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The effect of endotoxin on hepatocyte nuclear factor 1 nuclear protein binding: potential implications on CYP2E1 expression in the rat

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Abstract

The purpose of this study was to determine if changes in nuclear protein binding of hepatocyte nuclear factor 1 (HNF-1) occur after lipopolysaccharide (LPS) administration. In addition, the time-course of alterations in CYP2E1 regulation were evaluated. Rats were injected with 2.0 mg LPS and euthanized over a 72-h period. Nuclear protein binding to a consensus HNF-1 oligonucleotide was assessed by the electrophoretic mobility shift assay. CYP2E1 activity was analysed using chlorzoxazone as a substrate (6OH-CLZ), and CYP2E1 protein concentration was determined by enzyme-linked immunosorbent assay. Endotoxin treatment resulted in decreased nuclear protein binding to an HNF-1 element as early as 1 h after treatment and returned to control levels by 72 h. This reduced binding persisted for 24 h and returned to control values 48 h after LPS administration. In addition, the reduction in binding was primarily attributable to a HNF-1α immunoreactive protein. The observed reduction in HNF-1 binding was followed in the time-course by decreases in CYP2E1 activity and protein content with maximal decreases to 50 and 67% of control, respectively, at 48 h after LPS administration. Endotoxin is a potent inducer of the acute phase response (APR). The APR stimulation by endotoxin administration reduced HNF-1α binding and decreased the expression of CYP2E1 in the rat liver. The time-course of alterations in HNF-1 and CYP2E1 lend support to the possibility that HNF-1α may play a role in the down-regulation of genes that require HNF-1α for their constitutive expression. These data serve as an important precedent for future studies evaluating the direct association of decreased HNF-1α binding and reduced gene expression after LPS administration.

Introduction

Endotoxins (i.e. lipopolysaccharide; LPS) from Gram-negative bacteria are potent inducers of the acute phase response (APR) (Ramadori & Christ 1999). The APR is characterized by both an increase and decrease in the synthesis of a number of hepatic proteins known as positive and negative acute phase reactants. The primary role of the APR is to maintain homeostasis during inflammation and infection. Homeostatic mediators of the APR are cytokines such as tumour necrosis factor α (TNFα) and interleukins 1 and 6 (IL-1 and IL-6). Of these cytokines, IL-6 is thought to be the most important mediator of the hepatic APR because it alters the expression of multiple hepatic acute phase reactants (Ramadori & Christ 1999).

In addition to acute phase reactants, other important hepatic proteins are altered during inflammation, including the P450 enzymes (Shedlofsky et al 1994, 2000;

Morgan 1997, 2001; Roe et al 1998). The P450 family of enzymes is involved in the metabolism of both endobiotics (lipids, steroids and vitamins) and xenobiotics (drugs and environmental contaminants). Alterations in hepatic P450 enzymes by LPS as well as the cytokines TNFα, IL-1 and IL-6 are well documented (for a review see Morgan 2001). We have previously shown alterations in P450 form-specific activities, protein, mRNA levels and nuclear protein binding in rats (Roe et al 1998), and changes in probe drug clearance in humans (Shedlofsky et al 1994) after LPS treatment.

CYP2E1 is constitutively expressed in the liver, kidney, brain and other tissues, and is known to catalyse the oxidation of a variety of substrates including drugs, environmental contaminants and eicosanoids (Tanaka et al 2000). CYP2E1 is also important because of its high inducibility by ethanol and subsequent free radical production (Clot et al 1996; Fang et al 1998). CYP2E1 expression demonstrates multiple levels of transcriptional control. Both inhibition and induction of CYP2E1 occurs via various mechanisms at the gene, mRNA and protein levels. Chemical and disease states, including liver transplantation (Tirumalai et al 1998), traumatic injury (Tindberg et al 1996; Poloyac et al 2001) and obesity (Lucas et al 1998; Roe et al 1999), have been shown to affect the expression of this isoform at various regulation levels.

Although multiple disease states alter the transcription of the CYP2E1 gene, the critical transcription factors involved in these changes are largely unknown. Several transcription factor-binding sites have been identified in the regulatory region of the CYP2E1 gene, including binding sites for activator protein 1 (AP-1), NF-IL6, and hepatocyte nuclear factor 1α (HNF-1α) (Umeno et al 1988; Ueno & Gonzalez 1990; Liu & Gonzalez 1995; McGehee et al 1997). Of these known binding sites, Ueno & Gonzalez (1990) have demonstrated that HNF-1α is responsible for up to 90% of the constitutive expression of the CYP2E1 gene. Based on this background, the objectives of the present study were to evaluate changes in nuclear protein binding to an HNF-1 consensus sequence, immuno-identify the binding protein and assess alteration in CYP2E1 expression in the liver as a function of time after in-vivo LPS administration.

Materials and Methods

Animals

Male Sprague-Dawley rats (250–300 g) were fasted overnight and injected intraperitoneally with 2.0 mg LPS derived from *Escherichia coli* (serotype 0111:B4; DIFCO, Detroit, MI). Control animals received equivalent volumes of normal saline. Animals were euthanized after anaesthesia with 87 mg kg^{-1} ketamine (Ketaset, Aveco Co, Inc., Fort Dodge, IA) and 13 mg kg^{-1} xylazine (Rompun, Moboy Corp., Shawnee, KS), from 1 to 72 h after injection. Liver tissue was removed and immediately frozen in liquid nitrogen. All animal procedures were approved by the University of Kentucky Animal Care and Use Committee.

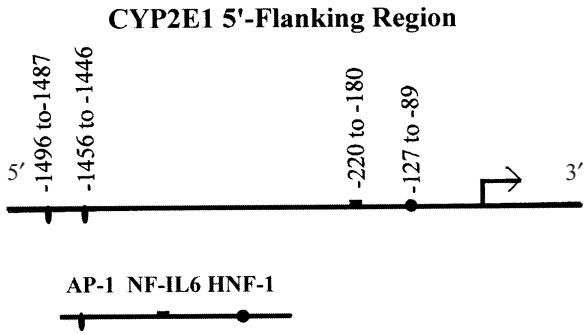

Figure 1 Schematic representation of the 5′-flanking region of the CYP2E1 gene. Computer analysis of the CYP2E1 5′-flanking region identified two putative AP-1 sites. Also included is a putative NF-IL6 binding site, and the previously identified HNF-1α regulatory site (Ueno & Gonzalez 1990; Liu & Gonzalez 1995).

Computer analysis of the CYP2E1 5′-flanking region

Computer analysis of approximately 1.5 kb of CYP2E1 5′-flanking region identified two putative AP-1 binding sites (-1456 to -1446 and -1496 to -1487), as well as the putative NF-IL6 site identified by McGehee et al (1997) and the HNF-1 site functionally identified by Ueno & Gonzalez (1990). These regulatory sites are represented in Figure 1.

Nuclear extract preparation

Nuclear protein was isolated according to the method of Dignam et al (1983) as modified by Roe et al (1996). Protein was quantitated by the Pierce Coomassie Plus Protein Assay (Pierce, Rockford, IL).

Electrophoretic mobility shift assay (EMSA)

The consensus HNF-1 oligonucleotide ($^{-62}$ GTG GTT AAT GAT CTA CAG TTA $^{-42}$), identified from the rat albumin promoter (Cereghini et al 1988; Raymondjean et al 1988) was synthesized by the University of

Kentucky Macromolecular Structural Analysis Facility (Lexington, KY). The consensus HNF-1 oligonucleotide was annealed to form a 21-bp double-stranded probe.

The NFκB and SP1 oligonucleotides were purchased from Promega Corporation (Madison, WI). The oligonucleotides used in the EMSA were end-labelled with γ-^{32}P-ATP (Amersham Corporation, Arlington Heights, IL) using T4 polynucleotide kinase (GIBCO/BRL) at 22°C for 20 min. The EMSA was carried out as described by Roe et al (1996). Sequence specificity of nuclear protein binding to the HNF-1 oligonucleotide was confirmed by competition studies in which nuclear extracts were incubated for 5 min at 22°C with 10-, 50- and 100-fold molar excess of unlabelled competitor oligonucleotide before the addition of radiolabelled oligonucleotide. Identification of proteins involved in the DNA-binding complex were determined by "supershift" EMSA in which an antibody specific for HNF-1α was added to the nuclear protein mix and incubated on ice for 20 min before the addition of radiolabelled oligonucleotide. Anti-HNF-1α was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

The EMSA was conducted using nuclear extracts from at least three separate animal experiments. Each liver nuclear extract preparation was from a single animal rather than using pooled samples from similar treatments. Therefore, intra-individual animal differences in nuclear protein binding may be evident. All EMSAs shown, although all from the same animal experiment, are representative of the findings in the three separate experiments.

Microsomal preparation, enzyme-linked immunosorbent assay (ELISA) and Western blotting

Hepatic microsomes were prepared as previously described (Blouin et al 1993). Microsomal samples (0.25 μg protein/well) were loaded onto 96-well microtiter plates. A standard curve of rat microsomes (Gentest Corporation, Woburn, MA) was also plated (1000–1 fmol P-450/microwell). Carbonate–bicarbonate buffer (10 μL; Sigma Chemical Co., St Louis, MO) was added to each well and stored overnight at 4°C. To determine CYP2E1 content, the buffer was removed and wells were blocked with 50% fetal bovine serum in phosphate-buffered saline, incubated with primary antibody (anti-rat CYP2E1 diluted 1:1000; Gentest), and subsequently incubated with anti-goat–alkaline phosphatase conjugate (1:50 000; Sigma). All incubations were for 1 h at 37°C. Plates were washed three times between each incubation with 0.05 M Tris-buffered saline with 0.1% Tween 20. K-Gold pre-mixed ELISA substrate (Neogen, Lexington, KY) was added and incubated for 30 min at 37°C. Absorbance at 405 nm was determined for samples and standards. ELISA assay linearity was demonstrated over the range 0.02–1.0 μg total microsomal protein/microwell (equiv. 7.8–500 fmol CYP2E1 protein/well).

Western blot analysis of microsomal protein was completed via a denaturing 10% polyacrylamide minigel. A total of 10 μg each of microsomal protein, molecular weight markers (Sigma) and purified CYP2E1 (Pan Vera, Madison, WI) were separated via electrophoresis using a mini Protean II apparatus (BioRad, Hercules, CA) and transferred onto nitrocellulose membranes (BioRad). Primary goat anti-rat CYP2E1 polyclonal antibody (dilution 1:1000; Gentest) was used to identify isoform protein migration. Alkaline phosphatase conjugated monoclonal rabbit anti-goat IgG antibody (dilution 1:50000; Sigma) was added for subsequent colorimetric analysis (BCIP-NBT; Boehringer-Mannheim, Indianapolis, IN).

CYP2E1-dependent activity

Detection of CYP2E1-dependent, 6OH-CLZ activity was determined by previously described methods (Peter et al 1990; Jayyosi et al 1995). Total microsomal protein concentration was determined by the method of Lowry et al (1951). Incubations were carried out at 37°C for 20 min in a shaking waterbath and included 400 μg total protein, 400 μM chlorzoxazone, NADPH regenerating system (containing 1 mM NADP, 10 mM glucose 6-phosphate, and 2 IU of glucose-6-phosphate dehydrogenase) in a final volume of 1 mL. The reaction was stopped with 50 μL 42.5% *o*-phosphoric acid followed by the addition of the internal standard, umbelliferone. Samples and standards were then extracted in 5 mL ethyl acetate, dried under nitrogen gas, and reconstituted in 200 μL of mobile phase. A total of 20 μL of each sample or standard was injected onto the HPLC system via a Shimadzu SIL-6B autoinjector. 6OH-CLZ and umbelliferone were seperated using a C-18 reverse-phase (5 μm, 150 mm × 4.6 mm) column (Supelco, Bellefont, PA) with a Shimadzu LC binary gradient HPLC system. The column was eluted with a mixture of 78% acetic acid (0.25%) and 22% acetonitrile at a constant flow-rate of 1 mL min^{-1} to yield retention times for umbelliferone, 6OH-CLZ and chlorzoxazone of 4.6, 6.0, and 18.0 min, respectively. Samples were monitored with UV detection at 287 nm

using a Shimadzu SPD-6AV spectrophotometric detector. The 6-OH-CLZ concentration was determined from the peak height ratios quantified using the Shimadzu CR501 integrator and 6-OH formation rates were determined.

Statistical analysis

Treatment and time factors for CYP2E1 activity and protein were statistically evaluated via a two-way analysis of variance. Fisher's LSD post-hoc test was used to assess statistical differences. Statistical comparisons were made using the SAS computer program (SAS Institute Inc., Cary, NC).

Results

EMSA analysis of nuclear proteins

Rat hepatic nuclear protein binding to a consensus HNF-1 oligonucleotide was altered after LPS treatment (Figure 2A). HNF-1 binding activity was constitutively present in nuclear extracts from control animals; however, a loss of HNF-1 binding activity occurred as early as 1 h after injection in nuclear extracts from LPS-treated animals. Nuclear protein binding to the consensus HNF-1 oligonucleotide sequence remained diminished up to 24 h after LPS treatment, but returned to control levels by 48 h after LPS treatment (Figure 2A).

The specificity of proteins binding to the consensus HNF-1 oligonucleotide was determined by a competition study using a molar excess of the unlabelled oligonucleotide. Nuclear protein binding to the consensus HNF-1 oligonucleotide was successfully competed with by as low as 10-fold molar excess of competitor oligonucleotide, but not by an unrelated SP-1 oligonucleotide (data not shown).

To further characterize the composition of the nuclear protein binding to the consensus HNF-1 oligonucleotide, an antibody specific for HNF-1α was added to rat hepatic nuclear protein before addition of radiolabelled oligonucleotide (Figure 2B). As indicated by the shifted band shown in Figure 2B, the majority of the binding complex consisted of the HNF-1α protein.

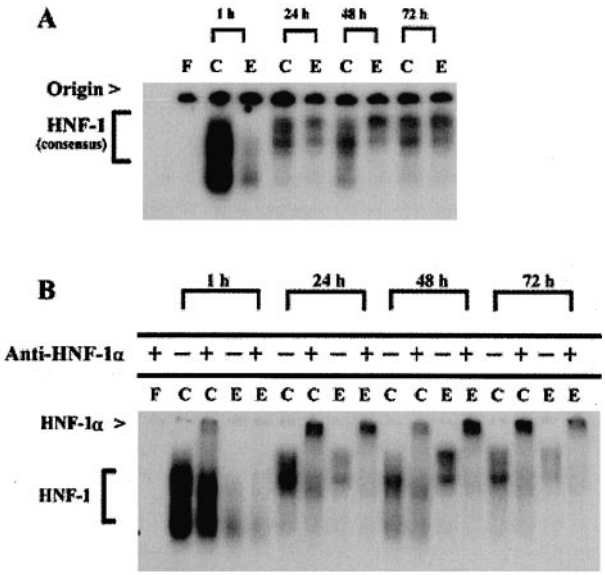

Figure 2 A. Nuclear protein binding to a consensus HNF-1 oligonucleotide in liver nuclear extracts from lipopolysaccharide-treated animals. Nuclear protein (2.5 μg) was incubated with the respective HNF-1 oligonucleotide. B. To identify the presence of HNF-1α in the DNA-binding complex, an antibody specific for HNF-1α was added to rat hepatic nuclear protein before the addition of radiolabelled oligonucleotide. Lanes: " + " indicates the addition of anti-HNF-1α; " − " indicates no addition of anti-HNF-1α. Sample characteristics: F, contains no nuclear protein; C, control; E, endotoxin and time points (1, 24, 48, and 72 h) are indicated. All EMSAs were carried out in probe excess.

Microsomal activity of CYP2E1

CYP2E1-dependent 6OH-CLZ activity decreased in endotoxin-treated animals as early as 4 h after endotoxin administration (Figure 3). Activity was maximally decreased to on average 50% of control levels for

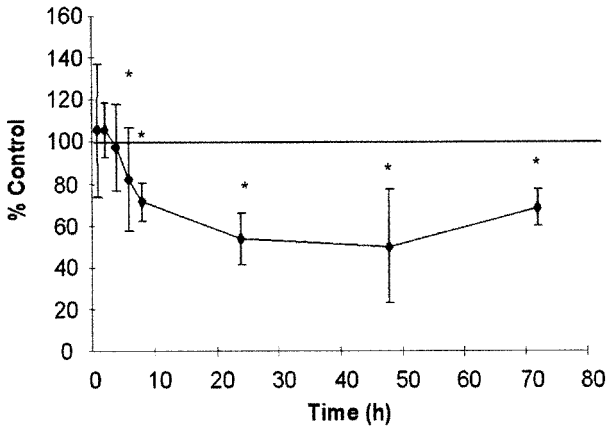

Figure 3 Total CYP2E1 activity, as measured by the 6OH-CLZ assay, decreased in endotoxin-treated animals as early as 4 h after endotoxin administration. Activity was maximally decreased to 50% of control levels for endotoxin-treated animals at 48 h after treatment, and remained significantly depressed at 72 h. Values are presented as mean ± s.d., n = 5. *$P < 0.05$, significant reduction compared with control.

endotoxin-treated animals at 48 h after endotoxin administration (control 1.16 ± 0.50 vs endotoxin 0.48 ± 0.16 nmol mg^{-1} min^{-1}) and remained significantly depressed up to 72 h.

ELISA and Western blot analysis of CYP2E1 protein

Total CYP2E1 protein concentrations decreased in endotoxin-treated animals at 24, 48 and 72 h after endotoxin administration (Figure 4A). The maximal decrease in CYP2E1 protein concentrations (on average 67% of control; $P < 0.01$) was observed 48 h after endotoxin administration (control 751.3 ± 69.3 vs endotoxin 476.0 ± 201.7 pmol mg^{-1}). In addition, Western blot analysis with the same primary and secondary antibody concentrations and incubation times demonstrated a single major band with identical migration as purified CYP2E1 (Figure 4B). The depicted Western blot from a single microsomal set demonstrates a clear reduction in CYP2E1 immunoreactivity at 24 h after endotoxin administration. This result was similar to that observed via ELISA analysis.

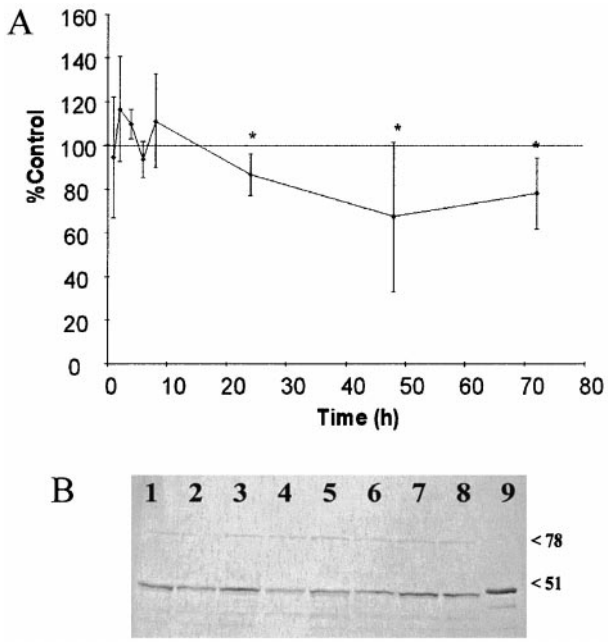

Figure 4 A. Analysis of hepatic CYP2E1 protein after lipopolysaccharide treatment. Microsomal protein (0.25 μg/well) was loaded onto a 96-well microtiter plate and CYP2E1 protein quantitated by ELISA. Values are presented as mean $\pm$ s.d., n = 5. $^*P < 0.05$, significant reduction compared with control. B. Representative Western blot of rat liver microsomes. Lanes 1, 3, 5 and 7 are control animals; lanes 2, 4, 6 and 8 are endotoxin-treated animals; and lane 9 is 0.5 μg purified CYP2E1. Lanes 1 and 2 are at 8 h after endotoxin administration; lanes 3 and 4 are at 24 h; lanes 5 and 6 are at 48 h; and lanes 7 and 8 are at 72 h. The Western blot presented (n = 1) demonstrated a single band and verifies the use of the antibody for ELISA quantitative analysis.

Discussion

Identification of the important transcription factors in the 5′-flanking regions of P450 genes is the first step in defining the molecular mechanisms involved in the down-regulation of these genes during an inflammatory response. In this study, we describe the effect of a single high dose of LPS (2.0 mg) in rats on the alteration of hepatic nuclear proteins potentially important in the regulation of the CYP2E1 gene.

The transcriptional control of CYP2E1 during mammalian development has been well documented (the CYP2E1 gene is activated within hours post partum). Ueno & Gonzalez (1990) have identified a positive element located between -127 and -89 in the 5′-flanking region of CYP2E1, which is responsible for 90% of in-vitro transcription in adult rat liver extracts. Later work in the same laboratory identified the *trans*-acting factor HNF-1α as the predominant nuclear protein involved in the activation of CYP2E1 gene expression using a CYP2E1 promoter/CAT reporter construct and an HNF-1α expression plasmid (Liu & Gonzalez 1995). McGehee et al (1997) have sequenced 1 kb of the CYP2E1 5′-flanking region and, using footprint analysis, identified 13 sequence-specific protected regions with rat liver nuclear extracts. Analysis of these protected regions for homology to consensus sequence transcription factor binding sites identified the HNF-1α site at -132 to -94, and one putative NF-IL6 site at -220 to -180.

Based on the CYP2E1 5′-flanking region analysis information from the above studies, we evaluated alterations in rat hepatic nuclear protein binding after LPS treatment, to a consensus HNF-1 binding site identified from the rat albumin promoter (Cereghini et al 1988; Raymondjean et al 1988). EMSA analysis of nuclear protein binding to an NFκB consensus element was used to demonstrate that the dose of LPS used in these studies did generate an APR (Roe et al 1998).

As shown in Figure 2A, LPS treatment of rats resulted in nearly a complete loss in nuclear protein binding to the consensus HNF-1 oligonucleotides as early as 1 h after injection, which remained diminished up to 24 h, but returned to control levels within 48 h.

The binding of hepatic nuclear proteins to the consensus HNF-1 oligonucleotide was shown to be specific using cross-competition EMSA with the CYP2E1 sequence-specific HNF-1 oligonucleotide identified by Ueno & Gonzalez (1990) (data not shown). Further identification of the nuclear proteins that bind the HNF-1 oligonucleotides revealed that the majority of the DNA-binding complex consisted of HNF-1α protein (Figure 2B). These data are consistent with the findings of Liu & Gonzalez (1995), who used transient co-transfection of an HNF-1α expression plasmid and the CYP2E1 promoter fused to the CAT reporter gene to identify liver-enriched transcription factors involved in the developmental expression of CYP2E1. Liu & Gonzalez (1995) noted that only HNF-1α, and not HNF-1β or other liver-enriched transcription factors, was able to activate the CYP2E1 promoter. In addition, removal or mutation of the HNF-1 binding sequence used in their studies led to inactivation of the CYP2E1 promoter in response to HNF-1α.

Previous studies evaluating other proteins which are down-regulated after initiation of the APR have also implicated an HNF-1 site. Trauner et al (1998), studying the regulation of the sodium-dependent bile acid transporter gene, *ntcp*, found that LPS treatment of rats (1.0 mg kg^{-1}) resulted in a 44% loss in nuclear protein binding to HNF-1. In another study, in which investigators were observing the in-vitro effects of LPS and cytokines (TNFα, IL-1, and IL-6) on regulation of the hepatic microsomal triglyceride protein (MTP) gene, an HNF-1 site was found to be critical for MTP gene expression (Navasa et al 1998). In that study, LPS, IL-1, and to a lesser extent TNFα treatment of HepG2 cells significantly reduced MTP mRNA levels. Mutation of the HNF-1 site in the MTP gene resulted in a marked decrease in basal expression of the MTP gene and the loss of the down-regulatory effect of IL-1.

LPS treatment also altered liver nuclear protein binding to AP-1 and NFκ-B consensus sequences (Essani et al 1996; Tuyt et al 1996; Roe et al 1998). We observed a time-dependent increase in both AP-1 and NFκB binding activity after high-dose LPS treatment, with the level of binding activity returning to normal by 72 h. It has been postulated that an AP-1 site located in the CYP2E1 gene may be involved in the up-regulation of CYP2E1 seen with IL-4 treatment, but the mechanism of cytokine regulation of this P450 form requires further study (Langouet et al 1995; Morgan 2001).

The high dose of LPS used in our studies resulted in changes in CYP2E1 protein and activity. Both CYP2E1 activity and protein were significantly depressed with maximal reduction at 48 h after injection of LPS (Figures 3 and 4). CYP2E1 activity was significantly reduced as early as 4 h after endotoxin administration, whereas reductions in CYP2E1 protein content were not observed until 24 h after endotoxin administration. One explanation for the discordance between the CYP2E1 protein and activity is the difference in assay variability observed between semi-quantitative protein determination and HPLC measurement of enzymatic activity. The increased variability associated with the protein measurement may not allow for detection of small changes in CYP2E1 protein which occur within 24 h after endotoxin administration. A second explanation may be owing to the fact that the protein analysis measures both functional (holoenzyme) and non-functional (apoenzyme) CYP2E1 protein. Therefore, degradation of the non-functional protein may be necessary to observe significant reductions in protein content. Supportive of the endotoxin-mediated reduction in CYP2E1 expression are data from several studies demonstrating reductions in mRNA, protein content and activity after endotoxin administration in rat liver and hepatocyte studies (Chen et al 1992; Nadin et al 1995; Sewer et al 1996; Maheo et al 1998; Morgan 2001). The current study evaluated a more detailed time-course of CYP2E1 down-regulation in comparison with observed changes in transcription factor binding.

In summary, these data demonstrate parallel decreases in HNF-1 binding and decreased CYP2E1 expression in the rat liver after endotoxin administration. This information, coupled with previous studies characterizing the transcription factors in the CYP2E1 5′-flanking region, implies that HNF-1α may play a role in the LPS-mediated down-regulation of CYP2E1. This data serves as an important precedent for future studies evaluating the direct association of decreased HNF-1α binding and reduced gene expression after LPS administration.

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