes P450 and metabolism of xenobiotics. *CMLS* **58:**737-747.

Barker CW, Fagan JB and Pasco DS (1992) Interleukin-1 beta suppresses the induction of P4501A1 and P4501A2 mRNAs in isolated hepatocytes. *J Biol Chem* **267:**8050-8055.

Barker CW, Fagan JB and Pasco DS (1994) Down-regulation of P4501A1 and P4501A2 mRNA expression in isolated hepatocytes by oxidative stress. *J Biol Chem* **269:**3985-3990.

Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT (1985) Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34:**3337-3345.

Cearley C, Churchill L and Krueger JM (2003) Time of day differences in IL1beta and TNFalpha mRNA levels in specific regions of the rat brain. *Neurosci Lett* **352:**61-63.

Chang GWM and Kam PCA (1999) The physiological and pharmacological roles of cytochrome P450 isoenzymes. *Anaesthesia* **54:**42-50.

Chow T, Imaoka S, Hiroi T and Funae Y (1999) Developmental changes in the catalytic activity and expression of CYP2D isoforms in the rat liver. *Drug Metab Dispos* **27:**188-192.

Della Fazia MA, Servillo G and Sassone-Corsi P (1997) Cyclic AMP signalling and cellular proliferation: regulation of CREB and CREM. *FEBS Lett* **410:**22-24.

Garcia Del Busto Cano E and Renton KW (2003) Modulation of hepatic cytochrome P450 during Listeria monocytogenes infection of the brain. *J Pharm Sci* **92:**1860-1868.

Gavrilyuk V, Dello Russo C, Heneka MT, Pelligrino D, Weinberg G and Feinstein DL (2002) Norepinephrine increases I kappa B alpha expression in astrocytes. *J Biol Chem* **277:**29662-29668.

Goralski KB and Renton KW (2004) Brain inflammation enhances 1-methyl-4-phenylpyridinium-evoked neurotoxicity in rats. *Toxicol Appl Pharmacol* **196:**381-389.

Gorski K, Carneiro M and Schibler U (1986) Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell* **47:**767-776.

Han X, Lee G, Hefner C, Maher JJ and Correia MA (2005) HEME-REVERSIBLE IMPAIRMENT OF CYP2B1/2 INDUCTION IN HEME DEPLETED RAT HEPATOCYTES IN PRIMARY CULTURE : TRANSLATIONAL CONTROL BY A HEPATIC eIF2{alpha} KINASE? *J Pharmacol Exp Ther*.

Iber H, Chen Q, Cheng PY and Morgan ET (2000) Suppression of CYP2C11 gene transcription by interleukin-1 mediated by NF-kappaB binding at the transcription start site. *Arch Biochem Biophys* **377:**187-194.

Ke S, Rabson AB, Germino JF, Gallo MA and Tian Y (2001) Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide. *J Biol Chem* **276:**39638-39644.

Kelicen P and Tindberg N (2004) Lipopolysaccharide induces CYP2E1 in astrocytes through MAP kinase kinase-3 and C/EBPbeta and -delta. *J Biol Chem* **279:**15734-15742.

Lee SH and Lee SM (2005) Suppression of hepatic cytochrome p450-mediated drug metabolism during the late stage of sepsis in rats. *Shock* **23:**144-149.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:**402-408.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with folin phenol reagent. *J Biol Chem* **193:**265-275.

Morgan ET (2001) Regulation of cytochrome p450 by inflammatory mediators: why and how? *Drug Metab Dispos* **29:**207-212.

Morgan ET, Li-Masters T and Cheng P-Y (2002) Mechanisms of cytochrome P450 regulation by inflammatory mediators. *Toxicology* **181-182:**207-210.

Nadeau S and Rivest S (2002) Endotoxemia prevents the cerebral inflammatory wave induced by intraparenchymal lipopolysaccharide injection: role of glucocorticoids and CD14. *J Immunol* **169:**3370-3381.

Nicholson TE and Renton KW (1999) Modulation of cytochrome P450 by inflammation in astrocytes. *Brain Res* **827:**12-18.

Nicholson TE and Renton KW (2001) Role of cytokines in the lipopolysaccharide-evoked depression of cytochrome P450 in the brain and liver. *Biochem Pharmacol* **62:**1709-1717.

Nicholson TE and Renton KW (2002) The role of cytokines in the depression of CYP1A activity using cultured astrocytes as an in vitro model of inflammation in the central nervous system. *Drug Metab Dispos* **30:**42-46.

Omura T and Sato R (1964) The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J Biol Chem* **239:**2370-2378.

Pan J, Xiang Q and Ball S (2000) Use of a novel real-time quantitative reverse transcription-polymerase chain reaction method to study the effects of cytokines on cytochrome P450 mRNA expression in mouse liver. *Drug Metab Dispos* **28:**709-713.

Park Y and Kemper B (1996) The CYP2B1 proximal promoter contains a functional C/EBP regulatory element. *DNA Cell Biol* **15:**693-701.

Renton KW (2001) Alteration of drug biotransformation and elimination during infection and inflammation. *Pharmacology & Therapeutics* **92:**147-163.

Renton KW and Nicholson TE (2000) Hepatic and central nervous system cytochrome P450 are down-regulated during lipopolysaccharide-evoked localized inflammation in brain. *J Pharmacol Exp Ther* **294:**524-530.

Rivest S (2003) Molecular insights on the cerebral innate immune system. *Brain, Behaviour, and Immunity* **17:**13-19.

Ruminy P, Gangneux C, Claeyssens S, Scotte M, Daveau M and Salier JP (2001) Gene transcription in hepatocytes during the acute phase of a systemic inflammation: from transcription factors to target genes. *Inflamm Res* **50:**383-390.

Shaw PM, Edigkaufer M, Doehmer J and Adesnik M (1996) Sequence of the rat PB-inducible CYP2B1 promoter. *Biochim Biophys Acta* **1305:**54-58.

Shimamoto Y, Kitamura H, Hoshi H, Kazusaka A, Funae Y, Imaoka S, Saito M and Fujita S (1998) Differential alterations in levels of hepatic microsomal cytochrome P450 isozymes following intracerebroventricular injection of bacterial lipopolysaccharide in rats. *Arch Toxicol* **72:**492-498.

Shimamoto Y, Kitamura H, Iwai M, Saito M, Kazusaka A and Fujita S (1999) Mechanism of decrease in levels of hepatic P450 isozymes induced by intracerebral endotoxin:

independence from sympathetic nervous and adrenocortical systems. *Arch Toxicol* **73:**41-49.

Terrazzino S, Perego C, De Luigi A and De Simoni MG (1997) Interleukin-6, tumor necrosis factor and corticosterone induction by central lipopolysaccharide in aged rats. *Life Sci* **61:**695-701.

Footnotes

This work was supported by a grant from the Canadian Institute for Health Research (CIHR). Dalya Abdulla was funded by an Eliza Ritchie doctoral research award. Kerry B. Goralski was funded by fellowships from CIHR, IWK Health Centre, and the Nova Scotia Health Research Foundation.

Figure Legends

Figure 1. **The upregulation of TNFα and IL-1β levels in the brain following the administration of LPS into the lateral cerebral.** Rats were injected i.c.v. with either 25 μg LPS or saline and brain homogenates were obtained 1 – 6 hours later. Levels of TNFα (A) and IL-1β (B) are shown as pg of cytokine per mg of protein present in the brain homogenates. The levels of these two cytokines in naïve rats that have not received any i.c.v. treatment are shown in each panel. Each bar represents the mean ± S.E.M. from 4 rats. * Cytokine level is higher compared to the respective saline treatment using an un-paired t-test ($p < 0.05$).

Figure 2. **The induction of an inflammatory response in the liver in response to the administration of LPS into the lateral cerebral.** Rats were injected i.c.v. with either 25 μg LPS or saline, and liver RNA was isolated at various time points following treatment. The ratio of the intensity of each band to its respective GAPDH was obtained by densitometry, and the results are plotted as % control of each respective saline group for IκBα (A) and MAPKK (B). The average absolute value for IκBα expression (with respect to GAPDH) was 0.29 for the 2 hour saline samples, 0.67 for the 4 hour saline samples, 0.64 for the 6 hour saline samples, and 0.50 for the 9 hour saline samples. The average absolute value for MAPKK expression (with respect to GAPDH) was 0.66 for the 2 hour saline samples, 0.55 for the 4 hour saline samples, 0.79 for the 6 hour saline samples, and 0.71 for the 9 hour saline samples. Each bar represents the mean ± S.E.M. mRNA expression from 4 rats. Representative blots for TNFα (C) relative to GAPDH are shown for the 2, 4, 6, and 9 hour time points following the induction of CNS inflammation. * mRNA expression is higher compared to corresponding saline treated rats using an un-paired t-test ($p < 0.05$).

Figure 3. **An increase in nitrite levels in plasma of rats occurs in response to the administration of LPS into the lateral cerebral ventricle.** Rats were injected with 25 µg LPS i.c.v. and 2 – 15 hours later rats were sacrificed and plasma was obtained. Nitrite levels in the plasma were detected using a commercially available kit that converts total nitrates into nitrites (both end products of nitric oxide). Each bar represents the mean ± S.E.M.total nitrite level from 4 rats. * Nitrite level is higher compared to corresponding saline treated animals using an un-paired t-test ($p < 0.05$).

Figure 4. **Rapid changes in the expression of CYP2D1/5, CYP2B1/2, and CYP1A1 occur following the administration of LPS into the lateral cerebral ventricle to induce CNS inflammation.** Animals were administered either 25 µg of LPS or 5 µL of saline, and liver RNA was isolated. Specific probes for CYP2D1/5 and CYP2B1/2 were utilized for northern blot analyses (A and B). Specific primers for CYP1A1 were utilized for the quantitative PCR in (C). LPS treated animals were compared to the respective group of saline treated animals at each time point, with each bar showing the mean results from 4 rats. For CYP2D1/5 (A) and CYP2B1/2 (B), the ratio of the intensity of each band to its respective GAPDH was obtained, and the results are plotted as % control of the respective saline groups. The $2^{-\Delta\Delta C}{}_{T}$ method was used to obtain the fold decrease of CYP1A1 (C) at each time point compared to its respective saline treated group. The average absolute value for CYP2D1/5 expression (with respect to GAPDH) was 1.53 for the 2 hour saline samples, 0.78 for the 4 hour saline samples, 2.23 for the 6 hour saline samples, and 1.83 for the 9 hour saline samples. The average absolute value for CYP2B1/2 expression (with respect to GAPDH) was 0.48 for the 2 hour saline samples, 0.23 for the 4 hour saline samples, 0.57 for the 6 hour saline samples, and 0.36 for the 9 hour saline samples.* CYP

expression is lower compared to respective saline treated animals using an un-paired t-test ($p < 0.05$).

Figure 5. **The binding of specific transcription factors in the liver is increased in response to the administration of LPS into the lateral cerebral ventricle.** Rats were injected with either 25 µg of LPS or 5 µl of saline i.c.v., and liver nuclear fractions were isolated 3 hours later and were used in electromobility shift assays using commercially obtained oligonucleotides (Table 1). Representative blots for NF-κB (A), CREB (B), and AP-1 (C) are shown, where the solid arrow indicates specific binding. Specific competitions (Spec comp) and non-specific competitions (Non-spec comp) indicate competitions performed with excess non-radioactive specific oligonucleotide and non-radioactive non-specific oligonucleotide, respectively. Each blot shows the results from 4 saline and 4 LPS treated rats.

Figure 6. **NF-κB plays a vital role in the regulation of CYP isoforms.** Rats were injected with either 25 µg LPS or 5 µl saline i.c.v., and liver nuclear fractions were isolated 3 hours later and were used in electromobility shift assays using commercially obtained oligonucleotides (Table 1). EMSA binding reactions were performed using single stranded oligonucleotides (Table 1) that were annealed according to a standard annealing procedure as outlined in the Methods section. An upregulation in NF-κB binding to an NF-κB responsive region on the promoter of CYP2D5 (A) and CYP2B1 (B) is shown, where the solid arrow indicates specific binding, and the dashed arrow indicates non-specific binding. Specific competitions (Spec comp) and non-specific competitions (Non-spec comp) indicate competitions performed with excess non-radioactive specific oligonucleotide and non-radioactive non-specific oligonucleotide, respectively. The binding of NF-κB was confirmed using supershift assays, where (C) and (D) show a super shift (indicated by the diamond head arrow) in binding to the CYP2D5 NF-κB

region and the CYP2B1 NF-κB region, respectively, observed following incubation with a p65 antibody. Each blot shows the results from 4 saline and 4 LPS treated rats.

Figure 7. **The roles of C/EBP and CREB in the regulation of CYP2B1 and CYP1A1.** Rats were injected with either 25 μg of LPS or 5 μl of saline i.c.v., and liver nuclear fractions were isolated 3 hours later. EMSA binding reactions were performed using single stranded oligonucleotides (Table 1) that were annealed according to a standard annealing procedure. The binding of C/EBP to a C/EBP responsive region on the promoter of CYP2B1 (A) and NF-κB to an NF-κB response element on the promoter region of CYP1A1 (B) is shown, where the solid line indicates specific binding. Specific competitions (Spec comp) and non-specific competitions (Non-spec comp) indicate competitions performed with excess non-radioactive specific oligonucleotide and non-radioactive non-specific oligonucleotide, respectively, as described in the Methods section. The binding of C/EBPα to the CYP2B1 C/EBP region was confirmed using a supershift assay as shown in (C), where the diamond head arrow indicates the shift following incubation with a C/EBPα antibody. Each blot shows the results from 4 saline and 4 LPS treated rats.

Figure 8. The non-involvement of several pathways in mediating the effects of LPS i.c.v. on hepatic CYP isoforms.

Rats were administered a cytokine cocktail consisting of TNFα, IL-1β, IL-1α, IL-6, and IFNγ, and total cytochrome P450 levels were measured 24 hours later (A). In a separate set of experiments, rats were injected with 25 μg of LPS i.c.v. in addition to etanercept (Enbrel^{TM}) given either as a dose of 40 μg i.c.v. (B) or 2.5 mg/kg i.p. (C) or the IL-1 inhibitor YVAD administered as a dose of 0.63 μg i.c.v. (D), and total P450 levels were measured 24 hours later as described in the Methods section. "*" is statistically different compared to respective saline

treated animals ($p < 0.05$, 2-WAY ANOVA performed for panels B, C, and D, t-test performed for panel A).

Figure 9. The levels of endotoxin detected in the serum of animals injected with 25 μg LPS i.p. or i.c.v. Animals were injected with 25 μg of LPS either i.p. or i.c.v. and serum was obtained at 15 minutes (15m), 30 minutes (30m), 2 hours (2h), 4 hours (4h), 6 hours (6h), 15 hours (15h), and 24 hours (24h) following injection. Endotoxin levels were measured according to a commercially available kinetic assay kit. The graph shown represents the results from 3 animals. In the 30 minute i.p. and the 2 hour i.p. groups, the presence of endotoxin was detected in only 2 out of the three animals. Non-injected rats served as control (ctrl) animals for the experiment.

Figure 10. A proposed mechanism by which LPS regulates hepatic cytochrome P450 following its administration into the lateral cerebral ventricle. (1) The i.c.v. injection of LPS into the lateral cerebral ventricles of rats is thought to activate Toll-like receptor 4 (TLR4). (2) Activation of TLR4, normally present on microglia and astrocytes in the brain, would lead to the upregulation of cytokine and transcription factors in the brain. (3) The rapid "leakage" of LPS from the CNS into the periphery plays a role in downregulation of hepatic CYPs. Although cytokines are elevated in the CNS during this model of LPS-induced CNS inflammation, it appears from our results that centrally produced cytokines are not important for the regulation of hepatic CYPs. (4) The presence of LPS in the periphery induces a peripheral inflammatory response characterized by the production of cytokines (from immune cells such as macrophages) and the induction of an acute-phase response in the liver. (5) Cytokines from peripheral sources, in addition to LPS present in the serum, act on the hepatocyte to differentially regulate CYPs. (6) Some of the intra-hepatic mechanisms by which CYP regulation occurs involve upregulation of various transcription factors such as NF-κB, AP-1, and C/EBP. These transcription factors can

then bind to the promoter regions on specific CYP isoforms, leading to changes in the transcription of these CYPs.

DMD Fast Forward. Published on July 8, 2005 as DOI: 10.1124/dmd.105.004564
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1 – List of the EMSA oligonucleotides utilised in this study

The underlined portion of each oligonucleotide in the last four rows refers to the response element for the transcription factor being observed.

Description	Oligonucleotide	Source
NF-κB	5′ AGT TGA GGG GAC TTT CCC AGG 3′	Santa Cruz
AP-1	5′ CGC TTG ATG ACT CAG CCG GAA 3′	Santa Cruz
CREB	5′ AGA GAT TGC CTG ACG TCA GAG AGC TAG 3′	Santa Cruz
CYP2D5 NF-κB	–654 5′ CCA ACG TAG GGA CTT CCC AAG ATC CT 3′ –680	Single Stranded oligonucleotides annealed according to standard procedure
CYP1A1 NF-κB	–746 5′GCG AGA GGA ATC TCC CAG GC 3′ –726	Single Stranded oligonucleotides annealed according to standard procedure
CYP2B1 NF-κB	–888 5′CCA GGG GTG GAA TTT CCC ACA GT 3′ –879	Single Stranded oligonucleotides annealed according to standard procedure
CYP2B1 C/EBP	–67 5′ ACA TGT GAA GTT GCA TAA CTG AGT 3′ –45	Single Stranded oligonucleotides annealed according to standard procedure

Table 2 – The effects of 25 μg LPS administered i.v. on the activity and expression of hepatic P450 isoforms and inflammatory mediators

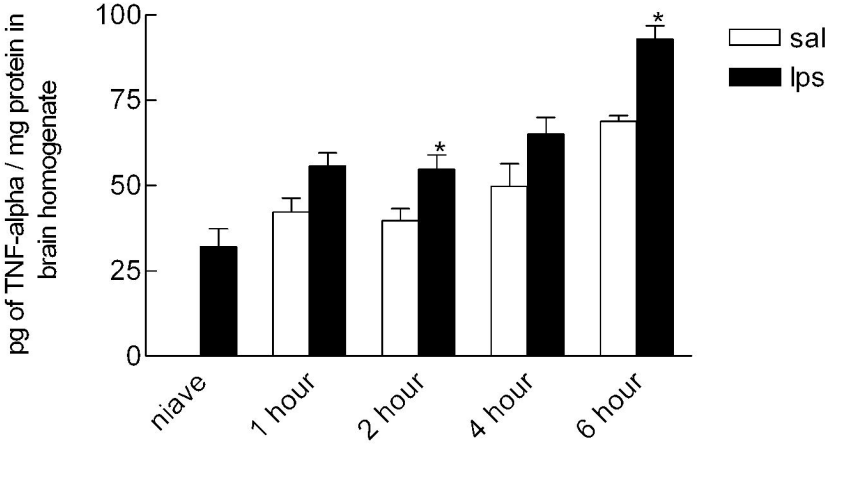
Assays were performed either 3, 6, or 24 hours following the i.v. injection of LPS. The values are presented as the pooled results from 4 different animals, and * is statistically different compared to corresponding saline treated animals ($p < 0.05$).

Assay description	Saline	25 μg LPS i.v.
CYP1A1 expression in liver relative to GAPDH (6 hour time point)	1.00 ± 0.39	0.069 ± 0.03*
CYP2B1/2 expression in liver relative to GAPDH (6 hour time point)	2.54 ± 0.63	1.18 ± 0.17*
TNFα expression in liver relative to GAPDH (3 hour time point)	0.38 ± 0.05	1.25 ± 0.37*
CYP2D1/5 expression in liver relative to GAPDH (6 hour time point)	87.80 ± 8.58	68.67 ± 2.07*
IκBα expression in liver relative to GAPDH (3 hour time point)	0.35 ± 0.13	3.50 ± 0.88*
IκBα expression in liver relative to GAPDH (6 hour time point)	0.72 ± 0.28	2.36 ± 0.26*
Total cytochrome P450 in nmol/mg protein (24 hour time point)	0.578 ± 0.043	0.400 ± 0.083*
CYP1A1/2 activity in pmol/mg protein/minute as measured by EROD assay (24 hour time point)	123.84 ± 40.22	71.58 ± 12.52*

CYP2B1/2 activity in pmol/mg protein/minute as measured by PROD assay (24 hour time point)	8.62 ± 3.61	4.51 ± 0.50*

Figure 1

(A)


pg of TNF-alpha / mg protein in brain homogenate
100
75
50
25
0
sal
lps
*
niave
1 hour
2 hour
4 hour
6 hour

(B)

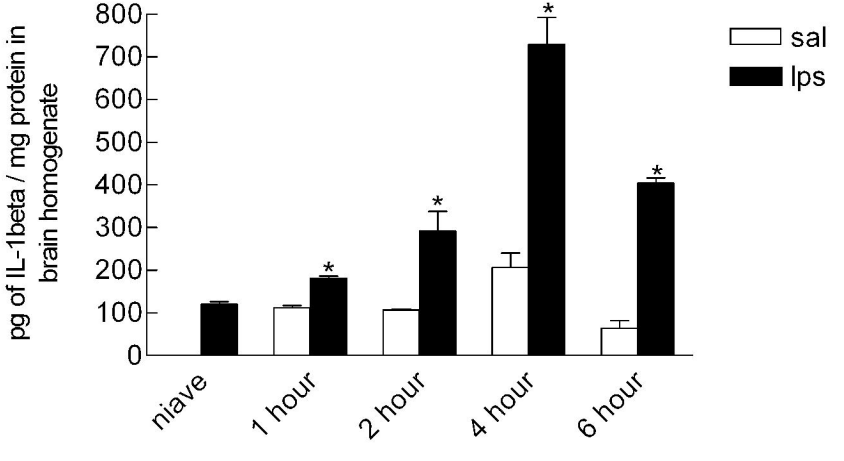
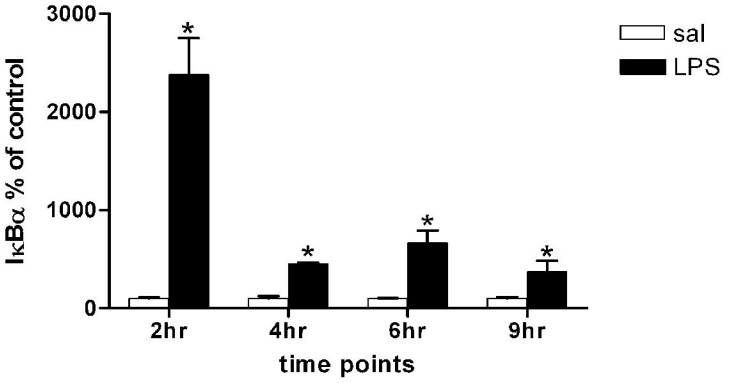
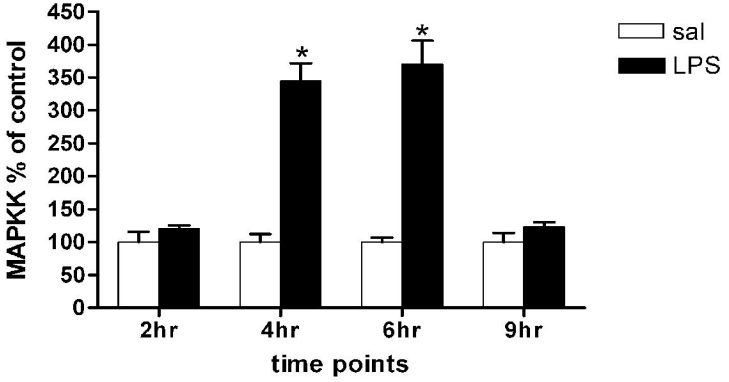

pg of IL-1beta / mg protein in brain homogenate
800
700
600
500
400
300
200
100
0
sal
lps
*
niave
1 hour
2 hour
4 hour
6 hour

Figure 2

(A)


3000
2000
1000
0
IκBα % of control
*
*
*
*
2hr
4hr
6hr
9hr
time points
sal
LPS

(B)


450
400
350
300
250
200
150
100
50
0
MAPKK % of control
*
*
2hr
4hr
6hr
9hr
time points
sal
LPS

(C)

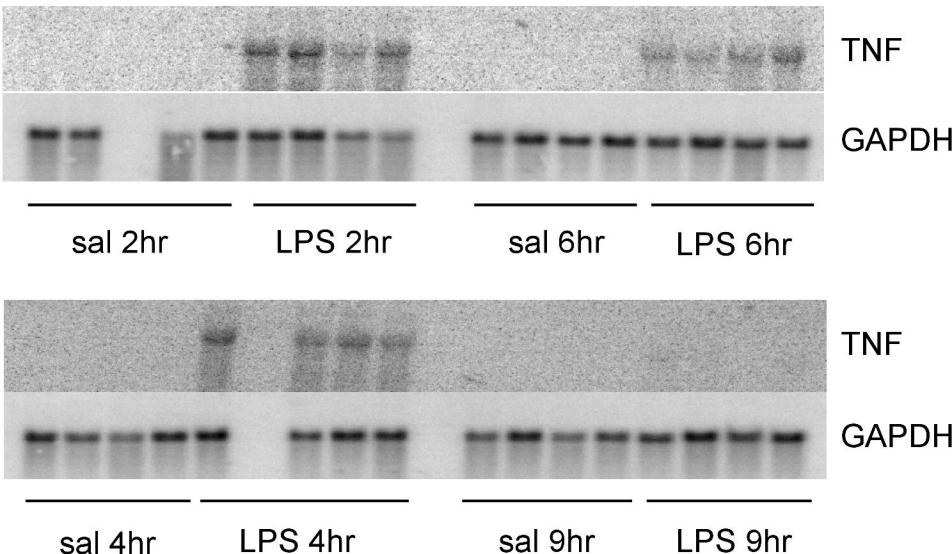
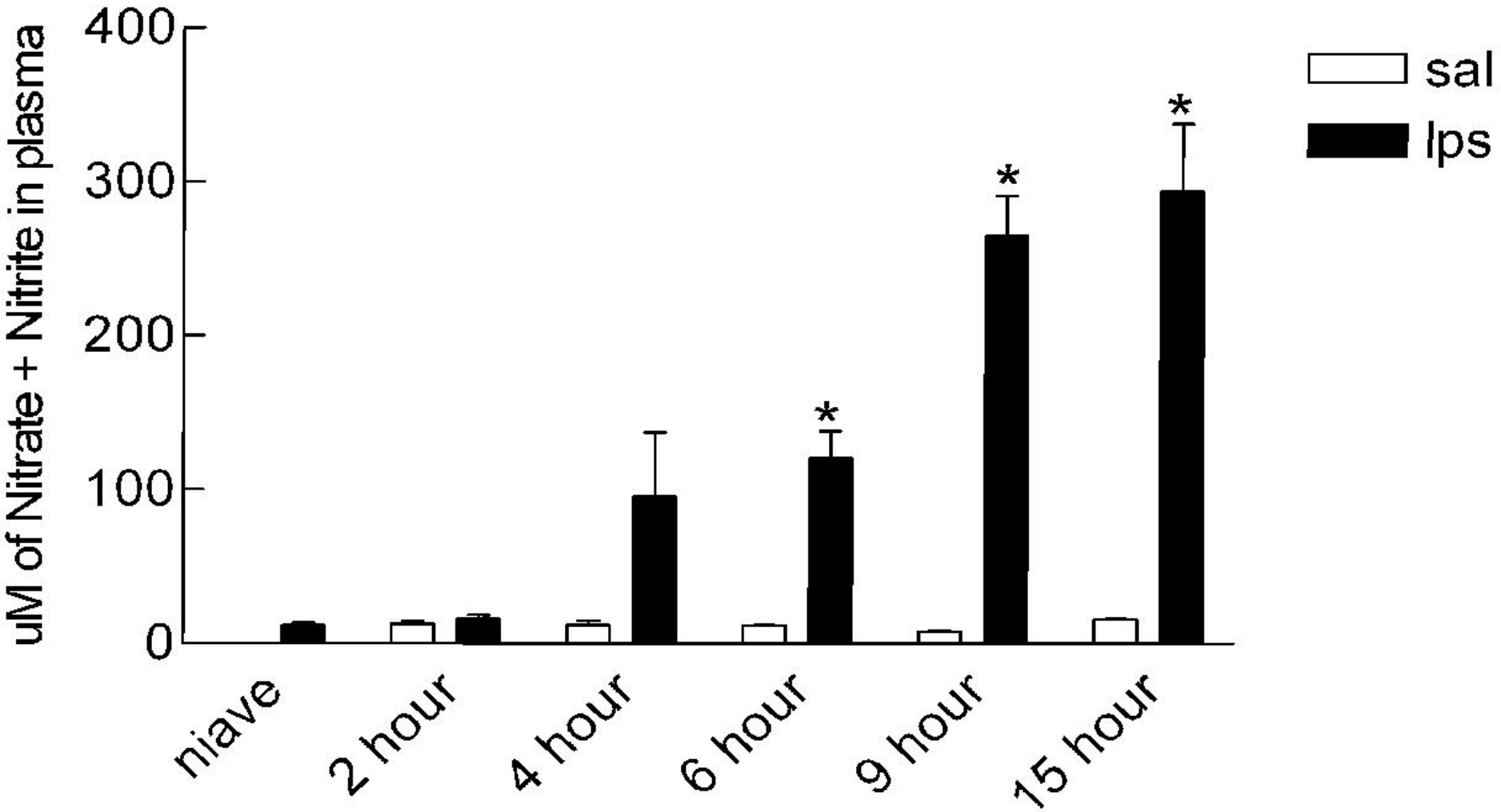
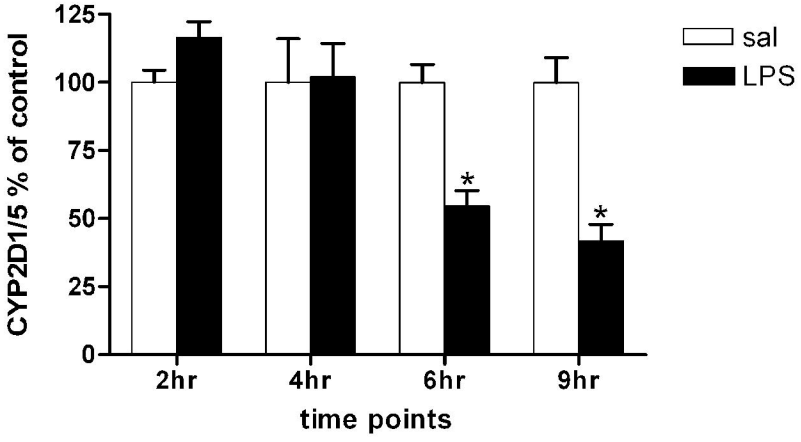

TNF
GAPDH
sal 2hr
LPS 2hr
sal 6hr
LPS 6hr
TNF
GAPDH
sal 4hr
LPS 4hr
sal 9hr
LPS 9hr

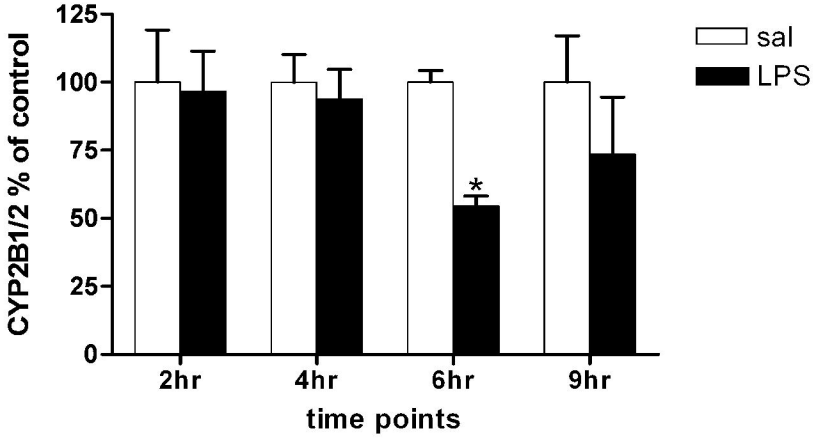
Figure 3


uM of Nitrate + Nitrite in plasma
400
300
200
100
0
sal
lps
*
*
*
niave
2 hour
4 hour
6 hour
9 hour
15 hour

(A)


CYP2D1/5 % of control
125
100
75
50
25
0
2hr
4hr
6hr
9hr
time points
sal
LPS
*
*

(B)


CYP2B1/2 % of control
125
100
75
50
25
0
2hr
4hr
6hr
9hr
time points
sal
LPS
*

(C)

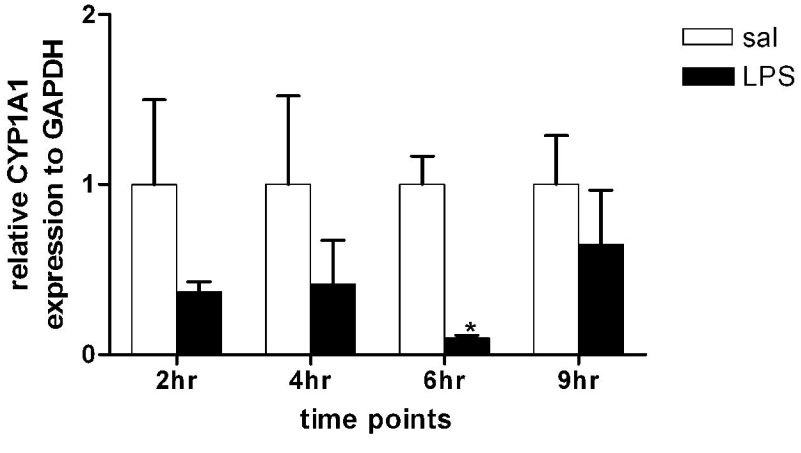
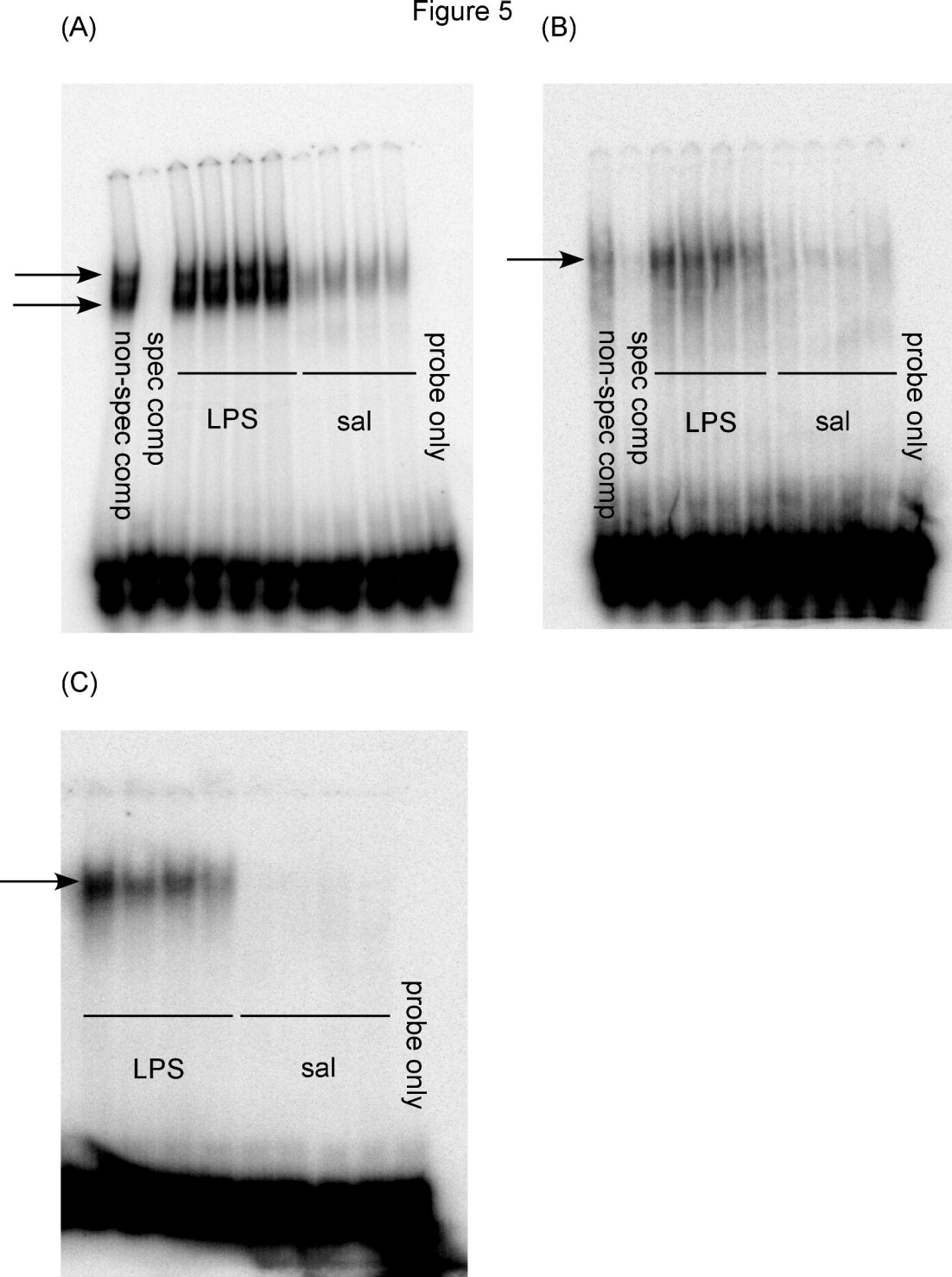

relative CYP1A1 expression to GAPDH
2
1
0
2hr
4hr
6hr
9hr
time points
sal
LPS
*

Figure 5


(A)
non-spec comp
spec comp
LPS
sal
probe only
(B)
non-spec comp
spec comp
LPS
sal
probe only
(C)
LPS
sal
probe only

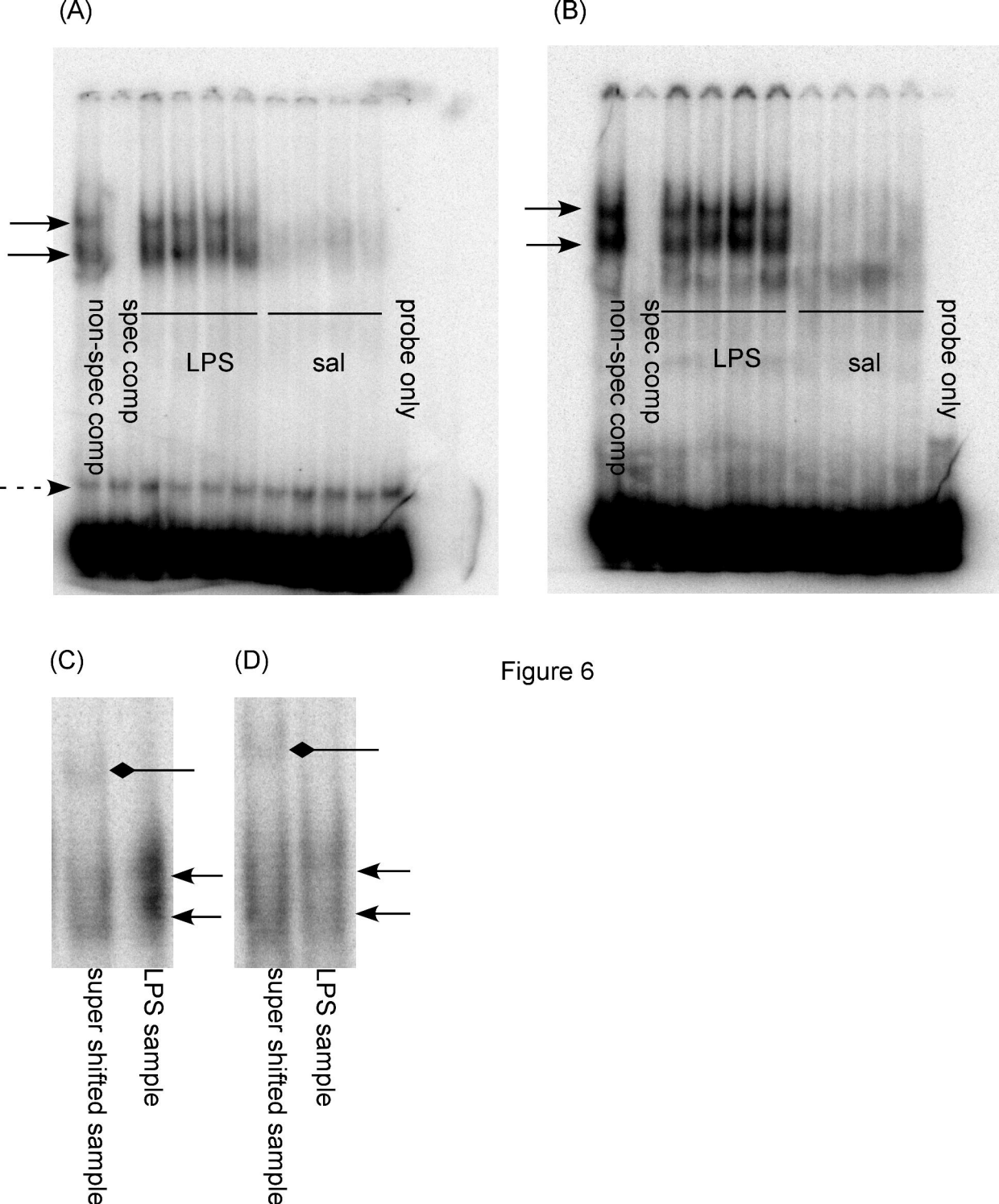
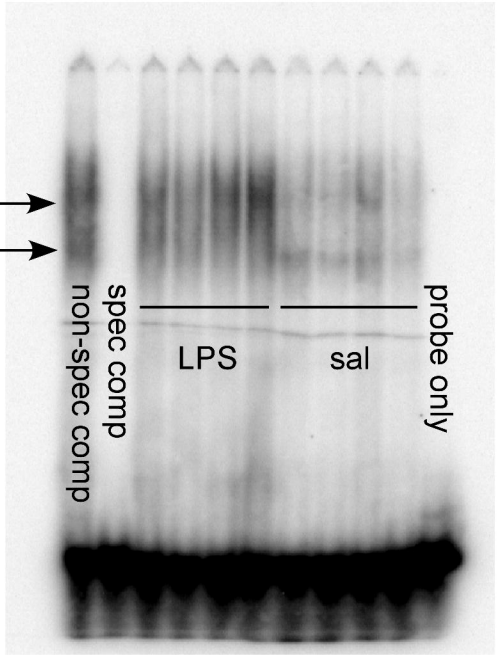
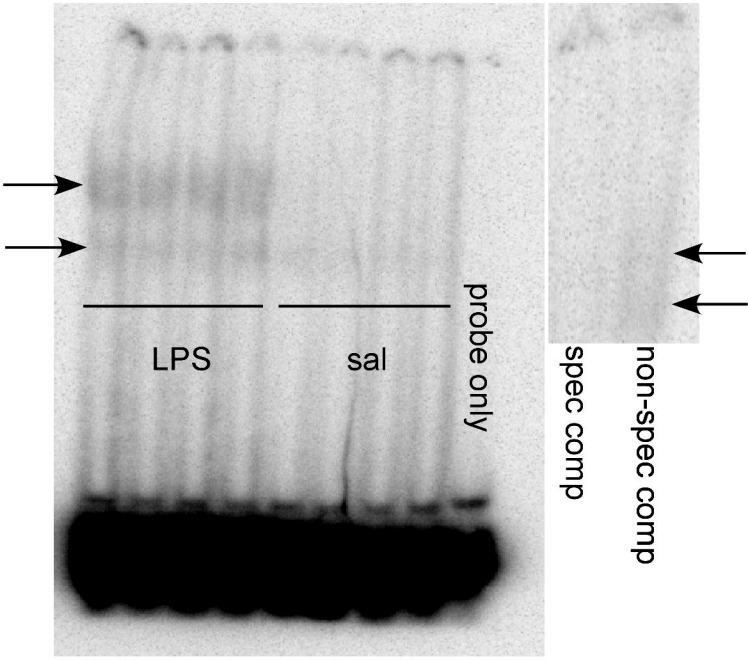

(A)
(B)
non-spec comp
spec comp
LPS
sal
probe only
(C)
(D)
super shifted sample
LPS sample

Figure 6

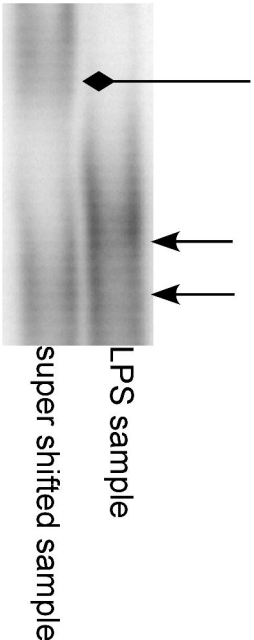
(A)


non-spec comp
spec comp
LPS
sal
probe only

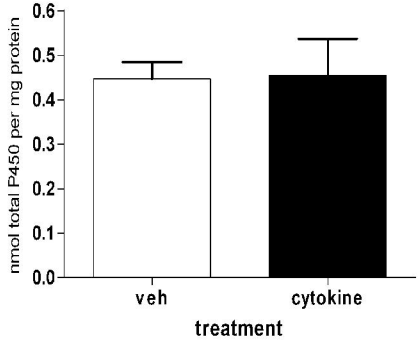
(B)


LPS
sal
probe only
spec comp
non-spec comp

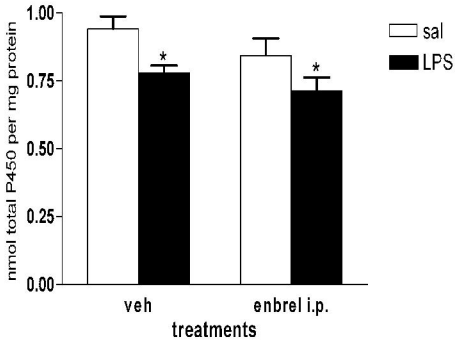
(C)


super shifted sample
LPS sample

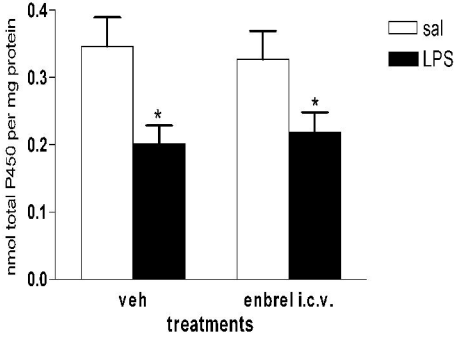
(A)


nmol total P450 per mg protein
0.6
0.5
0.4
0.3
0.2
0.1
0.0
veh
cytokine
treatment

(C)


nmol total P450 per mg protein
1.00
0.75
0.50
0.25
0.00
*
*
sal
LPS
veh
enbrel i.p.
treatments

(B)


nmol total P450 per mg protein
0.4
0.3
0.2
0.1
0.0
*
*
sal
LPS
veh
enbrel i.c.v.
treatments

(D)

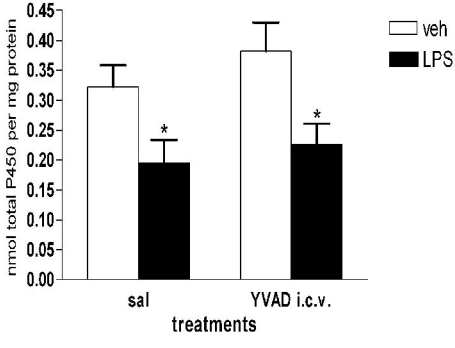

nmol total P450 per mg protein
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
veh
LPS
sal
YVAD i.c.v.
treatments

Figure 9

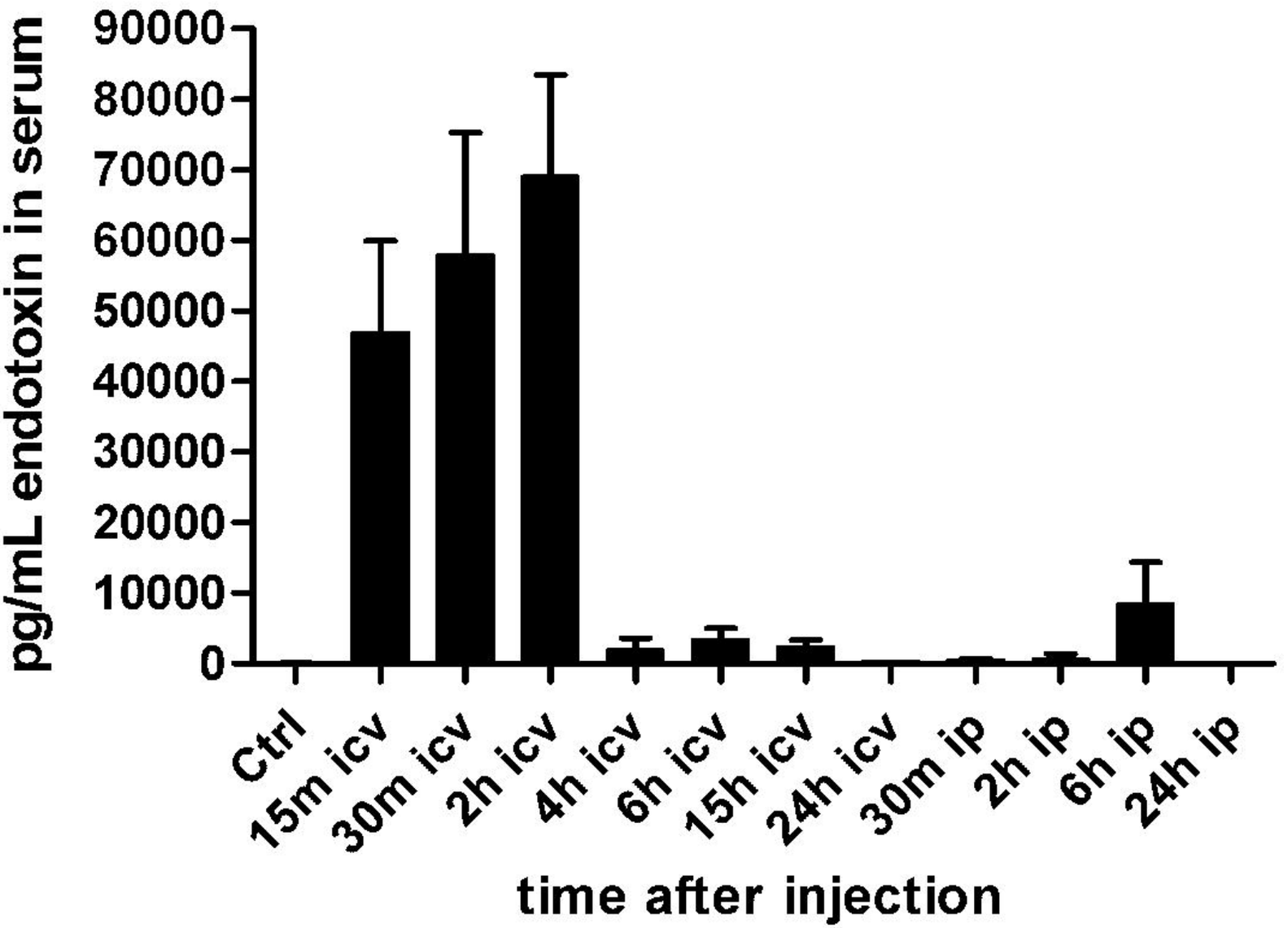
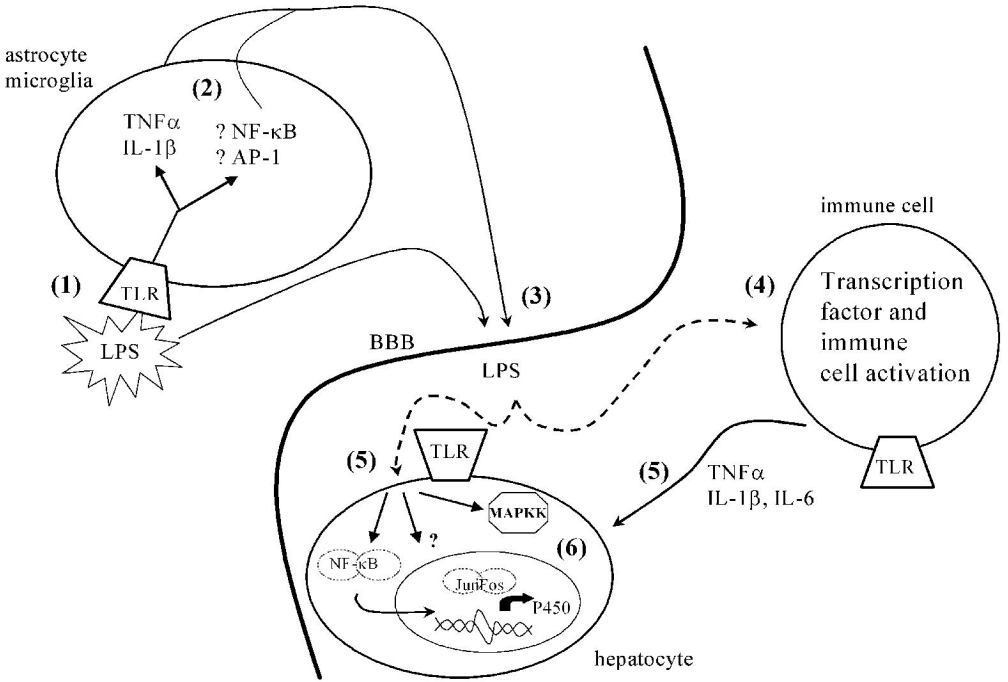

pg/mL endotoxin in serum
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Ctrl
15m icv
30m icv
2h icv
4h icv
6h icv
15h icv
24h icv
30m ip
2h ip
6h ip
24h ip
time after injection

Figure 10


astrocyte
microglia
(2)
TNFα
IL-1β
? NF-κB
? AP-1
(1)
TLR
LPS
(3)
BBB
LPS
immune cell
(4)
Transcription
factor and
immune
cell activation
(5)
TLR
(5)
TNFα
IL-1β, IL-6
TLR
MAPKK
?
(6)
NF-κB
Jun
Fos
P450
hepatocyte