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Symposium Article

TRANSCRIPTIONAL SUPPRESSION OF CYTOCHROME P450 GENES BY ENDOGENOUS AND EXOGENOUS CHEMICALS

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ABSTRACT:

This article is an invited report of a symposium sponsored by the Division for Drug Metabolism of the American Society for Pharmacology and Experimental Therapeutics held at Experimental Biology 2003 in San Diego, California, April 11–15, 2003. Several members of the cytochrome P450 (P450) superfamily are induced after exposure to a variety of chemical signals, and we have gained considerable mechanistic insight into these processes over the past four decades. In addition, the expression of many P450s is suppressed in response to various endogenous and exogenous chemicals; however, relatively little is known about the molecular mechanisms involved. The goal of this symposium was to critically examine our current understanding of molecular mechanisms involved in transcriptional suppression of *CYP* genes by endogenous and exogenous chemicals. Specific examples were drawn from the following chemical categories: polycyclic and halogenated aromatic hydrocarbon environmental toxicants, inflammatory mediators, the endogenous sterol dehydroepiandrosterone and peroxisome proliferators, and bile acids. Multiple molecular mechanisms are involved in transcriptional suppression, and these processes often involve rather complex cascades of transcription factors and other regulatory proteins. Mechanistic studies of *CYP* gene suppression can enhance our understanding of how organisms respond to xenobiotics as well as to perturbations in endogenous chemicals involved in maintaining homeostasis.

The cytochromes P450 (P450s)[1] constitute a superfamily of hemoproteins that play key roles in the biotransformation of xenobiotics and endogenous chemicals. The xenobiotic substrates include therapeutic agents, dietary constituents and environmental toxicants, and several of the endogenous substrates are important signaling molecules that are involved in the regulation of homeostatic processes. Many *CYP* genes are inducible, meaning that exposure to appropriate chemical signals leads to elevated levels of expression of the encoded gene product, a catalytically active P450 protein. The study of P450 induction has a rich history, spanning the past 45 years (Conney, 2003). Since the pioneering discoveries in the 1950s and 1960s that polycyclic aromatic hydrocarbons (PAHs) and barbiturates increase hepatic drug-metabolizing capacity, considerable insight has been gained into the molecular mechanisms by which *CYP* gene induction occurs. We now understand a great deal about how up-regulation of *CYP* gene transcription often involves activation of various cytosolic or nuclear receptors, including the aromatic hydrocarbon receptor (AHR), the constitutive androstane receptor, the pregnane X receptor (PXR), and the peroxisome proliferator-activated receptor-α (PPARα) (Honkakoski and Negishi, 2000).

Expression of *CYP* genes can also be decreased after exposure to a variety of chemical and pathophysiological signals (Riddick et al., 2003). Compared with our knowledge of P450 induction, the mechanisms involved in P450 suppression are poorly understood. This is at least partly attributable to the analytical hurdle associated with measuring down-regulation of gene expression in a quantitative and reproducible manner. Whereas several inducible P450s are expressed in the basal state at nearly undetectable levels and the magnitude of

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[1] Abbreviations used are: P450, cytochrome P450; ADIOL, androst-5-ene-3,17-diol; ADIONE, androst-5-ene-3,17-dione; AHR, aromatic hydrocarbon receptor; BARE, bile acid response element; bp, base pair(s); CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DRE, dioxin-responsive element; FTF, α-fetoprotein transcription factor; FXR, farnesoid X receptor; GH, growth hormone; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; HNF, hepatocyte nuclear factor; IκB, inhibitory protein of nuclear factor-κB; IL, interleukin; JNK, cJun N-terminal kinase; LCA, lithocholic acid; LIP, liver-enriched transcriptional inhibitory protein; LPS, bacterial lipopolysaccharide; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; MC, 3-methylcholanthrene; NF-κB, nuclear factor-κB; PAH, polycyclic aromatic hydrocarbon; PCN, pregnenolone 16α-carbonitrile; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; SHP, small heterodimer partner; STAT, signal transducer and activator of transcription; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TF, transcription factor; VDR, vitamin D receptor.

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induction can be several hundred-fold, measurement of P450 suppression is limited to the narrow and finite window in which a gene can be down-regulated from its constitutive level. Nevertheless, studies of P450 suppression can help us to understand the mechanisms by which organisms respond to physiological and pathophysiological signals. As summarized previously (Morgan, 2001), P450 suppression may be: 1) a pathophysiological response to stress signals, e.g., toxicants, inflammation; 2) an adaptive homeostatic response, allowing for controlled generation of reactive oxygen species, nitric oxide, or arachidonic acid metabolites; or 3) part of a tightly regulated physiological pathway, e.g., bile acid production.

The goal of this symposium was to provide a view of our current state-of-the-art understanding of molecular mechanisms involved in transcriptional suppression of *CYP* genes by endogenous and exogenous chemicals. Presentations highlighted work on the following classes of chemical signals that trigger P450 down-regulation: polycyclic and halogenated aromatic hydrocarbon environmental toxicants, inflammatory mediators, the endogenous sterol dehydroepiandrosterone (DHEA) and peroxisome proliferators, and bile acids. Studies of *CYP* gene suppression by these and other chemical signals are helping to uncover the mechanisms involved in negative transcriptional regulation. As summarized previously (Clark and Docherty, 1993), these include: 1) direct inhibition of transcription initiation (silencing); 2) interference with the DNA binding of a transcription factor (TF) by another factor binding to an adjacent or overlapping DNA sequence (steric hindrance); 3) sequestration of a TF in an inactive form via protein-protein interactions; 4) sequestration of factors involved in transmitting an activating signal to the polymerase complex (squelching); or 5) recruitment of corepressors and modulation of the histone code and chromatin structure. Examples of many of these processes were explored in this symposium.

Positive and Negative Transcriptional Regulation of Cytochromes P450 by Polycyclic Aromatic Hydrocarbons (D.S.R., C.L., A.B., Y.E.T.)

Halogenated aromatic hydrocarbons (e.g., polychlorinated dioxins, dibenzofurans, and biphenyls) are ubiquitous and persistent environmental contaminants that pose potential health risks to humans and wildlife species. The prototypical compound in this category is 2,3,7,8-tetrachorodibenzo-*p*-dioxin (TCDD) (Fig. 1). Several nonhalogenated PAHs are genotoxic carcinogens that undergo P450-catalyzed bioactivation to reactive metabolites that bind covalently to DNA and other macromolecules. The prototypical compound in this category is the laboratory chemical 3-methylcholanthrene (MC) (Fig. 1). Most of the toxic and adaptive responses to aromatic hydrocarbons such as MC and TCDD are mediated by the AHR, a ligand-activated TF. The AHR normally resides in the cellular cytoplasm in a multiprotein complex with the 90-kDa heat shock protein and other chaperones. Ligand binding triggers transformation of the AHR into its activated nuclear form, which is a heterodimer consisting of the AHR and the AHR nuclear translocator. The AHR·AHR nuclear translocator heterodimer binds to specific DNA enhancer sequences known as dioxin-responsive elements (DREs), generally located in the 5′-flanking region of multiple genes including *CYP1A1*. In this manner, exposure to aromatic hydrocarbons results in an increased rate of transcription of *CYP1A1* and several other genes encoding drug-metabolizing enzymes.

The role that the AHR plays in the induction of drug- and carcinogen-metabolizing enzymes such as CYP1A1 is well established. However, the expression of a number of genes of biological and toxicological significance is decreased in response to these chemicals (Riddick et al., 2003). Several genes that are down-regulated play

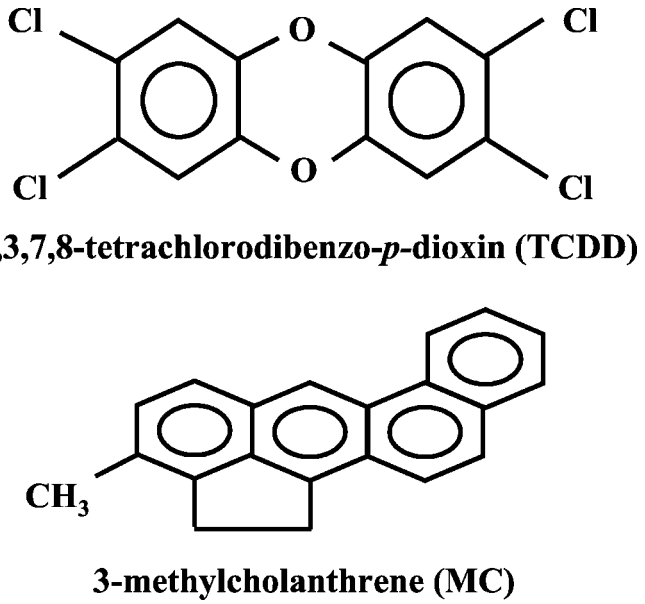


FIG. 1. *Structures of 2,3,7,8-tetrachlorodibenzo-p-dioxin and 3-methylcholanthrene.*

important roles in cellular growth and differentiation and may be important targets for the toxic effect of aromatic hydrocarbons (e.g., epidermal growth factor receptor, estrogen receptor, pS2, cathepsin D). As a model system, we have been examining the mechanisms by which aromatic hydrocarbons cause suppression of constitutive hepatic P450 enzymes, especially the predominant male-specific rat liver enzyme CYP2C11. The *CYP2C11* gene has been a focus for work on the endocrine control of drug and steroid metabolism because of its interesting sex-specific, hormone-modulated, and developmental regulation.

We (Jones and Riddick, 1996) and others (Yeowell et al., 1987; Shimada et al., 1989) showed that in vivo administration of MC to adult male rats caused suppression of hepatic CYP2C11 at the catalytic activity, protein, and mRNA levels. These results suggested that CYP2C11 is regulated by MC at a pretranslational level. Nuclear run-on analysis provided the first direct demonstration that the decrease in hepatic CYP2C11 mRNA caused by in vivo administration of MC to male rats is at least partially caused by a decreased rate of transcription (Lee and Riddick, 2000). We are particularly interested in determining whether the AHR mediates this negative transcriptional response. Our structure-activity relationship study with a series of anthracene derivatives showed that affinity for binding to the AHR and the potency for transforming the AHR to its DNA-binding form correlated with the ability of these compounds to down-regulate CYP2C11 protein expression in primary rat hepatocytes cultured on Matrigel (BD Biosciences, Bedford, MA) (Safa et al., 1997). In the same hepatocyte system, TCDD decreased the level of CYP2C11 mRNA without altering the half-life of the message, providing additional support for an effect on transcription (Bhathena et al., 2002).

In exploring a direct AHR-mediated mechanism of CYP2C11 down-regulation, we showed by electrophoretic mobility shift analysis that the AHR binds to a DRE-like sequence in the *CYP2C11* 5′-flank, located at position −1558 to −1539 relative to the transcription start site (Bhathena et al., 2002). However, in vitro DNase footprinting using hepatic nuclear extracts prepared from MC-treated rats did not reveal significant alterations in protein binding to several regions of the *CYP2C11* 5′-flank (Bhathena et al., 2002). We also developed luciferase reporter constructs containing specific segments of the

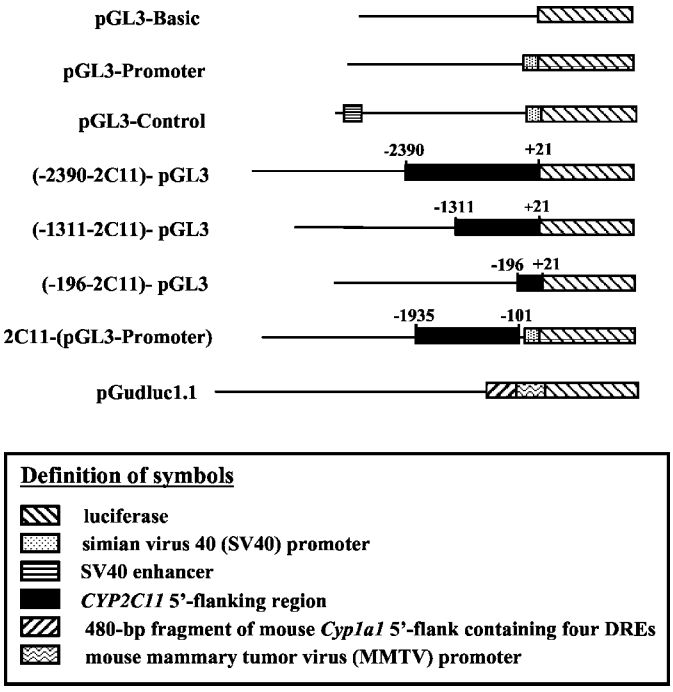


FIG. 2. *Schematic diagram of the luciferase reporter constructs used in transfection assays to study the regulation of CYP2C11 by aromatic hydrocarbons.*

Of direct relevance to the findings discussed in this article, we have identified and characterized a DRE-like sequence at position −1558 to −1539 (Bhathena et al., 2002) and a STAT5b-binding site at position −1179 to −1171 (Timsit and Riddick, 2002), relative to the *CYP2C11* transcription start site.

CYP2C11 5′-flank and promoter, or a defined region of the *CYP2C11* 5′-flank and a heterologous promoter. As shown schematically in Fig. 2, we studied two constructs that encompassed the putative DRE-like motif [(−2390-2C11-pGL3 and 2C11-(pGL3-Promoter)] and two constructs that did not [(−1311-2C11)-pGL3 and (−196-2C11)-pGL3]. However, the *CYP2C11* 5′-flank did not confer down-regulation in response to TCDD in transient transfection studies carried out in mouse, rat, and human liver-derived cell lines (Bhathena et al., 2002). The same reporter constructs were also not down-regulated by TCDD in transfection studies in primary rat hepatocytes cultured on Matrigel, under conditions in which TCDD decreased expression of endogenous CYP2C11 mRNA and the inflammatory cytokine interleukin (IL)-1β decreased both CYP2C11 mRNA and luciferase reporter expression (Bhathena et al., 2002). In all of these transfection experiments, the positive control reporter plasmid pGudluc1.1 (Fig. 2), which is under the control of four DREs from the mouse *Cyp1a1* gene, showed a robust induction (11- to 15-fold in primary hepatocytes and 13- to 214-fold in continuous cell lines) in response to TCDD, indicating that AHR was activated under these conditions. These studies suggest that aromatic hydrocarbons suppress *CYP2C11* by a negative transcriptional mechanism, not simply due to AHR binding to an identified DRE-like sequence. This mechanism is distinct from that used by inflammatory cytokines. We propose to follow up these studies in the following ways: 1) examine other regions of the *CYP2C11* gene for regulatory elements; 2) study protein-DNA interactions in a native chromosomal context via ligation-mediated polymerase chain reaction-based in vivo footprinting; and 3) study regulation of luciferase reporter plasmids in living rats.

The primary physiological regulator of hepatic *CYP2C11* is the male-specific pulsatile pattern of growth hormone (GH) secretion. In exploring an indirect endocrine-disrupting mechanism of *CYP2C11* suppression, we showed for the first time that MC interferes with the ability of GH to stimulate hepatic expression of CYP2C11 at the mRNA, protein, and catalytic activity levels in hypophysectomized male rats (Timsit and Riddick, 2000). A similar pituitary component was noted for the suppression of CYP2C11 by the simple aromatic hydrocarbon ethylbenzene (Serron et al., 2001). Our observation stimulated investigation of the molecular mechanisms by which aromatic hydrocarbons may interfere with hepatic GH signaling via a *Janus* kinase-signal transducer and activator of transcription (STAT) pathway. Although GH activates multiple intracellular signaling pathways in hepatocytes, there is substantial evidence that signaling via STAT5b is instrumental in mediating the sexually dimorphic effects of pulsatile GH on hepatic P450 expression (Park and Waxman, 2001). There is also precedent that foreign chemicals such as ethanol (Badger et al., 2003) and peroxisome proliferators (Shipley and Waxman, 2003) can interfere with GH signaling via STAT5b. Our in vivo work with hypophysectomized male rats demonstrated that MC does not interfere with the ability of GH to trigger STAT5b phosphorylation, nuclear localization, and binding to a canonical STAT5b DNA recognition sequence (Timsit and Riddick, 2002). Similarly, in vitro studies with the H4IIE rat hepatoma cell line showed a similar lack of effect of MC on GH-stimulated STAT5b nuclear localization and DNA binding (Timsit and Riddick, 2002). These results suggest that PAHs interfere with the ability of GH to stimulate hepatic *CYP2C11* expression; however, whether the STAT5b signaling pathway is important in this response requires further investigation. We propose to follow up these studies in the following ways: 1) examine other GH signaling pathways; 2) study the *trans*-activation function of STAT5b following aromatic hydrocarbon exposure; and 3) study regulation of other GH-regulated, STAT5b-dependent genes, e.g., mouse *Cyp2d9*.

The overall goal of these molecular mechanistic studies is to improve our understanding of how environmental contaminants of toxicological significance modulate the expression of genes encoding xenobiotic- and hormone-metabolizing enzymes.

Down-Regulation of Cytochromes P450 by Inflammatory Mediators (P.-Y.C., E.T.M.).

It has long been known that infectious or inflammatory stimuli elicit the down-regulation of a number of different hepatic P450 mRNAs and proteins (Morgan, 2001; Renton, 2001). The reason for this down-regulation is unclear, but it leads to an impairment of drug clearance that has obvious consequences for human therapy, particularly for drugs with a low therapeutic index. Although inflammation causes down-regulation of some P450s, not all P450 enzymes are suppressed. For example, members of the CYP4A subfamily are induced in rat liver in response to inflammation caused by injection of bacterial lipopolysaccharide (LPS) (Sewer et al., 1996).

Transcriptional and Post-Transcriptional Mechanisms. A major component of inflammation-evoked P450 down-regulation is pretranslational, since the reductions in P450 proteins are usually preceded by decreases in the corresponding mRNAs (Sewer et al., 1996). The ensuing discussion will be limited to pretranslational mechanisms, but it should be noted that kinetic evidence for post-translational regulation also exists. For example, we have found that CYP2B proteins are down-regulated more rapidly than CYP2B mRNAs in rat hepatocytes stimulated with LPS (Ferrari et al., 2001).

There are two potential mechanisms for down-regulation of hepatic P450 mRNAs consequent to inflammatory stimulation: suppression of transcription, and destabilization of P450 mRNAs. Despite the fact that the phenomenon of hepatic P450 mRNA suppression by inflammatory stimuli has been known for almost 15 years, there is little direct evidence to support either mechanism. The first evidence for

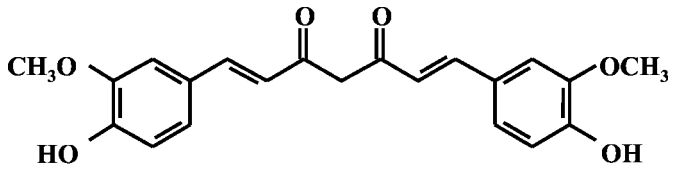
regulation of endogenous *CYP* genes by inflammatory stimuli was provided by Barker et al. (1992), who showed that monocyte-conditioned medium or IL-1 could suppress the TCDD-induced transcription of *CYP1A1* and *1A2* in cultured rat hepatocytes. This effect was subsequently demonstrated to be due to interaction of the AHR with the inflammatory TF, nuclear factor-κB (NF-κB), preventing the receptor from activating the *CYP1A* genes (Ke et al., 2001).

Using transcription run-on assays, we showed that transcription of *CYP2C11* was down-regulated to 5% and 23% of control levels in male rat livers 24 h after treatment with LPS or turpentine, respectively (Wright and Morgan, 1990). We subsequently demonstrated, using reporter gene assays in transfected rat hepatocytes, that ~200 bp of the *CYP2C11* promoter could confer transcriptional suppression by IL-1, IL-6, or LPS to 40 to 60% of control rates (Chen et al., 1995). In contrast, we found that the sterile irritant turpentine did not significantly affect transcription of *CYP2C12* in female rat liver (Wright and Morgan, 1990). We speculated that the lack of effect might in this case be due to a lack of probe specificity because the same probe failed to show sex-specific transcription of *CYP2C12*, whose mRNA is expressed specifically in female rat liver (Wright and Morgan, 1990). This explanation is probably correct, since subsequent studies showed that *CYP2C12* transcription is indeed sex-specific in rat liver (Legraverend et al., 1992).

Even in those studies in which regulation of endogenous *CYP* transcription has been demonstrated, transcription rates were measured after 24 h of treatment with inflammatory stimuli (Wright and Morgan, 1990; Barker et al., 1992). Since hepatic CYP2C11, 2E1, and 3A2 mRNAs are down-regulated to 5 to 25% of control levels within only 6 h of LPS treatment in rats (Sewer et al., 1996), the existing transcription data cannot discriminate between a primary and a secondary role for transcriptional suppression in the observed effects on the mRNAs. For this reason, we initiated a study to determine whether or not: 1) LPS alters transcription of multiple *CYP* genes whose mRNAs are known to be down-regulated in rat livers; and 2) the transcriptional suppression is of sufficient rapidity and magnitude to contribute significantly to the rapid down-regulation of the mRNAs. Rats were injected i.p. with 1 mg/kg *Escherichia coli* LPS, and their livers were harvested 1 h later for analysis. Hepatic nuclei were prepared, and effects of LPS treatment on the transcription rates of the *CYP2C11*, *2E1*, and *3A2* genes were compared with those of saline injection using nuclear run-on transcription assays. We found that transcription of all three *CYP* genes was suppressed by 70 to 90% only 1 h after LPS injection (Cheng et al., 2003). A second experiment in which transcription was measured 2 h after LPS injection confirmed these observations (Cheng et al., 2003).

These novel findings clearly support the hypothesis that transcriptional suppression is a primary mechanism for down-regulation of hepatic P450 mRNAs in the acute phase of infection and inflammation, and provide a rationale for studying the mechanism of this transcriptional suppression. It should be noted, however, that even this rapid and substantial transcriptional suppression is unlikely to fully explain the 75 to 95% in vivo down-regulation of P450 mRNAs within 6 h of LPS treatment (Sewer et al., 1996). This is much faster than can be explained by the measured half-lives of CYP2C11 and 2E1 mRNAs in cultured rat hepatocytes (Peng and Coon, 1998; Iber et al., 2001). This kinetic consideration strongly suggests that these hepatic P450 mRNAs are destabilized under conditions of inflammation or infection. This is a neglected area of research that deserves further study.

Effect of Curcumin on P450 Transcription. The demonstration that LPS treatment causes rapid transcriptional suppression of *CYP2C11*, as well as other *CYP* genes, prompted us to investigate whether this model could be used to study the mechanisms of transcriptional *CYP* suppression in vivo. We previously found that the *CYP2C11* gene contains a low-affinity binding site for NF-κB, and that mutation of this site blocked the down-regulation of a reporter gene by IL-1 and/or LPS (which activate NF-κB), but not by IL-6 (which does not activate NF-κB) (Iber et al., 2000). Therefore, we wanted to determine whether inhibition of NF-κB activation in rat liver could prevent the rapid suppression of *CYP2C11* gene transcription in vivo. NF-κB activation in response to inflammatory cytokines is achieved via the stimulated phosphorylation and subsequent proteasomal degradation of the inhibitory protein of NF-κB (IκB), a protein that binds and sequesters NF-κB in the cytoplasm. Curcumin (diferuloylmethane; Fig. 3) has been shown to prevent NF-κB activation in cells, probably by inhibiting IκB kinase (Jobin et al., 1999). In initial experiments, we established a protocol for optimum inhibition of hepatic NF-κB activation by curcumin. We then used this protocol to examine the role of NF-κB in the rapid suppression of *CYP2C11, 2E1,* and *3A2* transcription by LPS treatment. Injection of 1 mg/kg curcumin 1 h before LPS injection caused a substantial (but incomplete) inhibition of NF-κB activation in the liver. Treatment with curcumin alone did not affect *CYP* gene transcription, but when given 1 h before LPS injection, curcumin blocked the suppression of *CYP3A2* and partially blocked the suppression of *CYP2E1* (Cheng et al., 2003). However, these effects were apparently not related to inhibition of NF-κB activation by curcumin, because curcumin treatment failed to block the activation of angiotensinogen (Cheng et al., 2003), a known NF-κB-regulated gene. Although the mechanisms by which curcumin produced these effects are unknown, this observation indicates that curcumin may be a useful tool to investigate the mechanisms of suppression of *CYP3A2* and *CYP2E1*.



curcumin

FIG. 3. *Structure of curcumin.*

Interestingly, curcumin treatment did not significantly affect the suppression of *CYP2C11* transcription in this model. However, since curcumin also did not block the activation of the NF-κB-regulated gene, angiotensinogen, the degree of NF-κB inhibition produced by curcumin was obviously not sufficient to produce significant functional consequences. Therefore, the role of NF-κB in *CYP2C11* regulation remains to be determined.

Roles of Specific TFs. Recently, Jover et al. (2002) reported that increased liver-enriched transcriptional inhibitory protein (LIP), an N-terminal-truncated fragment of the CCAAT/enhancer binding protein-β, was responsible for the suppression of CYP3A4 mRNA by IL-6. Therefore, we investigated whether or not LIP abundance in rat liver nuclei was increased in response to LPS treatment. We found that nuclear LIP abundance was not significantly affected at 1 h after LPS injection, at a time when *CYP3A2* transcription was suppressed by about 70% (Cheng et al., 2003). Therefore, we concluded that LIP induction is not the cause of rapid *CYP* gene suppression by LPS, although it could potentially be important for sustained suppression at later time points. We also examined the abundance and DNA-binding activity of hepatocyte nuclear factor (HNF)-1α, which was proposed to be important for the down-regulation of *CYP2E1* by LPS (Roe et

al., 2001). We found that nuclear abundance of HNF-1α in rat liver was unaffected 1 h after LPS injection, whereas its DNA-binding activity was reduced by 27% (Cheng et al., 2003). This decrease is much smaller than the magnitudes of transcriptional suppression of *CYP2E1*, *3A2*, or *2C11* observed at the same time point. Therefore, decreased activity of HNF-1α alone cannot explain the observed effects on *CYP* transcription, although it may contribute, together with other changes in TF activities. We are currently investigating the effects of LPS treatment on other TFs that are important in basal *CYP* transcription.

Dehydroepiandrosterone Is Metabolized by and Modulates Expression of Multiple Cytochromes P450 (R.A.P., S.L.R., K.K.M.M.)

DHEA is a 19-carbon sterol formed in the human adrenal from cholesterol by a series of P450 monooxgenase and hydroxysteroid dehydrogenase-dependent reactions (Conley and Bird, 1997). In its sulfated form (DHEA-S), DHEA is the major circulating sterol that serves as a precursor to the sex steroids, estrogen and testosterone. Secretion of DHEA and DHEA-S from the adrenal varies widely throughout human life and initially is high in the fetus to provide estrogen precursor for the placenta. In neonatal life its secretion from the adrenal is low and rises in the second decade of life when secondary sexual characteristics are being developmentally fixed. There is a pronounced age-dependent decline for individuals aged 30 or more, which is strikingly different from other steroid hormones, suggesting that the mechanisms regulating DHEA formation in the adrenal are unique (Rainey et al., 2002).

In 1989, our laboratory demonstrated that DHEA feeding in rats and mice led to a number of biochemical alterations, including peroxisome proliferation and changes in the levels of several P450s and related enzyme activities, such as NADPH-cytochrome P450 oxidoreductase and palmitoyl CoA oxidase (Wu et al., 1989). The oxidoreductase was induced approximately 2-fold at concentrations above 50 mg/kg body weight, whereas total P450 levels were largely unaffected. Fatty acyl CoA oxidase activity increased 20- to 50-fold. CYP4A1 activity, protein, and mRNA were induced between 17- and 20-fold. Using androstenedione and testosterone as substrates, we observed that whereas overall sterol oxidation was not greatly different between liver microsomal fractions from control and DHEA-fed rats, there were pronounced changes in the metabolites formed. For androstenedione, 16α-hydroxylase activity decreased by over 80%, 16β-hydroxylase activity increased by 6- to 7-fold, and 6β-hydroxylase activity was unaffected. Similar results were observed when testosterone was used as a substrate; namely, 6β- and 16β-hydroxylase activities increased 2- to 6-fold, whereas 2α/β-hydroxylase and 16α-hydroxylase activities decreased by 70 to 80%. These results suggest that at least three differentially regulated steroid hydroxylase activities exist in rat liver microsomal fractions; one activity increases, one activity decreases, and a third remains constant. Clearly, expression of several P450s is altered by feeding DHEA, some induced and some repressed.

Our subsequent studies have addressed species differences in DHEA metabolism and how DHEA and its metabolites affect P450 expression. We have studied the metabolism of DHEA by liver microsomal fractions from rat, hamster, human, and pig and noted that there are considerable differences in metabolism of the sterol among species, in particular, when compared with human, a species that produces DHEA (Fitzpatrick et al., 2001; Michael Miller et al., 2004). For example, 7α- and 16α-hydroxylase activities for DHEA were observed in rodents and pig, whereas humans produced 7β-hydroxy-DHEA, in addition to 7α- and 16α-hydroxylated products. The rates of metabolism were also relatively high in rat, hamster, and at least one human (7–10 nmol/min/mg protein). Longer time courses of metabolism also produced 7-oxo-DHEA as a metabolite, involving interconversion of 7α- and 7β-hydroxy-DHEA and 7-oxo-DHEA (Fitzpatrick et al., 2001; Robinzon et al., 2003). Using purified P450s, we determined that rat CYP3A23 produces 7α-hydroxy-DHEA, whereas rat CYP2B1, 2C11, and 2D1 preferentially produce 16α-hydroxy-DHEA, but not 7-oxidized metabolites (Michael Miller et al., 2004). Human CYP3A4/5 produced 7α-hydroxy-, 7β-hydroxy-, and 16α-hydroxy-DHEA, whereas CYP3A7 forms only 16α-hydroxy-DHEA and low amounts of 7β-hydroxy-DHEA. Other human P450s (CYP2A6, 2B6, 2C8, 2C9, 2C19, and 2D6) did not metabolize DHEA appreciably (Michael Miller et al., 2004). The metabolite profiles discussed above suggest that, across species, even P450s with significant sequence identity display very diverse stereoselective and regioselective metabolism of this sterol.

Since little is known about whether DHEA or its oxidative metabolites have individual biological action, we studied their role in regulation of gene expression using transiently transfected reporter genes in HepG2 cells. Like others, we could show that peroxisome proliferators, such as nafenopin, and fatty acids can activate the murine PPARα in HepG2 cells, but that neither DHEA nor any of its metabolites could activate PPARα in this system. However, when we tested the role of DHEA and its metabolites in activating the PXR, we noted that DHEA and both its cytosolic metabolites, androstenedione (ADIONE; androst-5-ene-3,17-dione) and androstenediol (ADIOL; androst-5-ene-3,17-diol) could activate this receptor; however, ADIONE and ADIOL were better activators than DHEA. We had previously demonstrated that in vivo, ADIOL is a better inducer of *CYP4A1* than is DHEA; ADIONE is not a good activator of PPARα in rat hepatocytes. However, all three compounds induce CYP3A23 protein and mRNA expression in primary rat hepatocytes, whereas only DHEA and ADIOL induce CYP4A1 protein and mRNA expression (X.-D. Lei and R. A. Prough, unpublished observations). Peters et al. (1996) demonstrated that DHEA-S does not induce *Cyp4a* or fatty acyl CoA oxidase in PPARα-null mice, but Ripp et al. (2002) demonstrated that *Cyp3a11* induction by DHEA is not altered in PPARα-null mice, clearly showing the distinct role of PXR in DHEA induction of various genes in mice. Ripp et al. (2003) also demonstrated that DHEA feeding suppresses expression of rat CYP2C11 protein and mRNA.

The negative regulation of the *CYP2C11* gene by a variety of signals may involve disruption of the stimulatory effect of pulsatile GH on this gene. STAT5b is a key TF in the GH-dependent regulation of *CYP2C11*, and it appears that STAT5b is a component in a complex cascade of liver-enriched TFs involving inhibitory cross-talk between STAT5b and HNF-3β (Park and Waxman, 2001). Activation of the PPARα causes down-regulation of several GH-regulated hepatic genes including *CYP2C11* (Corton et al., 1998), and it appears that inhibition of STAT5b *trans*-activation function by ligand-activated PPARα may be an important mechanism in this response (Shipley and Waxman, 2003). As discussed in greater detail in a previous section, Morgan and coworkers (Wright and Morgan, 1990; Chen et al., 1995) have shown that ILs negatively regulate *CYP2C11* expression as well. Negative regulation in response to inflammatory cytokines such as IL-1β is thought to be due to a NF-κB site that overlaps the transcriptional start site (Iber et al., 2000). A glucocorticoid-response element half-site (-149 to -144, relative to the transcription start site) has also been identified, although its functional significance is unclear (Morishima et al., 1987; Strom et al., 1994). As discussed in greater detail in a previous section, environmental contaminants of the

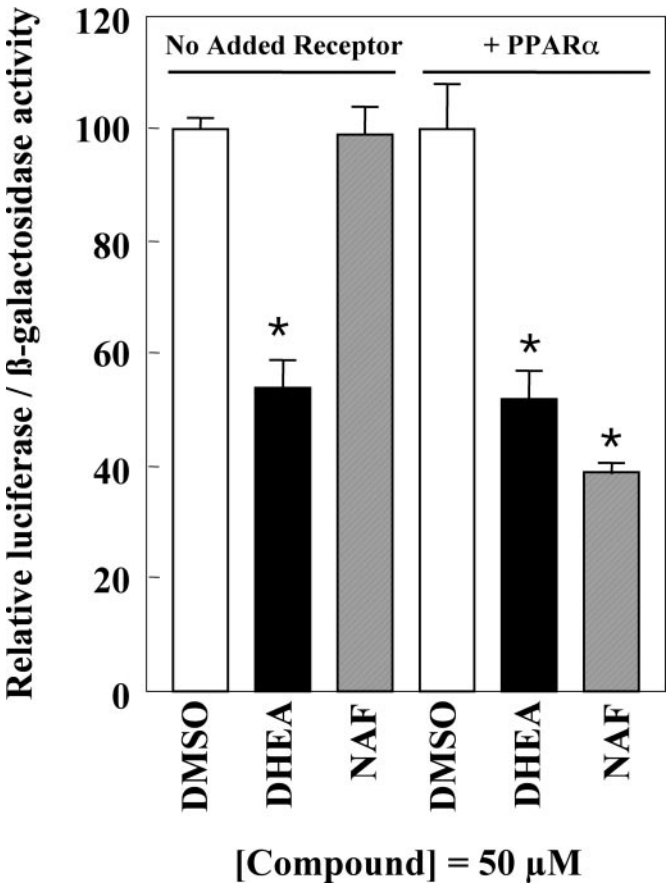


FIG. 4. *PPARα is not required for negative regulation of a CYP2C11-luciferase construct by DHEA.*

HepG2 cells were transiently transfected with 1300/2C11-Luc reporter plasmid that includes a portion of the 5′-flanking region of *CYP2C11* (−1287 to + 25, relative to the transcription start site) and pCMVβ in the absence and presence of a PPARα expression vector, and treated 24 h later with either dimethyl sulfoxide (DMSO), DHEA, or nafenopin (NAF). Each bar represents the mean ± S.D. of three experiments in triplicate. ⋆, significantly different ($p < 0.05$) from the respective dimethyl sulfoxide control.

aromatic hydrocarbon class also suppress *CYP2C11* (Bhathena et al., 2002).

To establish the mechanism of negative regulation of the rat *CYP2C11* gene, we conducted a series of experiments using a *CYP2C11*-luciferase reporter construct, 1300/2C11-Luc (containing positions −1287 to + 25, relative to the transcription start site), transiently transfected into HepG2 cells. DHEA treatment resulted in negative regulation of this gene, even in the absence of coexpressed murine PPARα (Fig. 4). However, with a peroxisome proliferator such as nafenopin, PPARα coexpression was required for negative regulation. A series of deletion and mutation analyses demonstrated that the responsive region for negative regulation by both DHEA and nafenopin was between −108 and −60 relative to the transcription start site; the most effective 2-bp mutation was at position −75. These studies also identified a sequence at −121 bp from the transcription start site as a perfect nuclear receptor-binding half-site (AGGTCA), which matches the consensus sequence for nuclear receptor binding (Mangelsdorf et al., 1995). This site appears to also be involved in constitutive expression of the *CYP2C11* reporter in HepG2 cells. These results suggest that DHEA may act through a signaling system different from PPARα to negatively regulate *CYP2C11* expression. Gel shift assays utilizing in vitro transcription and translation of expression plasmids for PPARα (commercially available kit from Promega, Madison, WI) demonstrated binding of PPARα/retinoid X receptor-α heterodimers to the peroxisome proliferator-responsive element of rat fatty acyl CoA oxidase, but not to the negative regulatory region or the constitutive regulatory region of *CYP2C11* (Ripp et al., 2003).

We have identified several other genes that are regulated by DHEA in vivo but are not regulated by nafenopin, a known peroxisome proliferator (Gu et al., 2003). Studies are in progress to identify the mechanism of this regulatory system for DHEA action, which appears not to involve PPARα. This process may explain some of the other biological actions of DHEA or its metabolites.

Bile Acid and Nuclear Receptor Regulation of Cytochrome P450 Gene Transcription (A.J., J.Y.L.C.)

Bile acids are the end products of cholesterol catabolism. About 15 enzymes are involved in catalyzing steroid ring modifications and side chain oxidation and cleavage to form the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) (Chiang, 1998). Four P450 enzymes play regulatory roles in bile acid synthesis in the liver. Microsomal cholesterol 7α-hydroxylase (CYP7A1) is the first and rate-limiting enzyme of the classic bile acid biosynthetic pathway to synthesize CA and CDCA in humans (Fig. 5). Microsomal sterol 12α-hydroxylase (CYP8B1) is involved in synthesis of CA. Mitochondrial sterol 27-hydroxylase (CYP27A1) catalyzes a sterol side chain oxidation reaction in the classic pathway and also hydroxylates cholesterol to 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, mainly in peripheral tissues and macrophages. Microsomal oxysterol 7α-hydroxylase (CYP7B1) converts these metabolites to 3β,7α-dihydroxy-5-cholestenoic acid, which may be converted to bile acids in the liver. Bile acids synthesized in the liver are conjugated with glycine or taurine and excreted into the bile. In the intestine, CA and CDCA are converted to the secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), respectively, by 7α-dehydroxylase in the bacterial flora. Bile acids emulsify dietary fats, cholesterol, and lipid-soluble vitamins for absorption and transport to the liver via the portal circulation. About 95% of bile acids are reabsorbed in the intestine, and the 5% lost in the feces are replenished by synthesis from cholesterol in the liver. Conversion of cholesterol to bile acids accounts for 90% of daily cholesterol catabolism; the remaining cholesterol is used for synthesis of steroid hormones. Hence, bile acid synthesis is the major route for cholesterol disposal and plays a pivotal role in regulation of cholesterol homeostasis.

Bile acid synthesis is feedback inhibited by bile acids returning to the liver via enterohepatic circulation. Much experimental evidence suggests that bile acids inhibit bile acid synthesis by inhibiting *CYP7A1* gene transcription. Transient transfection assays of *CYP7A1*-luciferase reporters in HepG2 cells were used to identify regions conferring bile acid repression. Two bile acid response elements (BARE-I and BARE-II) have been identified in rat and human *CYP7A1* gene promoters (Chiang, 2003). These BAREs contain several AGGTCA-like repeating sequences, which are potential binding sites for nuclear receptors; AGGTCA is the consensus half-site for nuclear receptor binding (Mangelsdorf et al., 1995). The BARE-II sequences in the rat and human *CYP7A1* are highly conserved. They contain an overlapping HNF-4 and α-fetoprotein TF (FTF; also known as *CYP7A1* promoter factor and liver-related homolog) binding site. The BARE-I in the rat gene contains a liver X receptor (LXR) binding site, which is absent in the human gene. It has been demonstrated that LXR induces *Cyp7a1* gene transcription to convert excess cholesterol to bile acids in mice fed a high cholesterol diet (Lehmann et al., 1997). This mechanism is absent in humans due to the lack of a LXR binding site in the human *CYP7A1* gene (Chiang et al., 2001;

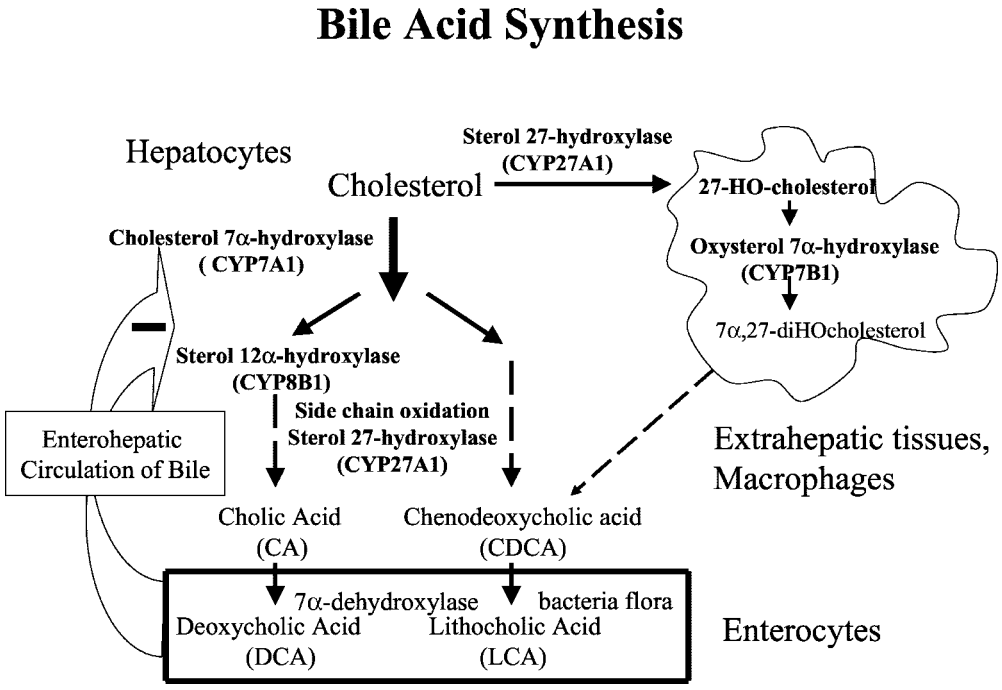


FIG. 5. *Bile acid biosynthetic pathways.*

The classic pathway is initiated by CYP7A1 to synthesize two primary bile acids, CA and CDCA, in the liver. CYP8B1 is involved in the synthesis of CA. CYP27A1 catalyzes a steroid side chain oxidation reaction in the classic pathway and also initiates the alternative (acidic) pathway in peripheral tissues and macrophages. CYP7B1 is involved in the acidic pathway for synthesis of CDCA. CA and CDCA are converted to DCA and LCA in the intestine by 7α-dehydroxylase in the bacterial flora. Bile acids are reabsorbed in the ileum and transported back to the liver via the portal circulation and inhibit *CYP7A1* and *CYP8B1* gene transcription.

Agellon et al., 2002). A receptor-mediated mechanism for bile acid inhibition of the *CYP7A1* gene has been proposed (Chiang, 2002). This model is supported by the recent identification of the farnesoid X receptor (FXR) as a bile acid receptor (Forman et al., 1995; Makishima et al., 1999; Wang et al., 1999). Cotransfection with FXR and retinoid X receptor expression plasmids enhanced the bile acid inhibitory effect by 80%. However, FXR does not bind to the BAREs. It was suggested that FXR inhibited the *CYP7A1* gene by an indirect mechanism, and liver-specific TFs other than FXR must be involved (Chiang et al., 2000). It appears that bile acid-activated FXR induces a negative nuclear receptor, small heterodimer partner (SHP), which subsequently interacts with FTF and inhibits its *trans*-activation of the *CYP7A1* gene (Fig. 6) (Goodwin et al., 2000). The *CYP8B1* gene is also extensively inhibited by bile acids. However, cotransfection with FXR does not enhance the inhibitory effect of bile acids (Zhang and Chiang, 2001). It seems that bile acids regulate the *CYP8B1* gene differently from the *CYP7A1* gene. The BARE identified in the human *CYP8B1* gene also contains HNF-4 and FTF binding sites. Results indicate that HNF-4 plays a much more important role than FTF in basal transcription of the human *CYP8B1* gene (Zhang and Chiang, 2001). Mutation of the HNF-4 site abolished bile acid inhibition of *CYP8B1* gene transcription, suggesting that HNF-4 also plays a role in mediating bile acid repression. SHP interacts with HNF-4 and inhibits *CYP8B1* gene transcription. However, the interaction between SHP and HNF-4 is much weaker than that with FTF. Other mechanisms may play a more important role in bile acid inhibition of *CYP8B1* gene transcription. These include bile acid inhibition of HNF-4 binding to DNA by decreasing HNF-4 protein, mRNA, and gene transcription (Zhang and Chiang, 2001).

More recent studies identified PXR and the vitamin D receptor (VDR) as bile acid-activated receptors (Staudinger et al., 2001). PXR is a promiscuous steroid and xenobiotic receptor. Previous studies demonstrated that pregnenolone 16α-carbonitrile (PCN) and dexamethasone strongly inhibited CYP7A1 activity, mRNA, and protein expression in rat liver (Li et al., 1990). These synthetic steroids are PXR ligands. In PXR knockout mice, CYP7A1 mRNA expression is not inhibited by PCN, indicating that PXR may mediate PCN inhibition of *Cyp7a1* gene transcription (Staudinger et al., 2001). PXR induces CYP3A, CYP2B, and CYP2C subfamilies of drug-metabolizing P450 enzymes in the liver and intestine (Kliewer et al., 2002). CYP3A4 converts CDCA and LCA to hyocholic acid (HCA) and

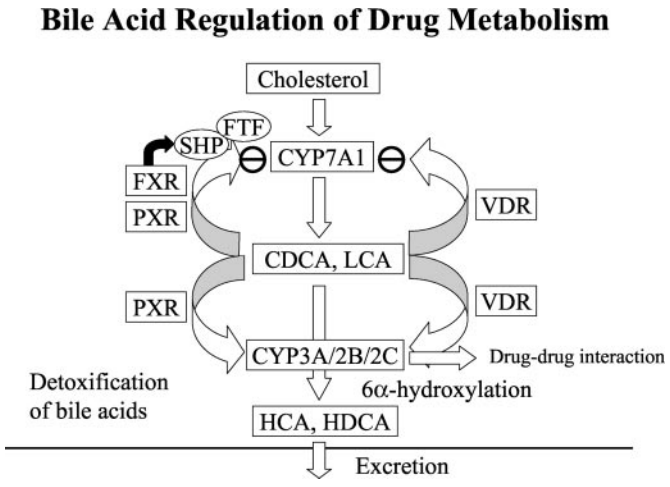


FIG. 6. *Bile acid regulation of drug metabolism.*

Bile acids are ligands of three nuclear receptors: FXR, PXR, and VDR. FXR inhibits *CYP7A1* gene transcription by an indirect mechanism; FXR induces SHP to interact with FTF and inhibit *CYP7A1* gene transcription. In the liver and intestine, PXR and VDR induce CYP3A, CYP2B, and CYP2C subfamilies of cytochrome P450 enzymes to catalyze 6α-hydroxylation of CDCA and LCA to HCA and HDCA, respectively. CYP3A metabolizes 60% of the drugs in clinical use and is involved in important drug-drug interactions.

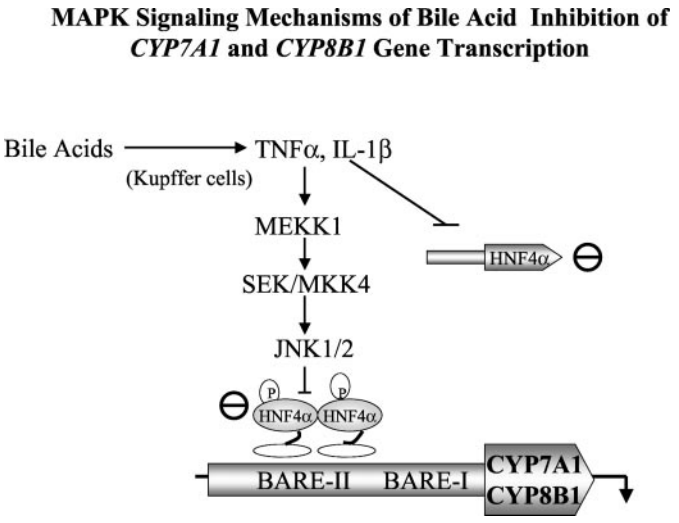


FIG. 7. *MAPK signaling mechanism of bile acid inhibition of CYP7A1 and CYP8B1 gene transcription.*

Bile acids induce the inflammatory cytokines tumor necrosis factor-α (TNFα) and IL-1β in hepatic macrophages (Kupffer cells) to stimulate MAPK signaling pathways leading to phosphorylation of JNK. JNK may phosphorylate HNF-4 and inhibit *trans*-activation of *CYP7A1* and *CYP8B1* gene transcription. Cytokines may also decrease HNF-4 protein, mRNA, and gene transcription and thereby inhibit transcription of genes under HNF-4 control.

hyodeoxycholic acid (HDCA), respectively. HCA and HDCA are soluble and nontoxic, and are excreted into urine. It has been suggested that when hydrophobic bile acids (e.g., CDCA and LCA) accumulate in the liver in cholestasis and other liver diseases, PXR is activated to detoxify these toxic bile acids, thus protecting the liver from the toxicity of bile acids. VDR is a specific LCA receptor. VDR also induces *CYP3A4* in the intestine and may protect the colon against bile acid-induced proliferation (Makishima et al., 2002).

In FXR knockout mice, CYP7A1 mRNA expression is not repressed and SHP mRNA is not induced by CA; this is consistent with SHP inhibition of *Cyp7a1* gene transcription (Sinal et al., 2000). However, in SHP knockout mice, CYP7A1 mRNA expression is inhibited by CA (Wang et al., 2002). One possible explanation for this discrepancy is that redundant mechanisms may exist to suppress the *Cyp7a1* gene in SHP knockout mice. Several FXR/SHP-independent mechanisms of bile acid feedback inhibition have been suggested (Chiang, 2002). PXR and VDR may suppress *CYP7A1* by SHP-independent mechanisms. PXR and VDR may bind to the BARE and directly inhibit *CYP7A1* gene transcription. Bile acids are known to induce inflammatory cytokines (tumor necrosis factor-α and IL-1β) in hepatic macrophages (Miyake et al., 2000), which stimulate mitogen-activated protein kinase (MAPK) signaling pathways leading to the activation of cJun N-terminus kinase (JNK) (Gupta et al., 2001) (Fig. 7). JNK may inhibit *CYP7A1* gene transcription by phosphorylation of TFs such as HNF-4. Since *CYP8B1* gene transcription is highly regulated by HNF-4, MAPK signaling pathways may play important roles in mediating bile acid feedback inhibition of *CYP8B1* gene transcription. IL-1β induces JNK, which may inhibit *CYP8B1* gene transcription by inhibiting HNF-4 binding to the *CYP8B1* gene. This may be the major mechanism for bile acid inhibition of *CYP8B1* gene transcription.

Concluding Remarks

This symposium summarized our current state of knowledge on the mechanisms involved in down-regulation of *CYP* gene transcription by endogenous and exogenous chemicals. Each of the preceding sections summarizes many recent findings that lead to our current understanding of how specific classes of chemicals and mediators trigger suppression of P450 expression at the transcriptional level. The salient findings discussed in each section of the symposium can be summarized as follows. 1) Aromatic hydrocarbon toxicants decrease the rate of transcription of the male-specific, GH pulse-regulated hepatic *CYP2C11* via an incompletely understood mechanism that appears to involve the AHR. 2) Although transcriptional suppression is a primary mechanism for down-regulation of hepatic P450s in the acute phase of infection and inflammation, the determination of the precise roles of NF-κB and other TFs in these processes remains a fertile area of investigation. 3) Although the endogenous sterol DHEA can regulate the expression of multiple hepatic P450s and cause peroxisome proliferation, the transcriptional suppression of *CYP2C11* by this chemical appears to occur via a PPARα-independent pathway. 4) Multiple receptors (e.g., FXR, PXR, VDR) are activated by bile acids, and these end products of cholesterol catabolism control their own synthesis via complex signaling pathways involving cascades of hepatic TFs that are coordinated to bring about transcriptional suppression of *CYP7A1* and *CYP8B1*.

In a broad and general sense, the following unifying themes emerged from this symposium. 1) Diverse chemical signals can trigger P450 suppression. 2) Multiple molecular mechanisms can be involved in transcriptional suppression. 3) These processes often involve rather complex cascades of TFs and other regulatory proteins. 4) A deeper understanding of the mechanisms involved in these processes will further our appreciation of how organisms respond to xenobiotics as well as to perturbations in endogenous chemicals involved in maintaining homeostasis.

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