The Effect of High Dose Endotoxin on CYP3A2 Expression in the Rat

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Purpose. The purpose of our research was two-fold: 1) to further characterize the downregulation of CYP3A2 mRNA, protein, and activity during an acute phase response (APR); 2) most importantly, to relate the time-dependent activation of nuclear proteins to putative DNA binding sequences within the CYP3A2 5'-flanking region, with the loss in CYP3A2 expression.

Methods. Rats were injected (2.0 mg/animal, i.p.) with LPS and sacrificed at 1, 2, 4, 6, 8, 24, 48, and 72 hours. Hepatic nuclear protein was isolated and analyzed for binding activity to AP-1, NFκB, and NF-IL6 consensus sequences. Hepatic CYP3A2 mRNA levels were determined by solution hybridization and CYP3A2 protein, CYP3A2 activity, and total P450 were measured in hepatic microsomes.

Results. Computer analysis of the 5'-flanking region of CYP3A2 revealed the presence of 5 NF-IL6 and 4 AP-1 putative DNA binding sites. The strongest increase in AP-1 binding activity occurred between 6 and 24 hr, and the alteration in binding complexes to an NF-IL6 oligonucleotide occurred between 4 and 24 hr. Maximum loss in CYP3A2 mRNA occurred at 8 hr post-LPS injection and remained lowered at the 24 hr timepoint. CYP3A2 protein was significantly decreased at 24, 48, and 72 hours post-LPS treatment with corresponding decreases in CYP3A2 activity and total P450.

Conclusions. The changes in NF-IL6 and AP-1 binding after LPS treatment, which appears to correlate with the changes in CYP3A2 mRNA, combined with the presence of putative NF-IL6 and AP-1 sites located in the CYP3A2 5'-flanking region, may indicate a potential role for NF-IL6 and AP-1 in CYP3A2 downregulation during an APR.

KEY WORDS: lipopolysaccharide (LPS); cytochrome P450 3A2 (CYP3A2); nuclear factor-interleukin 6 (NF-IL6); activator protein-1 (AP-1); nuclear factor kappa B (NFκB).

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ABBREVIATIONS: CYP, cytochrome P450; APR, acute phase response; LPS, lipopolysaccharide; NF-IL6, nuclear factor - interleukin 6; AP-1, activator protein-1; NFκB, nuclear factor kappa B; C/EBP, CAAT enhancer binding protein; IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumor necrosis factor; EMSA, electrophoretic mobility shift assay; ELISA, enzyme-linked immunosorbent assay.

INTRODUCTION

Bacterial endotoxins (i.e., LPS) from gram negative bacteria are potent inducers of the APR (21). The APR is characterized by alterations in the synthesis of a number of hepatic proteins known as acute phase proteins (APPs). The primary role of the APR is to maintain homeostasis during inflammation and infection. The principal homeostatic mediators of the APR are cytokines such as TNFα, IL-1, and IL-6; of these, IL-6 is thought to be the most important mediator of the hepatic APR (21).

Other important hepatic proteins altered during inflammation include the CYP450 enzymes (14,23,24). Alterations in hepatic CYP450 enzymes by LPS and cytokines are well documented (1,14–16,23–25). In our laboratories we have shown a decrease in CYP450 form-specific activities and proteins in humans and rodents treated with either LPS or IL-1 (24,25).

The CYP3A family of enzymes is responsible for the oxidation of a number of drugs and steroids (6,11). In the rat CYP3A2, and in the human CYP3A4, is constitutively expressed at relatively high levels (12). In the case of CYP3A2, the downregulation caused by both LPS and various cytokines occurs predominantly at the transcriptional level (1,15,16,23). Despite studies conducted on the effects of LPS and cytokines on CYP3A2 and all other CYP450 forms, no data exist to define the molecular mechanism(s) involved in the downregulation of CYP450s during an APR. The objective of our current research, beyond characterization of CYP3A2 expression (activity, protein, and mRNA), is to identify putative nuclear protein binding sites in the CYP3A2 gene that may be important in the regulation of CYP3A2 during an APR.

MATERIALS AND METHODS

Animals

Overnight-fasted, male Sprague-Dawley rats (250–300 g) were injected with 2.0 mg/rat i.p., LPS derived from *E. coli*, serotype 0111:B4, (DIFCO, Detroit, MI); control animals received equivalent volumes of normal saline. Animals were euthanized after anaesthesia with 87 mg/kg ketamine (Ketaset®, Aveco Co, Inc.; Fort Dodge, IA), and xylazine, 13 mg/kg (Rompun®, Moboy Corp., Shawnee, KS), at 1, 2, 4, 6, 8, 24, 48, and 72 hours post-injection. Liver tissue was removed and immediately frozen in liquid nitrogen.

Analysis of the 5'-Flanking Region of CYP3A2

Computer analysis of the CYP3A2 5'-flanking region identified several putative nuclear protein binding sites including 5 NF-IL6 and 4 AP-1 sites (Fig. 1). Although no NFκB binding sites were identified in the CYP3A2 sequences, EMSAs with an NFκB oligonucleotide were included to serve as a positive control for transcription factor binding induced by LPS.

Nuclear Extract Preparation

Nuclear protein was isolated as previously described by Roe et al. (19). Protein was quantitated by the Bradford method (7).
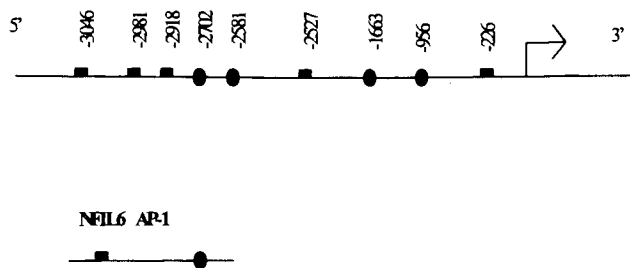
NFIL6 AP-1

Fig. 1. Schematic of 5'-flanking region of CYP3A2 gene. Computer analysis of the CYP3A2 5'-flanking region identified several putative nuclear protein binding sites including 6 NF-IL6 and 4 AP-1 sites.

Electrophoretic Mobility Shift Assay (EMSA)

The NFkB, AP-1, and SP1 oligonucleotides were purchased from Promega Corp. (Madison, WI), and an NF-IL6 consensus oligonucleotide identified by Toda et al. (27), was synthesized by the University of Kentucky Macromolecular Structural Analysis Facility (UKMSAF; Lexington, KY). All oligonucleotides used in the EMSAs were end-labeled with γ-^{32}P-ATP (Amersham Corp., Arlington Heights, IL) using T4 kinase (GIBCO/BRL) at 22°C for 20 minutes. EMSAs were carried out as described by Roe et al. (19). Sequence specificity of nuclear protein binding to all oligonucleotides tested was confirmed by competition studies in which nuclear extracts were incubated for 5 minutes at 22°C with 10-, 50-, and 100-fold molar excess unlabeled competitor oligonucleotide prior to the addition of radiolabeled oligonucleotide. To control for protein loading, EMSAs were first done with the SP1 oligonucleotide, since LPS treatment does not effect binding to the SP1 binding site.

RNA Isolation and Solution Hybridization

Total RNA was isolated from 100 mg liver tissue using commercially available Trizol reagent (GIBCO/BRL; Grand Island, New York). To specifically quantify CYP3A2 transcripts, a 40-mer probe complementary to rat CYP3A2 cDNA (+1690 to +1729) was synthesized by the UKMSAF and end-labeled with γ-^{32}P-ATP. Quantitation of CYP3A2 message was performed by solution hybridization to an excess of radiolabeled probe (5 × 10^4 cpm) at 55°C overnight under high stringency conditions (12 mM Tris, pH 7.5, 0.2% sodium dodecyl sulfate, 3 mM EDTA, and 300 mM NaCl). Digestion with S1 nuclease (100 U/reaction, Boehringer Mannheim; Indianapolis, IN) for 1 hr at 37°C digested all nonhybridized molecules. Hybridized complexes were precipitated with trichloroacetic acid for 1 hr, collected on nitrocellulose filters, and quantified using liquid scintillation spectroscopy. The abundance of CYP3A2 transcripts was determined from a standard curve of hybridizations using various concentrations of an oligonucleotide complementary to the radiolabeled probe.

Microsome Preparation and Spectral P-450 Analysis

Hepatic microsomes were prepared as previously described (5). Total CYP450 content of prepared microsomes was determined by the method of Omura and Sato (17).

Enzyme-Linked Immunosorbent Assay (ELISA)

Microsomal samples (0.5 μg protein/well) were loaded onto 96 well microtiter plates. A standard curve of Gentest supplied rat microsomes was also plated (1000-1 fmol CYP450/well). 100 μL of 10X carbonate-bicarbonate buffer (Sigma Chemical Co., St. Louis, MO) was added to each well and plates were stored overnight at 4°C. To assay CYP3A2 content, plating solutions were removed and wells were blocked with 200 μL of 50% horse serum in phosphate buffer at 37°C for 1 hr. The blocking agent was removed, and plates were incubated at 37°C for 1 hr with 200 μL primary antibody (anti-rat CYP3A2 diluted 1:1000, Gentest Corporation, Woburn, MA). Primary antibody was removed, and after washing plates 3 times, plates were incubated for 1 hr with 200 μL/well of anti-goat-alkaline phosphate conjugate (1:50,000, Sigma Chemical Co., St Louis, MO). The secondary antibody was removed and plates were washed at least 3 times. 150 μL of K-Gold premixed ELISA substrate (Neogen, Lexington, KY) was added and after 30 min each plate was read at 405 nm. Absorbance of sample containing wells was compared to a standard curve of known concentrations of CYP3A2.

Testosterone Metabolism Assay

The testosterone metabolism assay was performed as previously described by Blouin et al (5). The 2β-hydroxytestosterone and 6β-hydroxytestosterone metabolites of testosterone, indicative of CYP3A2 enzymes, were resolved and quantitated by HPLC as previously described by Blouin et al. (5). Each sample was spiked with 11α-hydroxytestosterone as an internal standard.

Statistical Analysis

To establish statistical significance between controls and endotoxin treated animals, a two-way ANOVA was performed to compare treatment vs. time. A Fisher LSD post-hoc test determined statistically significant differences for each time interval. Linear regression analysis was performed to determine correlation between the various CYP3A2 parameters (activity, protein, and mRNA).

RESULTS

EMSA Analysis of Nuclear Proteins

Nuclear protein binding to NFkB and AP-1 consensus elements was increased in a time-dependent manner in LPS-treated animals (Fig. 2). NFkB binding was increased as early as 1 hr post-injection and slowly returned to levels similar to control samples by 72 hr. A significant increase in AP-1 binding was apparent at 2 hr post-injection with strongest binding at 8 hr, which returned to control levels by 72 hr. Nuclear protein binding to a consensus NF-IL6 oligonucleotide was also altered after LPS treatment (Fig. 2). A loss in protein binding to the NF-IL6 element occurred as early as 1 hr post-injection, followed by a loss in only the upper complex of bands between the 4 and 8 hr time points with nuclear extracts from LPS-treated rats. By 24 hr, nuclear protein binding to the consensus NF-IL6 oligonucleotide was restored to control levels. The specificity of proteins binding to NFkB, AP-1, and NF-IL6 oligonucleotides was determined by competition studies using a molar
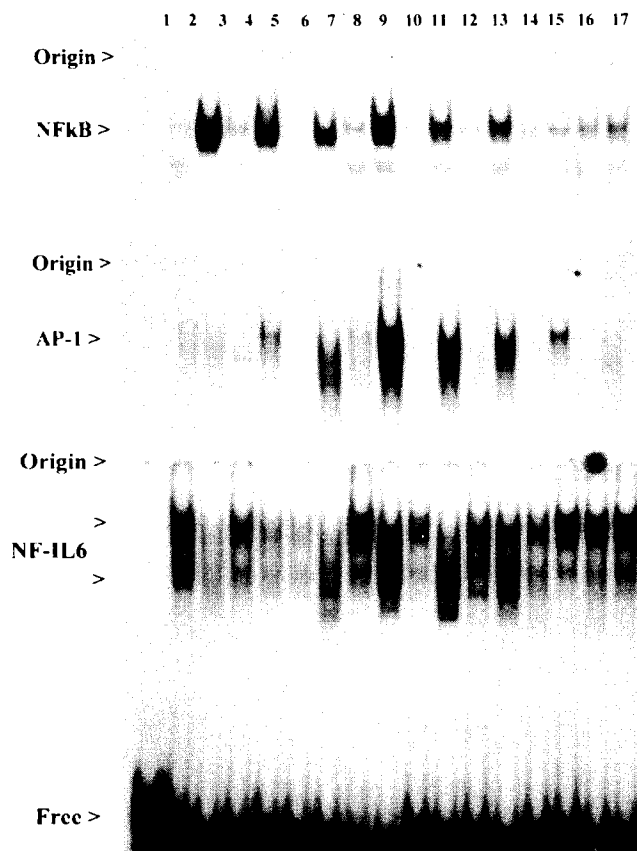
Fig. 2. Nuclear protein binding to NFκB, AP-1, and NF-IL6 consensus elements in liver nuclear extracts from LPS-treated animals. Nuclear protein (2.5 μg) was incubated with the respective radiolabeled consensus element. Lane 1, free probe without nuclear protein; Lane 2, Control 1 hr; Lane 3, LPS 1 hr; Lane 4, Control 2 hr; Lane 5, LPS 2 hr; Lane 6, Control 4 hr; Lane 7, LPS 4 hr; Lane 8, Control 6 hr; Lane 9, LPS 6 hr; Lane 10, Control 8 hr; Lane 11, LPS 8 hr; Lane 12, Control 24 hr; Lane 13, LPS 24 hr; Lane 14, Control 48 hr; Lane 15, LPS 48 hr; Lane 16, Control 72 hr; Lane 17, LPS 72 hr. The pattern of binding shown for all three consensus sequences is a representative EMSA of three separate animal experiments in which the results were similar in each case. Protein binding to the respective consensus sequences was specific in that all bands were successfully competed with as low as 10-fold molar excess of self-competitor but not by an unrelated element (data not shown). All EMSAs were carried out in probe excess.

excess of the respective unlabeled oligonucleotide. Protein binding to NFkB, AP-1, and NF-IL6 was successfully competed with as low as 10 fold molar excess of the respective oligonucleotide and not with an unrelated oligonucleotide (data not shown). EMSA analysis was conducted using nuclear extracts from three separate animal experiments. The EMSAs shown in Fig. 2, although all from the same animal experiment, are representative of the findings in the three separate experiments.

CYP3A2 mRNA Levels

Downregulation of CYP3A2 message occurred as early as 1 hr post-injection of LPS, with maximum loss at the 8 hr time point (70% of control) as determined by solution hybridization (Fig. 3). A steady increase in CYP3A2 mRNA occurred following the 8 hr timepoint.
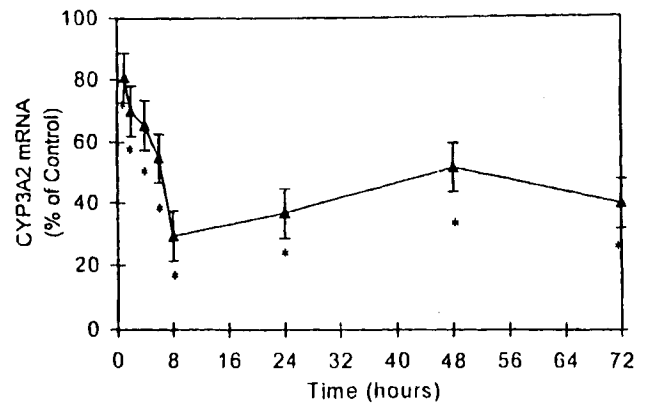

Fig. 3. Downregulation of CYP3A2 mRNA following high dose endotoxin treatment. Total RNA was isolated from liver tissue of both control and endotoxin-treated rats. CYP3A2 mRNA was determined by solution hybridization using a 40 bp oligonucleotide specific for CYP3A2 as described in Materials and Methods. Results expressed as means (N = 5) ± SD. *Significantly different from control animals, p < 0.01.

ELISA Analysis of CYP3A2 Protein

Total CYP3A2 protein concentrations decreased in endotoxin treated animals 24, 48, and 72 hrs following LPS administration (Fig. 4). Minimal protein concentrations of 28 ± 14% (p < 0.01) of control levels were observed 48 hrs post-injection.

Microsomal Activity of CYP3A2

Total CYP3A2 activity decreased in endotoxin treated animals 24, 48, and 72 hrs post-injection (Fig. 5). The decrease in rate of synthesis of both 2β hydroxytestosterone and 6β hydroxytestosterone indicated a decrease in total CYP3A2 activity to minimal levels of 57 ± 28% (2β p < 0.01) and 51 ± 11% (6β p < 0.01) of control levels for LPS treated animals at the 48 hr timepoint. Furthermore, the decrease in CYP3A2 protein concentration correlated with the decrease observed in CYP3A2 activity.
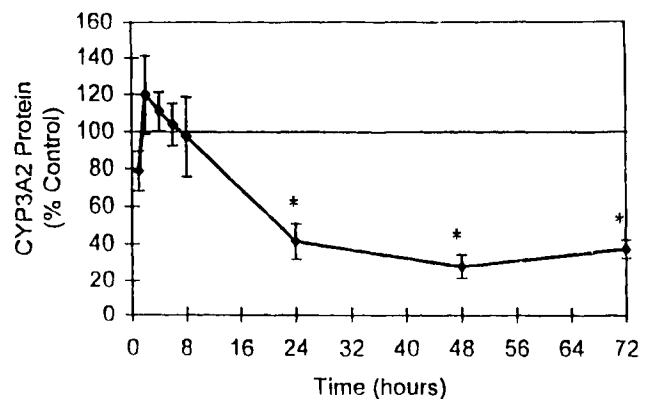

Fig. 4. Analysis of hepatic CYP3A2 protein following high dose endotoxin treatment. Microsomal protein (0.5 μg/well) was loaded to a 96 well microtiter plate and CYP3A2 protein quantitated by an ELISA as described in Materials and Methods. Results expressed as means (N = 5) ± SD. *Significantly different from control animals, p < 0.01.
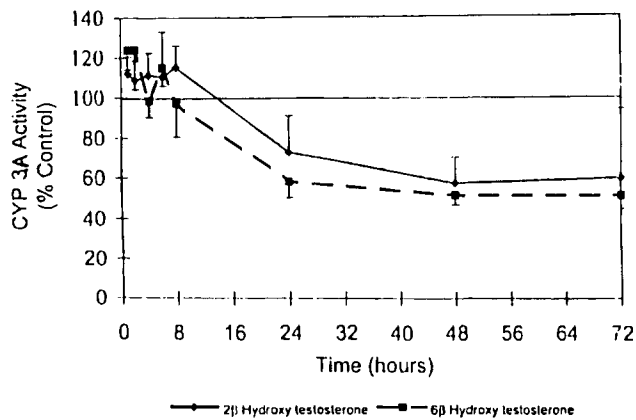
Fig. 5. Total CYP3A2 activity decreased in endotoxin treated animals 24, 48, and 72 hours following endotoxin administration. The decrease in rate of synthesis of both 2β hydroxytestosterone and 6β hydroxytestosterone indicate a decrease in total CYP3A2 activity to minimal levels of 57 ± 28% (2β p < 0.01) and 51 ± 11% (6β p < 0.01) of control levels for endotoxin treated animals 48 hours after endotoxin administration. Results expressed as means (N = 5) ± SD. *Significantly different from control animals, p < 0.01.

Spectral Analysis of Total Hepatic Microsomal P450

Total CYP450 concentrations decreased in endotoxin treated animals 24, 48, and 72 hrs following endotoxin administration (data not shown). A maximum reduction of total CYP450 levels occurred 48 hrs post-administration to levels at 60 ± 12% (p < 0.01) of control values.

DISCUSSION

Our studies describe the effect of a single, high dose of LPS in rats on CYP3A2 activity, protein, and mRNA expression. In addition, our study is the first to begin to analyze the 5'-flanking region of a CYP450 gene (CYP3A2), that is downregulated during an APR, for potential nuclear protein binding sites known to be activated during inflammation. The high dose of LPS used in our studies resulted in changes in total P450 as well as all CYP3A2 parameters tested. A maximum loss of 40% at 48 hrs post-injection was recorded for total P450. CYP3A2 activity and protein were also depressed in our studies by 43% (2β hydroxytestosterone), 49% (6β hydroxytestosterone) and 72% protein at 48 hrs, respectively. Loss in CYP3A2 mRNA levels was significant as early as 1 hr post-injection of LPS, with a maximum loss of 70% at 8 hrs. A positive correlation was identified between CYP3A2 activity (2β-, 6β-hydroxytestosterone) and CYP3A2 protein levels (r = 0.57, r = 0.73 respectively; p < 0.001) and CYP3A2 protein and CYP3A2 mRNA levels (r = 0.8, p < 0.001). Our results are similar to studies by Sewer et al. (23), who noted similar time-dependent reductions in total P450, CYP3A2 protein, CYP3A2 mRNA and CYP3A2-dependent activities following treatment of rats with LPS.

LPS is a potent inducer of the APR via the release of cytokines such as TNFα, IL-1, and IL-6 (21). There is strong evidence that the downregulation of CYP450s seen with LPS treatment may be mediated by cytokines. Several studies have shown that both *in vivo* and *in vitro* administration of cytokines results in downregulation of various CYP450 forms. Morgan

et al. (15) have studied the effects of cytokines on both male and female rats as well as primary hepatocytes in culture. In their studies, both *in vivo* and *in vitro* treatment with IL-1 suppressed expression of CYP3A2 at both the mRNA and protein level; however, IL-6 did not significantly effect CYP3A2 mRNA or protein level at a total dose of 15 ug/kg. Exposure of male rats to TNFα decreased CYP3A2 mRNA, protein, and activity levels as reported by Nadin et al. (16). Abdel-Razzak et al. (1) examined the effects of numerous cytokines on CYP450 expression including CYP3A2, in adult human primary hepatocytes. In their studies, IL-1β, IL-6, and TNFα were the most potent depressors of P450 enzymes after 72 hrs of treatment. *In vitro* exposure of cells to all three cytokines resulted in a decrease in CYP3A2 mRNA; however, only IL-1β and TNFα significantly decreased CYP3A2 activity. These results suggest that cytokines, IL-1β, IL-6, and TNFα, may be involved in the downregulation of CYP3A2 expression during an APR.

The major objective of our work was to begin to relate changes in CYP3A2 activity, protein, and mRNA, with activation of nuclear proteins involved in regulating a variety of genes important in the APR, that could be potentially involved in the downregulation of CYP3A2 as well. We used EMSA analysis to study binding of rat hepatic nuclear extracts to NFκB, AP-1, and NF-IL6 consensus sequences. These three trans-acting factors are among the most critical for expression of cytokines and many APR genes (2,13,28,29). In addition, computer analysis of the 5'-flanking region of CYP3A2 revealed the presence of multiple, putative NF-IL6 and AP-1 binding regions. Increased DNA binding activity of hepatic nuclear protein from LPS-treated rats to NFκB, AP-1, and NF-IL6 oligonucleotides occurred in a time-dependent manner. Other investigators have shown activation of NFκB, AP-1, and NF-IL6 by LPS *in vitro* (9,20,22,29) and Essani et al. (10) have shown activation of NFκB at 1 and 5 hrs after LPS treatment *in vivo*. We are the first to fully characterize the time-dependent binding of all three transcription factors after *in vivo* LPS treatment.

The rapid activation of binding of nuclear proteins to the NFκB response element (maximum within 1 hr post-LPS injection) reported here (Fig. 2) has also been documented by Essani et al. (10). In Essani's studies, rats were treated with 0.5 mg/kg (i.v.) *Salmonella enteritidis* endotoxin and euthanized 1 or 5 hr later. Nuclear extracts were isolated from either liver sections or isolated liver cells including hepatocytes, endothelial cells and Kupffer cells. EMSA analysis of the subsequent nuclear extracts revealed an increase in nuclear protein binding to an NFκB oligonucleotide, as early as 1 hr post-treatment with LPS, followed by a significant loss in binding at 5 hr. Tuyt et al. (29) using human monocytes, also reported a rapid increase in nuclear protein binding to an NFκB consensus element within 30 minutes of treatment *in vitro* (1 μg/ml LPS). Under the same conditions, these investigators also report increased AP-1 binding within 30 minutes and NF-IL6 activation within 60 minutes; however, no time point was investigated past 120 minutes in their studies. In our study, maximum AP-1 binding occurred at 6 hrs post-LPS injection; considerably later than NFκB. This may be contributed to the fact that induction of NFκB binding activity does not require protein synthesis whereas *fos* and *jun* are expressed at low levels in most cell types, but induced by a variety of extracellular stimuli (18,22).

The nuclear protein NF-IL6 is a member of the CCAAT/ enhancer binding protein (C/EBP) family, and is important for the regulation of a variety of genes involved in the APR including IL-6 (4). In other investigations in which nuclear protein binding to NF-IL6 sites was analyzed, LPS treatment typically resulted in an increase in binding. However, those studies are primarily *in vitro* studies using a wide variety of dosing regimens (9,28,29). As shown in Fig. 2, there are clearly alterations over time in nuclear protein binding from LPS-treated rats to the NF-IL6 oligonucleotide used in our study. Perhaps the loss in binding at the early time points (1, 2, and 4 hr) is a result of the physical interaction of members of the C/EBP family of transcription factors with the NFκB subunits, resulting in a loss in ability of the C/EBP proteins to bind to the consensus NF-IL6 oligonucleotide used in our study. Stein et al. (26) have shown that NFκB p65, p50, and Rel, functionally and physically interact with three members of the C/EBP family (C/EBPα, C/EBPβ, and C/EBPδ). Matsusaka et al. (13) have also shown a direct interaction between NF-IL6 and the p65 subunit of NFκB, and that this interaction resulted in a strong synergistic activation of an IL-6 promoter-reporter construct. The authors go on to hypothesize that since many promoters of APR genes contain binding sites for both NFκB and NF-IL6, perhaps the cooperation between these factors are responsible for activation of critical genes involved in the APR.

An alternative hypothesis is that the complex pattern of binding observed with the NF-IL6 consensus sequence is a result of alterations in the composition of the binding proteins. The *in vivo* expression of C/EBPα, C/EBPβ, and C/EBPδ is dramatically altered during the APR (3). Previous studies have shown that in mice, steady-state levels of C/EBPα mRNA decreases after treatment with LPS, whereas steady-state levels of C/EBPβ and C/EBPδ mRNA increased (3). Alam et al. (2) identified an acute phase response element (APRE) located from -127 to -104 in the promoter region of the mouse $\alpha 1$-acid glycoprotein gene that contained a consensus binding site for C/EBP proteins. These authors showed that in control animals C/EBPα was the predominant form that bound to the APRE, whereas in acute phase-induced animals C/EBPβ replaced C/EBPα in binding to this element. C/EBPδ did not play a role in binding to the APRE. Furthermore, EMSA analysis of the APRE using liver nuclear protein from mice dosed with 4.0 mg/kg (100 ug/animal) and sacrificed 12 hr later closely resembled the binding pattern we observed with rat liver nuclear extracts at 8 hrs post-injection using the NF-IL6 element.

The time-dependent activation, between 6 and 24 hr post-injection, of NF-IL6 and AP-1 observed in our study appeared to correlate with the maximum loss in CYP3A2 mRNA at 8 hr post-injection. These time-dependent alterations in nuclear protein binding may be important for several reasons. The APR is clearly a tightly regulated, time-dependent process e.g., various cytokines are initially upregulated by NFκB, AP-1, and NF-IL6 after an inflammatory stimulus. These trans-activating factors in turn, go on to activate many APPs (2,13,28,29). Perhaps the downregulation of CYP450 enzymes by LPS and cytokines is a result of a redistribution of nuclear binding proteins and the hepatic transcriptional machinery to more critical genes such as APPs. Sequence similarity searches of the 5'-flanking region of the CYP3A2 gene revealed several potentially critical regulatory elements including NF-IL6 and AP-1 binding sites. In much earlier work by Chen et al., the authors

suggest that the downregulation of CYP2C11 may involve transcriptions factors such as NFκB, STAT, and C/EBP binding proteins (8). In recent work by this same laboratory, the authors again mention the possibility of NFκB being involved in the regulation of CYP4A by cytokines and LPS, yet no attempt has been made to begin to analyze these critical nuclear binding proteins (23). Also, the majority of the previous studies have not analyzed changes in CYP450 parameters at early time points, i.e. less than 6 hours. As indicated by our results, changes in nuclear binding proteins as well as CYP3A2 mRNA may occur as early as 1 hour post-injection of LPS. Thus, to delineate the molecular mechanism(s) involved in regulation of a gene, analysis at early time points post-stimulus appears to be critical.

In summary, our work fully characterizes alterations in CYP3A2 activity, protein, and mRNA expression following high dose endotoxin treatment. More importantly, we have identified putative nuclear protein binding sites in the 5'-flanking region of the CYP3A2 gene, namely 5 NF-IL6 and 4 AP-1 binding sites. We have begun to correlate alterations in nuclear protein binding to NF-IL6 and AP-1 consensus sequences with the changes in CYP3A2 activity, protein, and mRNA expression herein reported. Based on our analysis of the CYP3A2 promoter and the data from our EMSA studies, in particular the NF-IL6 and AP-1 results, we are currently attempting to further identify the molecular pathway by which LPS and/or cytokines may downregulate CYP3A2 expression by the alteration of nuclear protein binding to these critical DNA regulatory sequences.

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