Role of NF-κB in Regulation of PXR-mediated Gene Expression

A MECHANISM FOR THE SUPPRESSION OF CYTOCHROME P-450 3A4 BY PROINFLAMMATORY AGENTS*

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It is a long-standing observation that inflammatory responses and infections suppress the biotransformation of drugs and decrease the hepatointestinal capacity of drug clearance. This results in alterations of therapeutic indices and increases the toxicity of certain administered drugs. Inflammatory responses also play important roles in liver pathological conditions such as drug-induced hepatitis and cholestatic diseases (1, 2). The mechanisms of these clinically important effects have not been well understood.

In human liver, the first pass of biotransformation is mainly carried out by cytochrome P-450 (CYP)² 3A4, which is the predominant isofrom of monooxygenases that are expressed in the adult hepatointestinal system. It is estimated that CYP3A4 is responsible for the metabolism of over 50% of drugs in use today, many of which are either metabolically activated and/or metabolically broken down (detoxified) through this enzyme. Therefore, transcriptional and post-transcriptional alterations of CYP3A4 activity have direct effects on the efficacy of drugs and detoxification of xenobiotics (reviewed in Refs. 3 and 4).

Recent molecular and pharmacological studies have demonstrated that transcriptional activation of cyp3a4 is mediated by the nuclear receptor PXR (pregnan X receptor). The rodent PXR (5) and its human homolog hPXR (6), also known as steroid and xenobiotic receptor (7) or hPAR (8), were identified as xenobiotic receptors that can be activated by certain xenobiotics and endobiotics. PXR regulates the expression of cyp3a4 by associating with its obligate partner RXR, and the heterodimer binds to the nuclear receptor response elements found in the regulatory regions of these genes. Genes that are regulated by PXR include multiple drug-resistant genes such as MDR1 (9) and MRP2 (10) as well as genes involved in metabolism and transport of endogenous molecules, including bilirubin, bile acids, thyroid hormone, fatty acids, and steroids (11–13). PXR-RXR can also interact with pathways regulated by other nuclear receptors such as the constitutive androstane receptor-RXR by mutual binding to the consensus regulatory DNA sequences, thus forming a redundant, compensatory network for the metabolism and disposition of xenobiotic and endobiotics (14).

The mechanisms of cyp3a4 suppression caused by inflammatory responses and infections have been investigated (15, 16). Several aspects of the transcriptional regulation may be involved including decreases of PXR and RXR mRNA levels or induction of the liver inhibitory protein, which suppresses cyp3a4 through a distal flanking region (17). It is likely that modulation of transcriptional activation by several pathways leads to down-regulation of PXR-regulated gene expression.

It has been shown that most inflammatory cytokines induced during sepsis and aseptic responses lead to suppression of CYP3A4 gene expression. We hypothesize that there may be immediate, early events at transcriptional level where the effects of the proinflammatory responses converge. One of the critical responses to acute infections and inflammations is the activation of NF-κB (18–20), which has pleiotropic functions and has been shown to down-regulate the transcriptional activity of multiple steroid/nuclear receptors (21). NF-κB regulates innate as well as adaptive immune systems. One of the pivotal functions of NF-κB is its swift activation in response to LPS or proinflammatory cytokines, which is an evolutionally conserved defensive mechanism against infections. The classic NF-κB consists of p65 (RelA)

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‡ The abbreviations used are: CYP3A4, cytochrome P-450 3A4; hPXR, human pregnane X receptor; hPXR, human PXR; RXR, retinoid X receptor; RIF, rifampicin; SR120916, super repressor IκBα; LPS, lipopolysacchride; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PIPE, 1,4-piperazinediethanesulfonic acid.
and p50 heterodimer, and it is activated in response to various stimuli including LPS, TNF-α, double-stranded RNA, and UV radiation. In this study, we investigate the role of NF-κB in regulation of the transcriptional activity of the PXR-RXRα complex in an attempt to address the mechanism of suppression of cyp3a4 by LPS and proinflammatory cytokine TNF-α. The results reveal that NF-κB plays an important role in suppression of PXR-RXRα-regulated gene expression by interfering with the binding of PXR-RXRα to the regulatory DNA sequences. The mechanism may have general implications in gene expressions regulated by nuclear receptors where RXRα is a common dimerization partner.

**MATERIALS AND METHODS**

Oligonucleotides as the PCR primers and ER6 EMSA probe, the DNA modifying enzymes, and Lipofectamine were from Invitrogen. Dulbecco’s modified Eagle’s medium was from Invitrogen or HyClone (Logan, UT), fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA). Plasmid DNA purification kits, rifampicin, lipopolysacride, and monoclonal antibody against the FLAG tag were from Sigma. Recombinant human TNF-α was purchased from Roche Applied Science (Indianapolis, IN). The polyclonal antibodies against RXRα and p65 were from Santa Cruz (Santa Cruz, CA). The human HepG2 cell line was purchased from the American Type Culture Collection (Manassas, VA).

**Plasmid Constructs**—The reporter plasmid pGL3–3A4–Luc was constructed via the following steps. First, the promoter module (–362/+53)-containing DNA fragment was generated by PCR amplification using human genomic DNA as the template with the primers (5′-CATTTGCTGGCCTGGTGTT-3′ and 5′-CATAAAGCTTGGTGCTCTTTCGCTGGAAGT-3′). The 1.13-kb PCR product was restricted with BglII and HindIII, and the resultant 415-bp fragment was cloned into pGL3-basic vector (Promega) to yield pGL3-3A4 (–362/+53). The DNA fragment corresponding to the XREM region (–7836 to –7208) (23) were generated by PCR with the primer oligonucleotides CYP3A4-3 (5′-GGGGTACCAATTCTAGAGATGGTGTT-3′ and CYP3A4-4 (5′-CCGCTGAGATCTTCTGCAA CAGGTAAAGGAG-3′). The DNA fragment containing the KpnI and BglII site were created by restriction digestion. The KpnI and BglII-restricted fragment was then inserted into the KpnI- and BglII-restricted pGL3-3A4 plasmid to yield the pGL3-3A4-Luc reporter gene.

The expression vector for hPXR, pCI-hPXR, and FLAG-PXR was generated as follows. DNA fragment corresponding to the coding region of hPXR (amino acids 1–434) was generated by reverse transcription-PCR using total RNA from HepG2 cells. For pCI-hPXR, the PCR primers were 5′-GGGATTCCACAGGAGGTGAGACCCCAAAGAAGC TGG-3′ and 5′-GGGTGCAGGGGGCGTCCAGCTACTGTTGAT-3′; for FLAG-PXR, the PCR primers were 5′-CCGTCGAGATCTTCTGCAA CAGGTAAAGGAG-3′). The 1.13-kb PCR product was modified with EcoR I and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI; the PCR product was modified with EcoRI and NotI or with EcoRI and NotI. The 1.13-kb PCR product was digested with EcoRI and NotI and was subcloned into the vector pCI-neo (Promega) and p3XFLAG-myc-CMV-26 vector (Sigma).

**Cell Culture and Transient Transfection**—For primary human hepatocyte culture, cell suspension was purchased from Cambrex BioScience (Walkersville, MD). The donor of the human hepatocytes was a 26-year-old male without heart disease or hypertension. Serological tests showed negative for human immunodeficiency virus, types 1 and 2, hepatitis B surface antigen (HBsAg), hepatitis C virus, human T-cell lymphotropic I/II virus, and syphilis. Upon arrival the cells were resuspended in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, antibiotics, 4 μg/ml insulin, and 1 μmol/liter dexamethasone, plated in collagen-coated plate for attachment, and then maintained in Williams’ E medium containing ITS+ (insulin, transferrin, selenium, borine serum albumin, and linoleic acid), 0.1 μmol/liter dexamethasone and antibiotics overnight for recovery, and then the cells were treated with Me₂SO, RIF, RIF+LPS, and RIF+TNF-α. 24 h after the treatment, the cells were harvested for isolation of total RNA for real time reverse transcription-PCR analysis. HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B) in 5% CO₂ at 37 °C.

For transient transfection, HepG2 cells were seeded in 12-well plates at 30% confluence. On the next day transfection was performed using Lipofectamine (Invitrogen). 6 h after transfection, cells were treated with RIF and other reagents. 48 h later, cells were harvested to determine the luciferase activity using the luciferase assay system (Promega). Conclusions were made based on three or more independent transfection experiments.

**In Vitro Transcription Coupled to Translation and EMSA**—Human PXR and human RXRα polypeptides were generated by in vitro transcription coupled to translation using TNt-coupled reticulocyte lysate system (Promega, Madison, WI). Oligonucleotides used for EMSA were the ER6 consensus sequences in cyp3a4 promoter region as described (14). The double-stranded oligonucleotide was labeled with [α-32p]dCTP using Klen now enzyme (USB Corp., Cleveland, OH). For EMSA assay, PXR and RXRα and recombinant p65 (Promega) and p65 (produced by baculoviral expressions) in various combinations were incubated for 30 min in a reaction mixture containing 40 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA, 0.5 mM diethiothreitol, 20 mM Hepes, pH 7.9, 4% Ficoll (400 K), and ~30000 cpm of radiolabeled double-stranded oligonucleotide probe. After incubation for 30 min at room temperature, the reaction mixtures were separated by electrophoresis in 4.5% nondenaturing polyacrylamide gel. The results were recorded by autoradiography.

**Real Time Quantitative PCR**—For real time quantitative PCR, total RNA samples were reverse-transcribed by using Moloney murine leukemia virus reverse transcriptase (Invitrogen), and the cDNA samples were used for quantification by PCR. Amplifications were performed in the ABI Prism 7900HT (Applied Biosystems) by using SYBR Green Master Mix (Applied Biosystems). The PCR primers used were: PXR, 5′-GGCCCATCTGGCTATCATCTTCA-3′ and 5′-TTCTAGGGCCCTCCTGAAA-3′; RXRα, 5′-TAAATGGCGTCTCTGAGTCT-3′ and 5′-TTGGCTGAAGGGCCCTGTC-3′; CYP3A4, 5′-CCACAAAGGCTGTTGCGTCC-3′ and 5′-GGTTGATGATGATGACT-3′; ATGGTAGAAGGTGTTGTCGAGACG-3′ and 5′-GGTTGATGATGATGACT-3′. The β-actin was used as a housekeeping gene for normalization with the rest of the samples.

**Immunocytochemistry**—Primary human hepatocytes growing in 24-well plates were treated with LPS or TNF-α for 1 h. The cells were washed three times with cold PBS and then fixed with fresh 4% formaldehyde in PBS for 10 min at room temperature. After washing three times with PBS, the cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After washing with PBS (three times for 5 min each), the cells were blocked with 5% bovine serum albumin in PBS/Tween 20 for 1 h at room temperature. Then primary antibody against p65 (Santa Cruz, sc-109X) diluted (1:500) in PBS/Tween 20 was added, and the reaction was incubated at room temperature for 1 h. After three washes with PBS/Tween 20, 10 min each, secondary antibody conjugated with Alexa Fluor-568 (Molecular Probe, A11011) diluted in PBS/Tween 20 (1:1000) was added and incubated for 1 h at
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room temperature. The cells were washed with PBS/Tween 20 three times for 10 min each. 4′,6′-Diamino-2-phenylindole was added to stain the cells. The images were visualized, and representative views of the cells were recorded by fluorescence microscopy with an Olympus IX71 microscope.

**GST Pull-down Analysis**—The GST pull-down assay was essentially as described (22). [35S]Methionine-labeled full-length p65 protein was generated with a TNT-coupled reticulocyte lysate system (Promega) using the T7 promoter-driven cDNA plasmid as the template. PCR-generated cDNA fragments of RXRα corresponding to the domains of RXRα (see Fig. 5A for details and the sequences of PCR primer used are available upon request) were inserted into pGEX-5X-3 (Amersham Biosciences), yielding the expression plasmids for GST-RXRα fusion peptides. The plasmids were expressed in *Escherichia coli* (BL21), and fusion polypeptides were purified with the glutathione-Sepharose 4B affinity matrix (Amersham Biosciences) according to the manufacturer’s instructions. Ten micrograms of each fusion polypeptide (estimated by comparison with bovine serum albumin in an SDS-PAGE gel with Coomassie staining) was incubated with 20 μl of radiolabeled p65 in a total of 250 μl of binding reaction buffer (20 mM Hepes, pH 7.9, 1% Triton X-100, 20 mM dithiothreitol, 0.5% bovine serum albumin, and 100 mM KCl) for 2 h at 4 °C. After incubation, the beads were washed with the same buffer without bovine serum albumin five times. The bound proteins were eluted by boiling in the SDS-PAGE sample buffer and resolved by 8% SDS-PAGE gel electrophoresis. The signals were detected by autoradiography.

**Chromatin Immunoprecipitation (ChIP) Assay**—The ChIP assay was based on published procedure with modification (11). HepG2 cells were transfected with FLAG-tagged PXR and pGL3-3A4-Luc and were maintained in 10-cm plates under standard cell culture conditions. At 95% confluence formaldehyde was added directly to tissue culture plates (0.125M). The plates were incubated for 15 min at room temperature on a rocker. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The plates were incubated at room temperature for 5 min. The plates were then rinsed twice with ice-cold phosphate-buffered saline. The cells were scraped off the plates and collected into 50-ml conical tubes by centrifugation (600 × g for 5 min at 4 °C), and the pellet was washed once with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and resuspended in 2 ml of cell lysis buffer (5 mM PIPES, pH 8, 1 mM EDTA, 0.5 mM EGTA, 85 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 5 μg/ml each of leupeptin and aprotinin) and incubated for on ice for 10 min. The cells were homogenized on ice using an B type pestle by processing in a Dounce homogenizer 200 times to aid the release of nuclei. The nuclei were collected by centrifugation (5000 × g for 10 min at 4 °C) and then resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 0.5 mM EGTA, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 μg/ml each of leupeptin and aprotinin) and incubated again on ice for 10 min. The samples were sonicated into DNA fragments of 0.5–1.5 kilobase pairs (checked by agarose gel electrophoresis/ethidium bromide staining) and microcentrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was cleared by centrifugation at 12,000 × g for 5 min to the clean tubes. Appropriate antibodies (1 μg each) were added to the aliquots and then 25 μl of precleared 50% protein A/G beads (Amersham Biosciences) was added. The final volume of each sample was adjusted to no more than 500 μl with the same amount of immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 167 mM NaCl, 100 μg/ml sonicated salmon sperm DNA) as the nuclei lysis buffer. The mixtures were incubated on the rotating platform at 4 °C, overnight. After incubation, the beads were collected by centrifugation at 5000 rpm for 1 min in a microcentrifuge, and pellets were washed once with 1 ml of 1× dialysis buffer (2 mM EDTA; 50 mM Tris-Cl, pH 8.0) with 100 μg/ml sonicated salmon sperm DNA, twice with 1× dialysis buffer and three times with 1 ml of immunoprecipitation wash buffer (100 mM Tris-Cl, pH 9.0, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid) for 10 min with rotation. After the wash, 200 μl of protein kinase digestion buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 mg/ml proteinase K) was added to each sample, and the reaction was incubated at 55 °C for 3 h and then at 65 °C for 6 h to reverse the cross-linking. The sample was extracted once with phenol-chloroform-isomyl alcohol and precipitated with ethanol in the presence of 20 μg of glycogen overnight. The precipitated pellets were collected by centrifugation at 14,000 × g in microcentrifuge, and the pellets were resuspended in 20 μl of TE buffer. Aliquots from each tube were amplified by PCR, and PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The PCR primer pairs were 5′-TTGAGCTCCCCAGTAAACATTG-3′ and 5′-TGATGGAGGTTCTT- CCTGC-3′ for amplifying the cyp3a4 promoter region and 5′-ACTCAT- GTCCCCAATTAAGGTC-3′ and 5′-TTTCTTTGTCAAGAGTTCCA- C-3′ for amplifying the enhancer module.

**RESULTS**

**Suppression of PXR-mediated Gene Activation by LPS and TNF-α in Human Liver Cells**—The effects of LPS and TNF-α on the expression of PXR, RXRα, and CYP3A4 were investigated in a primary human hepatocyte cell culture model by quantitative real time PCR. Treatment of the hepatocytes with the prototypical human PXR agonist RIF induced a 34-fold increase in CYP3A4 mRNA; the RIF-induced CYP3A4 mRNA levels were suppressed by more than 50 and 90% after cotreatment with either TNF-α (2 ng/ml, 24 h) and LPS (5 μg/ml, 24 h), respectively (Fig. 1A). In contrast, PXR mRNA levels were unchanged by TNF-α treatment, and there was an approximately 30% decrease in hPXR mRNA levels after treatments with either LPS or TNF-α (Fig. 1C). Activation of NF-κB by LPS or TNF-α was confirmed by immunocytochemistry for p65 nuclear translocation (Fig. 1D). The RNA samples were also analyzed by microarray profiling, and the results were consistent with those obtained by the quantitative PCR, with respect to the changes of PXR, RXRα levels, and the suppression of cyp3a4 by LPS or TNF-α (data not shown).

To further investigate the effects of proinflammatory agents on the transcriptional activity of PXR and to avoid donor variability in PXR-regulated genes in the human primary hepatocytes, we constructed the luciferase reporter gene driven by PXR-responsive enhancer modules for analysis of the PXR-regulated gene expression in a human hepatoma cell line (HepG2) based on the published information (Fig. 2A) (23). HepG2 cells were transiently cotransfected with pGL3-3A4-Luc and hPXR expression plasmids pCI-hPXR. The transfected cells were then treated with RIF alone or cotreated with RIF and LPS or RIF and TNF-α. TNF-α and LPS caused significant suppressions of the luciferase gene expression (Fig. 2B) that are consistent with the results from the primary human hepatocyte culture model (Fig. 1). These results using the HepG2 cell line also confirmed the utility of the HepG2 cell culture model in analysis of PXR-regulated transcription.
NF-κB Plays a Critical Role in Down-regulation of cyp3a4 Expression by Inflammatory Mediators—NF-κB is an immediate early gene, which is activated in response to various stress stimuli including infections and inflammatory responses. NF-κB plays a pivotal role in mediating the pathological effects of TNF-α and LPS. It has been demonstrated that NF-κB regulates several nuclear/steroid receptors through physical and function interactions, resulting in transrepression of the gene expressions regulated by these receptors (24) (reviewed in Ref. 21). To test the role of NF-κB in mediating the suppression of PXR transcriptional activity, we first transiently cotransfected NF-κB p65 with PXR-driven luciferase reporter gene in HepG2 cells. Coexpression of NF-κB p65 potently suppressed PXR-driven luciferase reporter gene activity, suggesting a role for NF-κB in mediating suppression (Fig. 3). To further demonstrate that NF-κB is specifically involved in the suppression of cyp3a4 expression, we coexpressed the NF-κB super repressor, SRIκBα, in transient transfection assays and analyzed the effects of NF-κB inhibition on TNF-α- and LPS-treated cells. SRIκBα is a mutant of IκBα with a serine to alanine mutation at residues 32 and 36. These mutations render the IκBα unable to be phosphorylated at serines 32 and 36 and therefore resistant to degradation by the proteosome pathway, thus causing constitutive inhibition of NF-κB. In transient transfection assays, HepG2 cells were cotransfected with plasmids pCI-PXR, pGL3-3A4-Luc reporter gene and increasing amounts of SRIκBα expression plasmid. As expected, activation of NF-κB by either TNF-α or LPS caused suppression of the reporter gene activity. However, the LPS or TNFα-induced suppression of reporter gene was reversed by coexpression of SRIκBα (Fig. 3), indicating that NF-κB activation was directly responsible for the suppression of the PXR-regulated gene expression. NF-κB Regulates PXR Transcriptional Activity by Disrupting the Association between PXR-RXRα Complex and DNA Sequences—It has been shown that NF-κB regulates the transcriptional activity of steroid/nuclear receptors through direct protein-protein interaction. Na et al. (25) reported that NF-κB directly interacts with RXR. The association of NF-κB with nuclear receptors may potentially have a functional impact on the transcriptional activity of the PXR-RXRα complex. One possible effect is that the binding of p65 with RXRα may interfere with the formation of the enhancerome consisting of the PXR-RXRα complex and consensus DNA sequences. To test this hypothesis, we performed EMSA. PXR and RXRα proteins were generated through in vitro transcription coupled to translation. PXR and RXRα bound to the ER6 probe as dimer (Fig. 4, lanes 5 and 6). Addition of the recombinant p65 protein disrupted the binding of PXR-RXRα to the consensus ER6
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A

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\begin{array}{c}
\text{pGL3-3A4-Luc (5832 bp)} \\
\text{pCI-hPXR (6744 bp)} \\
pCMV \\
pXFlag-hPXR (7658 bp)
\end{array}
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B

![Diagram of interaction between PXR and NF-κB](image)

FIGURE 3. NF-κB activation is responsible for the suppression of PXR-regulated gene expression by inflammatory agents. HepG2 cells were seeded in 12-well plates, and the cells were transiently cotransfected with plasmids of pCI-hPXR (0.2 μg), pGL3-3A4-Luc (0.5 μg). The transcriptional activity of PXR was either suppressed by coexpression with p65 or treatment with LPS or TNF-α. HepG2 cells were cotransfected with SRIkBα as indicated. The cells were harvested 48 h after the TNF or LPS treatment for luciferase activity determination. # and *, statistically significant difference (p < 0.01 and p < 0.05, respectively) compared with RIF treatment. All of the data are the means ± S.D. of triplicate transfections representative of three independent experiments.

FIGURE 2. Suppression of PXR-mediated CYP3A4 reporter gene expression by LPS and TNF-α. A, luciferase reporter gene pGL3-3A4-Luc and human PXR expression plasmids used for the analysis of PXR-regulated gene expression. The pGL3-3A4-Luc directed by the distal upstream enhancer module XREM was constructed based on the published information (23). B, suppression of PXR-mediated pGL3-3A4-Luc luciferase reporter gene activity by TNF-α and LPS. HepG2 cells were transiently cotransfected with pCI-hPXR and pGL3-3A4-Luc reporter plasmids, and after 6 h, the transfected cells were cotreated with either RIF + TNF-α or RIF + LPS for 24 h, respectively. The luciferase activity was assayed 48 h after the treatments. # and *, statistically significant difference (p < 0.01 and p < 0.05, respectively) compared with RIF treatment. All of the data are the means ± S.D. of triplicate transfections representative of three independent experiments.

DISCUSSION

CYP3A4 is a predominant human liver monoxygenase metabolizing more than half of the drugs in use today. Transcriptional and posttranscriptional regulations of the expression of this enzyme are of great importance in therapeutic application as well as the development of therapeutics. Recent studies have demonstrated that the ligand-dependent transcription factor hPXR plays a pivotal role in coordinated regulation of cyp3a4, conjugation enzymes, and transporters at the transcriptional level (reviewed in Refs. 26 and 27), therefore, it is impor-
tional activity. Using real time quantitative PCR and microarray profiling with LPS- and TNF-α-treated primary human hepatocytes, we found that a slight decrease of PXR mRNA and RXRα mRNA level was essentially unchanged (Fig. 1). The maximal decrease in PXR mRNA may not account for the dramatic suppression of the CYP3A4 mRNA by LPS and TNF-α (Fig. 1). Using PXR- and PPARα-deficient mice, Richardson and Morgan (30) have shown that endotoxin caused approxi- mately the same levels of suppression of P-450 in KO mice as in the wild type, suggesting that nuclear receptors PXR and PPARα are not required for regulating the LPS-imposed suppression of the cytochromes P-450 including CYP3A, at least in the animals whose P-450s are required for regulating the LPS-imposed suppression of the cytochrome P-450 by LPS (34, 35). These clinically important phenomena have been investigated extensively. Several mechanisms have been proposed to explain the infection- and inflammation-induced suppression of cyp3a4 expression. For example, it has been observed that LPS treatment down-regulates the PXR mRNA level in cells and animals (29), and this may potentially result in suppression of cyp3a4 expression. However, the levels of the nuclear receptors may not be an accurate gauge in evaluating their transcriptional activity. Using real time quantitative PCR and microarray profiling with LPS- and TNF-α-treated primary human hepatocytes, we found that a slight decrease of PXR mRNA and RXRα mRNA level was essentially unchanged (Fig. 1). The maximal decrease in PXR mRNA may not account for the dramatic suppression of the CYP3A4 mRNA by LPS and TNF-α (Fig. 1). Using PXR- and PPARα-deficient mice, Richardson and Morgan (30) have shown that endotoxin caused approxi- mately the same levels of suppression of P-450 in KO mice as in the wild type, suggesting that nuclear receptors PXR and PPARα are not required for regulating the LPS-imposed suppression of the cytochromes P-450 including CYP3A, at least in the animals whose P-450s have not been induced by exogenous agents. However, because there have been extensive cross-talks between nuclear receptors, the compensatory roles of other nuclear receptors in mediating the LPS-induced suppression remains to be investigated. This is especially true in view of the current finding that the NF-κB-mediated suppression of the nuclear receptors may be through a general mechanism where the functions of a common partner (RXR) for nuclear receptors is being compromised upon NF-κB activation.

Recent studies have shown that the DNA sequences at approximately –5.95 kilobase of cyp3a4 regulatory region contains CCAAT/enhancer sequences, which can be regulated by liver inhibitory protein (17), thereby causing suppression. Liver-enriched transcription factor has also been shown to mediate the LPS suppressive effects of the organic anion transporting peptide 4 (31). In our current studies, we found that PXR-directed luciferase reporter gene without the CCAAT/enhancer sequences was also suppressed by NF-κB activation (Fig. 2), and inhibition of NF-κB alleviated the suppression (Fig. 3), suggesting that disruption of the binding of PXR-RXR complex to the consensus sequences (Figs. 4–6) is an important mechanism in addition to the regulation by liver inhibitory protein. It is highly likely that more than one mechanism may be responsible for the suppression of cyp3a4 gene expression.

A common transcriptional response to the challenges of infection and inflammation is the induction of immediate early genes. One of these genes is the pleiotropic transcription factor NF-κB, which is activated in response to various proinflammatory stimuli. NF-κB has been shown to interact with the nuclear/steroid receptor, Ah receptor (21, 24, 32) and modulates the transcriptional activity of these receptors (reviewed in Refs. 21 and 33). In the mouse LPS-induced CNS inflammation model, it has been shown that Toll-like receptor regulates the suppression of the hepatic cytochromes P-450 by LPS (34, 35). These studies suggested that NF-κB is involved in regulation of the hepatic P-450. Although it is unknown whether NF-κB activation plays a role in
the transcriptional activity of PXR, it was found that the common dimerization partner RXR for the nuclear receptors interacted with NF-κB (25). We hypothesized that NF-κB may play a direct role in suppression of cyp3a4 expression and developed a PXR-driven luciferase assay using HepG2 cell culture model for the analysis of transcriptional regulation of PXR by proinflammatory agents. In comparison with human primary hepatocyte culture, the magnitudes of PXR activation by rifampicin or transrepression of PXR by NF-κB activation were lower, which may be due to the clonal nature of the immortalized cell line. It is well known that hepatocytes lose certain aspects of xenobiotic responses in ex vivo culture conditions. These quantitative differences notwithstanding, the HepG2-based culture model has allowed us to analyze the transcriptional regulation by PXR and overcome certain drawbacks associated with using human primary hepatocyte culture, such as the donor variability and cost.

In this study, the important role of NF-κB in the suppression of cyp3a4 is demonstrated based on the following results: (i) TNF-α and LPS treatments of human primary hepatocytes resulted in activation of the NF-κB and coincided with the down-regulation of cyp3a4, and in luciferase reporter gene assay, activation of NF-κB suppressed the PXR-driven luciferase reporter gene activity; (ii) TNF-α- and LPS-imposed repression of cyp3a4 promoter activity was reversed by the NF-κB super repressor (SRκBα), thus demonstrating the specific involvement of NF-κB.

To further elucidate the mechanism underlying the suppression of cyp3a4 by NF-κB, we performed EMSA, GST pull-down, and ChIP assays to test the interaction between NF-κB and PXR-RXRα complexes. Using EMSA assay, we found that binding of PXR-RXRα heterodimer to the ER6 consensus sequences was inhibited by p65. The inhibitory effects of p65 on the binding of PXR-RXRα to ER6 were alleviated by p50, which is the cognate partner for p65, suggesting that the inhibitory effect of p65 could be competitively decreased by p50, which is consistent with the hypothesis that p65 interferes with the association of PXR-RXRα with DNA sequences (Fig. 4). This notion was further strengthened by the observation that the association between RXR and NF-κB p65 was mediated through the RXR DNA-binding domain as determined by GST pull-down assay (Fig. 5).

Furthermore, using the ChIP assay, we found that the association of RXRα with the regulatory regions of cyp3a4 was disrupted upon activation of NF-κB by either by LPS treatment or transient expression of p65, suggesting that the association between PXR-RXRα complex with DNA sequences was disrupted by NF-κB in vivo (Fig. 6).

FIGURE 6. Effects of NF-κB activation on the associations of PXR and RXRα with the regulatory regions of cyp3a4 determined by the ChIP assay. The HepG2 cells were cotransfected with 3×FLAG-tagged hPXR(Fig. 2A), pGL3–3A4-Luc. The NF-κB was activated by either cotransfection with p65 or treatment of the cells with LPS or TNF-α. The cells were formaldehyde cross-linked, and associations of FLAG-tagged PXR, RXRα with DNA sequences were determined by ChIP assay. The regions of PCR amplification are indicated in the lower panel. Three independent ChIP assays were performed, and typical results are shown. I.P., immunoprecipitation.

FIGURE 7. Schematic illustration of the suppression of CYP3A4 gene expression by NF-κB. Upon activation of NF-κB by TNF-α or LPS, NF-κB p65 translocates into the nucleus and disrupts the binding of the PXR-RXRα heterodimer to its regulatory sites by interacting with RXRα, which is the obligate partner of PXR, thereby suppressing cyp3a4 expression.
Transcriptional activation of gene expression consists of multiple interconnected yet distinct steps involving a constellation of transcriptional factors at different steps. For example, in regulation of cyp1a1 gene expression, the regulatory steps that have been investigated include histone remodeling and modifications (32, 36), recruitments of coactivator (37) and mediator complexes (38), and recruitment of the positive transcriptional elongation factor, which leads to phosphorylation of the C-terminal domain of the large subunit of RNA polymerase II (22). It is highly likely that transcriptional regulation of cyp3a4 is also subjected to regulation at these critical steps by various signaling mechanisms including NF-κB activation.

Taken together, these in vitro and in vivo results suggest that activation of NF-κB results in disruption of the interaction of the PXR-RXRα complex with the consensus DNA sequences in the regulatory regions of cyp3a4, thus providing a mechanistic explanation for the observed suppression of cyp3a4 by LPS, proinflammatory cytokines, and other stress signals that are known to induce NF-κB. The mechanism is depicted showing that NF-κB activation by physiological and pathological stimuli leads to its translocation into the nucleus where it interrupts the binding of PXR-RXRα complex to the cognate consensus DNA sequences, thereby causing transcriptional suppression (Fig. 7). Because RXRα binding is interfered with by NF-κB, this mechanism of suppression by NF-κB may be extended to other nuclear receptor-regulated systems where RXRα is a dimerization partner.

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REFERENCES

1. Lehmann, V., Freudenberg, M. A., and Galanos, C. (1987) J. Exp. Med. 165, 657–663
2. Pirovino, M., Meister, F., Rubli, E., and Karlaganis, G. (1989) Gastroenterology 96, 1389–1395
3. Guengerich, F. P. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 1–17
4. Quattrrochi, L. C., and Guzelian, P. S. (2001) Drug Metab. Dispos. 29, 615–622
5. Kliwer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterstrom, R. H., Perlman, T., and Lehmann, J. M. (1998) Cell 92, 73–82
6. Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T., and Kliwer, S. A. (1998) J. Clin. Invest. 102, 1016–1023
7. Blumberg, B., Sabbagh, W., Jr., Jugunu, H., Bolado, J., Jr., van Meter, C. M., Ong, E. S., and Evans, R. M. (1998) Genes Dev. 12, 3195–3205
8. Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., Ohlsson, R., Postlind, H., Blomquist, P., and Berkenstam, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 95, 12208–12213
9. Synold, T. W., Dussault, I., and Forman, B. M. (2001) Nat. Med. 7, 584–590
10. Kast, H. R., Goodwin, B., Tarr, P. T., Jones, S. A., Anisfeld, A. M., Stoltz, C. M., Tontonoz, P., Kliwer, S., Willson, T. M., and Edwards, P. A. (2002) J. Biol. Chem. 277, 2908–2915
11. Goodwin, B., and Moore, J. T. (2004) Trends Pharmacol. Sci. 25, 437–441
12. Guo, G. L., Staudinger, J., Ogura, K., and Klaassen, C. D. (2002) Mol. Pharmacol. 61, 832–839
13. Kullak-Ublick, G. A., Steiger, B., and Meier, P. J. (2004) Gastroenterology 126, 322–342
14. Xie, W., Barwick, J. L., Simon, C. M., Pierce, A. M., Safe, S., Blumberg, B., Guzelian, P. S., and Evans, R. M. (2000) Genes Dev. 14, 3014–3023
15. Morgan, E. T. (1997) Drug Metab. Rev. 29, 1129–1188
16. Renton, K. W. (2004) Curr. Drug Metab. 5, 235–243
17. Martinez-Jimenez, C. F., Gomez-Lechon, M. J., Castell, J. V., and Jover, R. (2005) Mol. Pharmacol. 67, 2088–2101
18. Aggarwal, B. B. (2004) Cancer Cell 6, 203–208
19. Karin, M., and Greten, F. R. (2005) Nat. Rev. Immunol. 5, 749–759
20. Xiao, C., and Ghosh, S. (2005) Adv. Exp. Med. Biol. 560, 41–45
21. McKay, L. I., and Cidlowski, J. A. (1999) Endocr. Rev. 20, 435–459
22. Tian, Y., Ke, S., Chen, M., and Sheng, T. (2003) J. Biol. Chem. 278, 44041–44048
23. Goodwin, B., Hodgson, E., and Liddle, C. (1999) Mol. Pharmacol. 56, 1329–1339
24. Tian, Y., Ke, S., Denison, M. S., Rabson, A. B., and Gallo, M. A. (1999) J. Biol. Chem. 274, 510–515
25. Na, S. Y., Kang, B. Y., Chung, S. W., Han, S. J., Ma, X., Trinchieri, G., Im, S. Y., Lee, J. W., and Kim, T. S. (1999) J. Biol. Chem. 274, 7674–7680
26. Hohoscini, C., and Meyer, U. A. (2003) Pharmacol. Rev. 55, 649–673
27. Honkoski, P., Sueyoshi, T., and Negishi, M. (2003) Ann. Med. 35, 172–182
28. Morgan, E. T., Li-Masters, T., and Cheng, P. Y. (2002) Toxicology 181–182, 207–210
29. Beigneux, A. P., Moser, A. H., Shigenaga, J. K., Grunfeld, C., and Feingold, K. R. (2002) Biochem. Biophys. Res. Commun. 293, 145–149
30. Richardson, T. A., and Morgan, E. T. (2005) J. Pharmacol. Exp. Ther 314, 703–709
31. Li, N., and Klaassen, C. D. (2004) Mol. Pharmacol. 66, 694–701
32. Ke, S., Rabson, A. B., Germinio, J. T., Gallo, M. A., and Tian, Y. (2001) J. Biol. Chem. 276, 39638–39644
33. Tian, Y., Rabson, A. B., and Gallo, M. A. (2002) Chem. Biol. Interact 141, 97–115
34. Abdulla, D., Goralski, K. B., Garcia Del Busto Cano, E., and Renton, K. W. (2005) Drug Metab Dispos. 33, 1521–1531
35. Goralski, K. B., Abdulla, D., Sinal, C. J., Arsenault, A., and Renton, K. W. (2005) Am. J. Physiol. Gastrointest Liver Physiol. 289, G343–G443
36. Wang, S., and Hankinson, O. (2002) J. Biol. Chem. 277, 11821–11827
37. Reischlag, T. V., Wang, S., Rose, D. W., Torchio, J., Reisz-Forszas, S., Muhammad, K., Nelson, W. E., Probst, M. R., Rosendall, M. G., and Hankinson, O. (2002) Mol. Cell. Biol. 22, 4319–4333
38. Wang, S., Ge, K., Roeder, R. G., and Hankinson, O. (2004) J. Biol. Chem. 279, 13593–13600
39. Bairoch, A., Apweiler, R., Wu, C. H., Barker, W. C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M. J., Natale, D. A., O’Donovan, C., Redaschi, N., and Yeh, L. S. (2005) Nucleic Acids Res. 33, 154–159