Functional Inhibitory Cross-talk between Constitutive Androstane Receptor and Hepatic Nuclear Factor-4 in Hepatic Lipid/Glucose Metabolism Is Mediated by Competition for Binding to the DR1 Motif and to the Common Coactivators, GRIP-1 and PGC-1α*

The role of the constitutive androstane receptor (CAR) in xenobiotic metabolism by inducing expression of cytochromes P450 is well known, but CAR has also been implicated in the down-regulation of key genes involved in bile acid synthesis, gluconeogenesis, and fatty acid β-oxidation by largely unknown mechanisms. Because a key hepatic factor, hepatic nuclear factor-4 (HNF-4), is crucial for the expression of many of these genes, we examined whether CAR could suppress HNF-4 transactivation. Expression of CAR inhibited HNF-4 transactivation of CYP7A1, a key gene in bile acid synthesis, in HepG2 cells, and mutation of the DNA binding domain of CAR impaired this inhibition. Gel shift assays revealed that CAR competes with HNF-4 for binding to the DR1 motif in the CYP7A1 promoter. TCPOBOP, a CAR agonist that increases the interaction of CAR with coactivators, potentiated CAR inhibition of HNF-4 transactivation. Furthermore, inhibition by CAR was reversed by expression of increasing amounts of GRIP-1 or PGC-1α, indicating that CAR competes with HNF-4 for these coactivators. Treatment of mice with phenobarbital or TCPOBOP resulted in decreased hepatic mRNA levels of the reported genes down-regulated by CAR, including Cyp7a1 and Peepck. In vivo recruitment of endogenous CAR to the promoters of Cyp7a1 and Peepck was detected in mouse liver after phenobarbital treatment, whereas association of HNF-4 and coactivators, GRIP-1, p300, and PGC-1α, with these promoters was significantly decreased. Our data suggest that CAR inhibits HNF-4 activity by competing with HNF-4 for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1α, which may be a general mechanism by which CAR down-regulates key genes in hepatic lipid and glucose metabolism.

Although CAR-mediated induction of microsomal P450 enzymes is generally protective for liver function, it can be harmful if toxic metabolites are produced, which was shown by the observation that CAR null mice treated with acetaminophen were resistant to the liver toxicity induced by acetaminophen metabolites (3). In addition to its primary role as a xenosensor for foreign compounds, CAR also plays a critical role in the protection of the liver by detoxification of toxic hepatic endogenous compounds. CAR was shown to be critical for the elimination of bilirubin from the body by activating five known components of the bilirubin clearance pathway (4). In addition, CAR and other nuclear receptors, such as PXR, FXR, and vitamin D receptor, are activated by elevated levels of toxic secondary bile acids such as lithocholic acids (5–7). These nuclear receptors protect the liver not only by inducing expression of P450s that metabolize the toxic bile acids but also by suppressing the expression of CYP7A1 and CYP8B1, two key hepatic enzymes involved in bile acid biosynthesis (8). These effects have been exploited by treating cholestasis patients with phenobarbital (PB) or rifampicin to induce CAR or PXR, respectively, which results in increased metabolism and decreased synthesis of toxic bile acid levels in the liver (9).

Recent gene arrays studies showed that phosphoethanolamine carboxykinase (PEPCK) and carnitine palmitoyltransferase (CPT) or enoyl-CoA isomerase (ECI) genes, which are involved in gluconeogenesis and fatty acid β-oxidation, respectively, were down-regulated by PB treatment in wild type mice but not in CAR null mice (10, 11). Further CAR activation disrupted thyroid hormone homeostasis during fasting, suggesting an unexpected role in energy homeostasis (12). Therefore, these recent studies extend the diverse function of CAR to the negative regulation of genes involved in hepatic glucose and lipid metabolism.

Unlike classical nuclear receptors that are activated by their cognate ligands, CAR is transcriptionally active in the absence of ligand (13). PB and TCPOBOP promote the transcriptional activity of CAR by further enhancing its interaction with coactivators such as GRIP-1, SRC-1, and PGC-1α (14–17). Conversely, inverse agonists of CAR, such as androstanol and androstenol, inhibit the constitutive activity of CAR (18). CAR is present in the cytoplasm in the liver in vivo, and agonists elicit nuclear translocation of CAR, which is essential for the activation of CAR target genes (19). In the nucleus, CAR interacts with its heterodimer partner, RXR, binds to the direct repeat 3 (DR3), DR4, inverted repeat 6 (ER6), ER7, or ER8 motifs in the regulatory region of target genes, and activates their transcription (6, 20). Ligand-independent nuclear localization mediated by interaction with the coactivator GRIP-1 has also been observed (15). In contrast to hepatocytes in vivo, in cultured hepatic HepG2 cells, CAR is primarily located in the nucleus in the absence of agonists.
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Whereas the mechanisms of induction of gene expression by CAR are well known, the mechanisms by which the expression of genes is down-regulated by CAR have not been studied extensively. It has been shown that CAR and PXR coordinately regulate genes that encode enzymes involved in drug metabolism and hepatic gluconeogenesis by inhibitory cross-talk with an insulin responsive forkhead transcription factor, Foxo-1 (21). Recently, we demonstrated that ligand-activated PXR inhibits HNF-4 transactivation of CYP7A1 and CYP8B1 by competing for binding to a common critical coactivator, PGC-1α (22). PGC-1α is a key transcriptional coactivator in glucose and energy homeostasis that is induced during nutritional fasting and CAR has been also shown to interact with PGC-1α (16). However, the mechanisms by which CAR suppresses transcription of these genes are not known. Most of these genes are down-regulated by CAR contain a functional HNF-4 site in their promoters and HNF-4 transactivation is critical for their promoter activities (23-25), so that interference with HNF-4 transactivation is a possible mechanism for CAR suppression of these genes.

Hepatic nuclear factor-4 (HNF-4), a member of the nuclear receptor superfamily, is expressed in liver, pancreas, kidney, and intestine (26). HNF-4 binds to DR1 motifs as a homodimer and regulates expression of numerous key genes involved in cholesterol, fatty acid, and glucose metabolism (6, 27, 28). A recent chromatin immunoprecipitation and microarray study showed that HNF-4 is functionally associated with hundreds of native promoters in liver and pancreatic β-cells, implying the importance of HNF-4 as a master transcription regulator in the liver and pancreas, particularly for lipid and glucose metabolic pathways (29). HNF-4 is also a key transcriptional regulator of CYP7A1 and CYP8B1 (8, 30). Previous studies, including analysis of conditional HNF-4 null mice, demonstrated that HNF-4 is an essential in vivo positive regulator of genes involved in lipid homeostasis and hepatic glucose production, in contrast to CAR, which negatively regulates these genes (10, 11, 31). Transcriptional coactivators such as GRIP-1, SRC-1, and PGC-1α are known to promote HNF-4 activity, and are also known coactivators of CAR (14-17, 32, 33). The importance of the HNF-4/PGC-1α pathway, in coordination with Foxo-1, in the induction of the hepatic gluconeogenic genes PEPCk and G6P during fasting is well established (33, 34). HNF-4 also has been shown to be critically involved in the regulation of liver CPT gene expression in combination with PGC-1α and the cAMP response element-binding protein during fasting (25). HNF-4 is, therefore, a key hepatic activator for genes involved in a variety of metabolic pathways in the liver.

We, therefore, have examined the hypothesis that CAR inhibits expression of key genes involved in lipid and glucose metabolic pathways by antagonizing HNF-4 transcriptional activity. Here, we report that HNF-4 activation of key genes involved in lipid and glucose metabolism was substantially inhibited by CAR via two mechanisms. First, CAR inhibits HNF-4 transactivation by competing with HNF-4 for binding to the DR1 motif, a consensus HNF-4 binding site, in the CYP7A1, CYP8B1, and PEPCk promoters. Second, ligand-activated CAR competes with HNF-4 for binding to common coactivators, including GRIP-1 and PGC-1α, and thus, causes dissociation of these coactivators from HNF-4 target promoters in mouse liver.

EXPERIMENTAL PROCEDURES

Animal Experiments—BALB/c male mice (10–12 weeks old) were maintained on a 12-h light and 12-h dark cycle. Mice were randomly divided into groups that were fed normal chow ad libitum or fasted for 12 h. Animals were then injected intraperitoneally with PB (100 mg/kg body weight), TCPOBOP (0.3 mg/kg body weight), or isotonic saline as a control.

Plasmids and Plasmid Construction—The reporter plasmids, −1887 human CYP7A1-luc, −371 human CYP7A1-luc, and −416 rat Cyp7a1-luc, were supplied by Dr. J. Chiang (35, 36), the expression plasmid for Foxa-2 (HNF-3B) by Dr. R. Costa (37), and the expression plasmid for GRIP-1 by Dr. M. Stallcup (38). The mammalian expression plasmids, pCMV-HNF-4 (22), pcDNA3 mouse CAR (39), and pcDNA3-PGC-1α (22), and the bacterial expression plasmids 6 histidine-tagged (His6) CAR (pET28aCAR), FLAG-tagged RXR, and 6His-HNF-4 were described previously (14, 22, 39). Mutation of Cys-21 and Cys-24 in the CAR DNA-binding domain to Ala was made using the QuickChange site-directed mutagenesis kit (Stratagene) with the primers 5′-TATGGGGCCGAGGAACGCTGTGGTGGCAGAC-3′ and 5′-CTGTGCGCCGTTCTCCAGCACCACAGCGTTCC-3′. The mutation was confirmed by DNA sequencing.

Cell Culture—Human hepatic HepG2 cells (ATCC HB8065) were maintained in 5% CO2 at 37 °C in Dulbecco’s modified Eagle’s medium/F-12 phenol-red free media supplemented with 100 units/ml penicillin G-streptomycin sulfate and 10% heat-inactivated charcoal dextran-treated fetal bovine serum.

Transient Transfection and Reporter Assay—HepG2 cells were cotransfected with DNA of human or rat CYP7A1-luc, DNA of pCMV-HNF-4, or pcDNA3CAR as indicated in the figure legends. For Gal4 reporter assays, COS-1 cells were transfected with DNA of the Gal4-TATA-luc reporter and DNA of Gal4DBD or Gal4DBD HNF-4 and PGC-1 in the presence of absence of CAR. Empty vector DNA was added as needed so that the same amounts of CMV expression vector DNA were present in each transfection. Ten ng of pRL-SV40 DNA, expressing Renilla luciferase, was cotransfected as an internal control. Transfection was carried out using Lipofectamine 2000 in 24-well plates. Twenty-four hours after transfection, cells were incubated with fresh media containing 0.25 μM TCPOBOP or Me3SO for 12 h. Luciferase activities were determined by the dual luciferase assay (Promega Inc.) and the firefly luciferase activities were divided by Renilla activities to normalize for transfection efficiency.

Gel Mobility Shift Assay—6His-CAR, FLAG-RXR, or 6His-HNF-4 were partially purified as described (22, 39, 40). Gel mobility shift assays were performed as previously described (14, 22, 39). Briefly, reactions contained 5,000 to 10,000 cpm of 32P-labeled oligonucleotides with sequences for either the DR1 motif containing the CYP7A1 promoter region or the DR4 motif containing the enhancer region of PB responsive CYP2B1, various amounts (10–20 ng) of HNF-4, CAR, and RXR, and 0.5 μg of poly-(dIdc) in a final volume of 20 μl of reaction buffer (30 mM KCl, 1 mM MgCl2, 15 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10% glycerol, 4 μM dithiothreitol). After a 15-min incubation at room temperature, the protein-DNA complexes were detected by non-denaturing polyacrylamide gel electrophoresis. For antibody supershift assays, 0.5 μg of RXR antibody (Santa Cruz Biotechnology, sc-553) was added to the reactions. For competition assays, a 25–100-fold molar excess of unlabeled double-stranded oligonucleotides containing the wild type or mutated DR1 motif in the CYP7A1 promoter were added for 5 min before the addition of the probe.

Chromatin Immunoprecipitation (ChiP) Assays in Mouse Liver—Mice were treated with either 100 mg/kg PB or saline for 90 min before the animals were sacrificed. Livers were finely minced and incubated in phosphate-buffered saline solution containing 1% formaldehyde at room temperature for 10 min with rotation to cross-link the DNA and proteins, and the reaction was stopped by adding glycine to 125 mM for 5 min. Cells were resuspended in hypotonic buffer (10 mM KOH-Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.2% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose) and lysed by...
RESULTS

CAR Inhibits HNF Transactivation of CYP7A1 Genes—To investigate whether CAR inhibits HNF-4 transactivation of CYP7A1, we performed transfection reporter assays in HepG2 cells. Cells were cotransfected with reporter plasmid DNA for −416 rat Cyp7a1-luc or −371 human CYP7A1-luc and increasing amounts of expression plasmids for HNF-4 and CAR as indicated. Empty vector was added so that the same amounts of CMV vector DNA were present in each transfection. The firefly luciferase activities were divided by the DNA amounts present in each transfection. The firefly luciferase activities to normalize for transfection efficiency. Data from a triplicate transfection are observed from two to four triplicate assays.

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FIGURE 1. CAR inhibits HNF-4 transactivation of the CYP7A1 promoter. A–C, HepG2 cells were cotransfected with 250 ng of DNA of rat (r) or human (h) CYP7A1-luc reporter plasmids as indicated, 50 (+) or 100 (++) ng of DNA of pCMV-HNF-4, and 15 (+) or 50 (++) ng of pcDNA3-CAR, and 10 ng of DNA of pRL-SV40 DNA, expressing Renilla luciferase as an internal control. Differences between the reactions with HNF-4 and CAR added and those with only HNF-4 added were analyzed by the Student’s t test. *, indicates p < 0.05, and **, p < 0.01. D, HepG2 cells were cotransfected with 250 ng of DNA of −1887 human CYP7A1-luc, 50 ng of DNA of CMV-Foxa2, and the indicated amounts of pcDNA3-CAR DNA. E, HepG2 cells were cotransfected with 250 ng of DNA for −371 hCYP7A1-luc wild type or a mutant reporter plasmid, in which the HNF-4 DR1 binding site was mutated, together with 50 ng of expression plasmid DNA for HNF-4 and CAR as indicated. Empty vector was added so that the same amounts of CMV vector DNA were present in each transfection. The firefly luciferase activities were divided by the Renilla luciferase activities to normalize for transfection efficiency. Data from a triplicate transfection are shown with the S.E. and consistent results were observed from two to four triplicate assays.

homogenization was performed with a Dounce homogenizer. The cell lysate was gauze-filtered and layered on a sucrose cushion buffer (10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose). Nuclei were pelleted by centrifugation at 5,000 × g for 1 min and resuspended in sonication buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.85% SDS). The samples were sonicated to reduce DNA length to between 200 and 1,000 bp. After centrifugation at 13,000 × g for 10 min, 2 volumes of dilution buffer (2 mM EDTA, 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate) were added. The chromatin sample was pre-cleared by incubation of 800 μl of chromatin solution with 80 μl of 25% slurry of protein A- or G-agarose that had been previously equilibrated with 30 μg of sonicated salmon sperm DNA and 100 μg of bovine serum albumin per ml of 25% slurry. The pre-cleared chromatin solution was incubated with 2–5 μg of antisera (HNF-4, sc-8987), SRC-1 (sc-6098), GRIP-1 (sc-8996), p300 (sc-5858), PGC-1α (sc-13067), or CAR (39), or normal rabbit or goat serum at 4 °C overnight. The immune complex was collected by centrifugation after incubation with 45 μl of 25% slurry of protein A- or G-agarose. The beads were washed, the bound chromatin was eluted, and the genomic DNA was purified as described above for the HepG2 cells. The DNA was used as a template for semi-quantitative PCR. Sequences of the primers used for ChIP assays are available upon request.

Expression of HNF-4 increased human CYP7A1 promoter activity (Fig. 1, lanes 1, 2, and 5). Expression of increasing amounts of CAR had little effect on basal activity (Fig. 1A, lanes 1–3), but progressively inhibited the HNF-4-mediated transactivation of both promoters with the activities at the highest concentrations of CAR reduced to those without exogenous HNF-4 added (Fig. 1, A and B). Similar results were observed with the −1887 human CYP7A1 reporter (Fig. 1C). To determine whether CAR inhibition of HNF-4-mediated transactivation of CYP7A1 was specific for HNF-4, we examined the effects of CAR on the activities of the CYP7A1 promoter by a forkhead transcription factor, Foxa-2 (HNF-3β). Foxa-2 has been shown to directly bind to the CYP7A1 promoter and activate the transcription (42). Expression of Foxa-2 in HepG2 cells resulted in about a 10-fold increase in human CYP7A1 promoter activity (Fig. 1D). In contrast to the inhibition of HNF-4 transactivation of the CYP7A1 gene, increasing amounts of CAR did not inhibit Foxa-2 transactivation of CYP7A1 (Fig. 1, C and D). These results indicate that the inhibition by CAR is not a general suppression of the promoter activity, but is specific for HNF-4 activity.

In addition, mutation of the DR-1 HNF-4 site, which eliminated binding of HNF-4 to the human promoter (data not shown), abolished the CYP7A1 promoter activity (Fig. 1E). These functional reporter assays demonstrate that HNF-4 is required for the activation of the CYP7A1 promoter in HepG2 cells and CAR inhibits HNF-4 transactivation of CYP7A1 genes.

CAR Competes with HNF-4 for Binding to the DR1 Motif in the CYP7A1 Promoter in Vitro—To delineate the mechanism by which CAR antagonizes HNF-4 transactivation, we first examined if CAR
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FIGURE 2. CAR competes with HNF-4 for binding to the DR1 motif in the CYP7A1 promoter in vitro. A, the sequence of the CYP7A1 promoter region containing the DR1 motif is shown. The wild type (wt) DR1 sequence is underlined on the left and the mutated DR1 sequence is underlined on the right with mutations (mt) indicated by asterisks. B, gel mobility shift assays were performed using 32P-labeled oligonucleotide probes containing the DR1 motif from the CYP7A1 promoter or the DR4 motif from the CYP2B1 enhancer region. Partially purified HNF-4, CAR, and RXR were added to the reaction in the absence or presence of RXR antibody as indicated and protein complexes were analyzed by nondenaturing gel electrophoresis as described under “Experimental Procedures.” The position of the CAR/RXR/DNA complex (C/R) is indicated by an arrow. C, partially purified CAR and RXR were preincubated for 5 min with increasing amounts (25, 50, or 100 molar excess) of unlabeled oligonucleotides containing either the wild type DR1 or the mutated DR1. The 32P-labeled probe, which contained the DR1 motif from the CYP7A1 promoter, was then added to the reaction and complexes C/R analyzed by electrophoresis on native polyacrylamide gels. D, partially purified HNF-4 (10 ng) was added to the reaction in the presence of 32P-labeled DR1 from the CYP7A1 promoter. Either 10 (+) or 20 ng (+) of CAR/RXR were added to the reaction. The incubation was continued for 15 min and complexes were analyzed by electrophoresis on native polyacrylamide gels. The positions of HNF-4 and CAR/RXR/DNA (C/R) complexes are indicated by arrows.

FIGURE 3. Mutation of the CAR DNA binding domain impairs CAR-mediated inhibition of HNF-4 transactivation. A, to construct a DBD mutant of CAR, two Cys in the first zinc finger in the DBD domain were mutated to Ala. B, wild type CAR (wt) or mutant CAR (mt) was synthesized by in vitro transcription and translation. 32P-Labeled oligonucleotide containing the DR4 motif was incubated with either the wild type CAR or the DBD mutant and analyzed by gel mobility shift assays. The position of the CAR/RXR/DNA (C/R) complex is indicated by an arrow. C, HepG2 cells were cotransfected with 250 ng of DNA of the reporter 1887 hCYP7A1-luc, 75 ng of DNA for HNF-4, and 15 ng (+) of DNA for wild type CAR or the DBD mutant expression vectors. Twenty-four hours after transfection, luciferase activities were determined as described in the legend to Fig. 1. The S.E. was calculated from a triplicate transfection and consistent results were obtained from 2 triplicate assays. Statistical significance of the differences between the reactions with HNF-4 and wild type or mutant CAR added and those with only HNF-4 added were analyzed by the Student’s t test (lanes 2 and 3, lanes 6 and 7). *, indicates p < 0.05.

Inhibits HNF-4 binding to the DNA by gel mobility shift assays. CAR is a promiscuous binding protein, which binds to both direct and everted nuclear receptor binding sites with different spacing between the half-sites (6, 20). Two oligonucleotide probes were used that contained either the DR1 motif, an HNF-4 binding site, from the CYP7A1 promoter (Fig. 2A), or the DR4 motif from the CYP2B1 enhancer region (39, 43). CAR or RXR alone did not bind to these probes but addition of CAR and RXR together resulted in a shifted protein-DNA complex as expected for the CYP2B1 promoter (Fig. 2B, lane 7) and surprisingly also for the CYP7A1 promoter (Fig. 2B, lane 4). Addition of antibody against RXR supershifted the complex for the CYP7A1 probe (Fig. 2B, lane 5), suggesting that CAR/RXR binds to the DR1 motif in the CYP7A1 promoter.

To examine if CAR binds to the DR1 motif in the CYP7A1 promoter, we examined if oligonucleotides with wild type and mutated DR1 motifs (Fig. 2A) could compete for binding of CAR to the CYP7A1 promoter fragment. Mutation of the DR1 site eliminated binding to HNF-4 (data not shown). Whereas addition of increasing amounts of the oligonucleotide containing the wild type DR1 efficiently reduced the CAR/RXR binding (Fig. 2C, lanes 1–4), addition of increasing amounts of the mutated oligonucleotide did not compete for CAR/RXR binding to the probe (lanes 5–7). Mutation of the sequences adjacent to the DR1 motif did not markedly affect binding of CAR/RXR to the probe (data not shown). These results indicate that CAR specifically binds to the DR1 motif in the CYP7A1 promoter.

We then tested whether CAR/RXR competes with HNF-4 for binding to the DNA. HNF-4 bound to the probe containing the DR1 motif (Fig.
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2D, lane 2), and addition of either CAR or RXR alone did not alter binding of HNF-4 (Fig. 2D, lanes 3 and 4). However, addition of approxi- mate equimolar concentrations of CAR/RXR and HNF-4 resulted in a decrease in the HNF-4:DNA complex by about 50% (Fig. 2D, lane 5) and the appearance of a second complex with the same mobility as the CAR-RXR:DNA complex (Fig. 2D, lanes 5 and 7). An increased amount of CAR/RXR further reduced HNF-4 binding (Fig. 2D, lane 6). These in vitro binding studies indicate that CAR binds to the DR1 motif in the CYP7A1 promoter in vitro with an affinity near that of HNF-4 and, thus, competes with HNF-4 for binding to the DNA. The dissociation of HNF-4 from the promoter may underlie the inhibition of HNF-4 transactivation of CYP7A1 by CAR.

Mutation of the CAR DNA Binding Domain Impairs CAR-mediated Inhibition of HNF-4 Transactivation—The in vitro DNA binding studies described above suggest that CAR antagonizes HNF-4 activity by competing with HNF-4 for binding to DNA. If this is true, then DNA binding of CAR should be required for the inhibition of HNF-4 transactivation. We, therefore, mutated two Cys in the first zinc finger of the DNA binding domain of CAR to Ala (Fig. 3A), which completely abolished its ability to bind to the DNA in gel shift assays (Fig. 3B). Whereas expression of wild type CAR significantly suppressed HNF-4 transactivation on the human CYP7A1 promoter (Fig. 3C, lanes 2 and 3), the mutant CAR no longer inhibited HNF-4 transactivation (Fig. 3C, lanes 6 and 7). These results demonstrate that DNA binding of CAR is required for inhibition of HNF-4 transactivation, and with the binding studies, strongly indicate that CAR interferes with HNF-4 transactivation by competing for binding to the DR1 motif in the CYP7A1 promoter.

TCPOBOP, a CAR Agonist, Potentiates the Inhibition of HNF-4 Transactivation by CAR—In HepG2 cells, CAR is constitutively in the nucleus so that the effects of an agonist, TCPOBOP, on CAR activity have been shown to result from the increased interaction of CAR with p160 coactivators including GRIP-1 and PGC-1 (15, 40). To examine if TCPOBOP enhances binding of CAR to the DNA, increasing amounts of CAR and RXR were incubated with an oligonucleotide probe containing a DR4 motif from CYP2B1 in the presence of Me2SO or TCPOBOP. Addition of TCPOBOP did not affect binding of CAR to the DNA (Fig. 4A). These data suggest that effects of CAR agonists are confined to increased interactions with coactivators and do not enhance DNA binding of CAR/RXR.

Next we examined if TCPOBOP potentiates the inhibition of HNF-4 transactivation by CAR in HepG2 cells. CAR suppressed HNF-4 trans...
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Activation and this inhibition was potentiated by TCPOBOP (Fig. 4B, lanes 3 and 4). Because TCPOBOP does not affect DNA binding of CAR, these results suggest that CAR may inhibit HNF-4 transactivation by competing with HNF-4 for common coactivators, as well as, by inhibition of HNF-4 binding to DNA.

CAR Inhibition of HNF-4 Transactivation Is Reversed by Exogenous Expression of the Common Coactivators, GRIP-1 or PGC-1α—Coactivators, GRIP-1 and PGC-1α, have been reported to enhance transactivation of both CAR and HNF-4 (14–17, 32, 33). If CAR interferes with HNF-4 signaling by competing for binding to these coactivators, then expression of CAR should inhibit coactivator-mediated enhancement of HNF-4 transactivation and overexpression of the coactivators should reverse the CAR inhibition. Coexpression in HepG2 cells of either GRIP1 (Fig. 4C) or PGC-1α (Fig. 4D) enhanced HNF-4 transactivation of the CYP7A1 promoter. Expression of increasing amounts of CAR progressively inhibited the transactivation and this inhibition was largely reversed by overexpression of GRIP1 (Fig. 4C, lanes 4–8) or PGC-1α (Fig. 4D, lanes 4–8). If CAR affected only the DNA binding of HNF-4, then the increasing amounts of coactivators should not partially reverse the inhibition of CAR as observed. Therefore, these experiments suggest that CAR can also interfere with HNF-4 transactivation by competing with CAR for binding to common coactivators.

To directly determine whether CAR inhibits HNF-4 transactivation by competing with coactivators, we utilized the Gal4 reporter system. Gal4HNF-4 can bind to and activate a reporter gene that contains Gal4 DNA binding sites. Because binding of HNF-4 to DNA is mediated by the Gal4 DNA binding domain (DBD) of Gal4HNF-4, inhibition by CAR of binding of Gal4HNF-4 to DNA is highly unlikely in this system. COS-1 cells were transfected with the 5Gal4-TATA-luc reporter along with either Gal4DBD or Gal4DBDHNF-4 with expression plasmid DNA for PGC-1α and increasing amounts of expression plasmid DNA for CAR. Whereas expression of PGC-1α or CAR did not affect the Gal4DBD activity (data not shown), PGC-1α substantially activated HNF-4 activity and expression of increasing amounts of CAR inhibited HNF-4/PGC-1α activation in a dose-dependent manner (Fig. 4E, lanes 4–6). Consistent with our hypothesis, TCPOBOP, which enhances interaction with coactivators, potentiated the CAR inhibition of the HNF-4/PGC-1α activation (Fig. 4E, lanes 7–9). These results indicate that in addition to interfering with HNF-4 activity by inhibiting binding of HNF-4 to the DR1 motif, CAR can inhibit HNF-4 activity by competing with coactivators, including GRIP-1 and PGC-1α.

Treatment of Mice with PB or TCPOBOP Decreased mRNA Levels of Endogenous Cyp7a1 and Cyp8b1 Genes in Mouse Liver—In vivo, treatment with CAR agonists PB or TCPOBOP resulted in both translocation of CAR to the nucleus (19) and increased interaction with coactivators (14, 15, 18, 40). If our studies, HepG2 cells are an accurate model for the effects of CAR on HNF-4 transactivation in vivo, then treatment with a CAR agonist should result in decreased expression of endogenous genes suppressed by CAR and decreased binding of HNF-4 to the promoters of those genes. We first tested if treatment with PB or TCPOBOP inhibits the expression of two key hepatic genes involved in bile acid biosynthesis, Cyp7a1 and Cyp8b1, in mice, and as a control whether expression of the PB-inducible gene Cyp2b10 is increased. PB (Fig. 5A) or TCPOBOP (Fig. 5B) treatment markedly decreased mRNA levels of mouse Cyp7a1 and Cyp8b1 by about 50%, as determined by either semi-quantitative RT-PCR (Fig. 5A) or real time RT-PCR (Fig. 5B). In contrast, the mRNA levels of Cyp2b10, a well known PB inducible CAR target gene (1, 39, 43), were substantially increased by treatment with these ligands.

Treatment with TCPOBOP Decreases mRNA Levels of PEPCK, Glc-6-P, CPT, and ECI in the Fasted Mice—In addition to the Cyp7a1 and Cyp8b1 genes studied above, recent studies of activation of CAR in wild type and CAR null mice by DNA array analysis suggested that CAR down-regulates genes involved in gluconeogenesis, such as PEPCK and Glc-6-P, and in fatty acid β-oxidation, such as CPT and ECI (10, 11). Each of these genes is induced upon nutritional fasting and the HNF-4/Foxo-1/PGC-1α pathway has been shown to be critical in the activation of gluconeogenic genes during fasting (33, 34). If the inhibitory effects of CAR on HNF-4 transactivation are generally true, then fasting-induced expression of each of these genes should be suppressed by treatment with CAR agonists.

To examine the effects of CAR activation on the expression of these genes, mice were fed ad libitum or fasted for 12 h and then treated with TCPOBOP or saline for 24 h. Total RNA was isolated from the livers and mRNA levels of PEPCK, Glc-6-P, CPT, and ECI were measured by real time PCR. As controls, we also measured mRNA levels of Cyp2b10, CAR, and SREBP-1c, a transcriptional activator of hepatic lipogenesis, such as SREBP-1c, a well known PB inducible SREBP-1c expression. Intriguingly, mRNA levels of CAR were increased more than 3-fold by fasting, and decreased binding of HNF-4 to the promoters of those genes. We first tested if treatment with PB or TCPOBOP inhibits the expression of two key hepatic genes involved in bile acid biosynthesis, Cyp7a1 and Cyp8b1, in mice, and as a control whether expression of the PB-inducible gene Cyp2b10 is increased. PB (Fig. 5A) or TCPOBOP (Fig. 5B) treatment markedly decreased mRNA levels of mouse Cyp7a1 and Cyp8b1 by about 50%, as determined by either semi-quantitative RT-PCR (Fig. 5A) or real time RT-PCR (Fig. 5B). In contrast, the mRNA levels of Cyp2b10, a well known PB inducible CAR target gene (1, 39, 43), were substantially increased by treatment with these ligands.

### FIGURE 5. Treatment of mice with PB or TCPOBOP decreases mRNA levels of Cyp7a1 and Cyp8b1 in the liver. A, two groups of two mice each were treated with 100 mg/kg PB or saline for 24 h. The mice were sacrificed and total RNA was isolated from the livers. The mRNA levels were determined by real time RT-PCR using primer sets specific for Cyp7a1, Cyp8b1, and β-actin. B, two groups of mice each were treated with 0.3 mg/kg TCPOBOP or saline for 24 h. The mice were sacrificed and total RNA was isolated from the livers. The mRNA levels were determined by real time RT-PCR using primer sets specific for Cyp7a1, Cyp8b1, Cyp2b10, and β-actin. The mRNA levels of Cyp7a1, Cyp8b1, or Cyp2b10 were normalized by dividing by the β-actin mRNA levels. Differences between control and TCPOBOP samples were analyzed by the Student’s t test. * indicates p < 0.05, and **, p < 0.01.
suggest that HNF-4 is not the primary activator of these genes in the fed state because TCPOBOP has little effect on their expression.

Treatment with PB Causes Recruitment of Endogenous CAR and Dissociation of Endogenous HNF-4 and Coactivators at the Cyp7a1 and PEPCK Promoters in Mouse Liver—If CAR suppresses these genes by competing with HNF-4 for binding to DNA and coactivators, then treatment with CAR agonists should result in recruitment of CAR and dissociation of HNF-4 and coactivators at the native target promoters. To test this possibility in vivo, we examined the effect of PB treatment on the association of endogenous CAR, HNF-4, and coactivators with the promoters of the Cyp7a1 and Pepek genes in mouse liver. CAR accumulates in the nucleus as short as 30 min after PB treatment (19). Therefore, mice were treated with either PB or saline for 90 min. Association of these proteins with the mouse Cyp7a1 or Pepek promoters was determined by ChIP assay using antibodies against CAR, HNF-4, and the coactivators, including SRC-1, GRIP-1, p300, and PGC-1α, and semi-quantitative PCR was performed.

Binding of endogenous HNF-4 to the Cyp7a1 and Pepek promoters was detected and treatment of PB inhibited in vivo binding of HNF-4 by more than 30% from 6 independent ChIP assays (Fig. 7). In contrast, recruitment of CAR to these promoters was detected after PB treatment (Fig. 7, A and B). Association of HNF-4 and CAR with the control glyceraldehyde-3-phosphate dehydrogenase coding region was not detected (Fig. 7C). The amounts of the coactivators, GRIP-1, SRC-1, PGC-1α, p300, associated with the promoters, were reduced after PB treatment (Fig. 7D), although the decrease in PGC-1α at the Pepek promoter was not statistically significant (Fig. 7E). In parallel experiments, we reproducibly observed that PB treatment induced recruitment of coactivators, including GRIP-1 and CBP, to the PB-inducible Cyp2b10 gene enhancer region that contains CAR/RXR binding sites in mouse liver.3 These ChIP studies provide crucial in vivo evidence to support our conclusions that CAR inhibits HNF-4 transactivation by competing with HNF-4 for binding to the DNA and common coactivators.

DISCUSSION

The inhibitory cross-talk between CAR and HNF-4 further extends the diverse xenobiotic function of CAR in the metabolism and elimination of toxic foreign compounds, bilirubin clearance, and drug-induced liver toxicity, to the regulation of cholesterol/bile acid, fatty acid, and glucose metabolic pathways. In this report, we present evidence that CAR can suppress HNF-4 transactivation by two distinct mechanisms (Fig. 8). In vitro gel shift assays indicated that CAR/RXR competes for the binding of HNF-4 to its DR1 site in the CYP7A1 promoter. Expression of CAR inhibited HNF-4 transactivation in HepG2 cells and binding of CAR to DNA was required for the inhibition. These results strongly indicate that CAR inhibits HNF-4 transactivation by competing for binding to the DR1 site (Fig. 8A). Consistent with the in vitro DNA binding studies, ChIP studies revealed that binding of HNF-4 to the CYP7A1 and CYP8B1 promoters was inhibited when CAR was ectopically expressed in HepG2 cells (not shown). The CAR agonistic ligand, TCPOBOP, has little effect on the binding of CAR/RXR to DNA but increases the interaction of CAR with coactivators (14, 15, 18, 40). The potentiation of CAR inhibition of HNF-4 transactivation by TCPOBOP and the results from the Gal4 reporter assays indicate that CAR competes with HNF-4 for binding of common coactivators. Furthermore, in ChIP assays in mouse liver, we observed that association of HNF-4 and coactivators with the Cyp7a1 and Pepek promoters was reduced, whereas CAR binding was increased, after PB treatment. Together, our data strongly support a model in which CAR competes with HNF-4 both for its DNA binding site and for interaction with coactivators (Fig. 8B). Both of these mechanisms may contribute to inhibition by CAR of HNF-4 transactivation in some genes, or one or the other might be predominant, depending on the context of the binding sites in the target genes.

PXR, like CAR, is a xenobiotic nuclear receptor, which induces the expression of xenobiotic metabolizing P450 enzymes. Interestingly, PXR also inhibits HNF-4 transactivation and mediates cross-talk between regulation of xenobiotic metabolism and cholesterol/bile acid, glucose, and fatty
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Acid metabolism. However, we found previously that PXR/RXR, which is a promiscuous nuclear receptor that binds to DR3, DR4, ER6, and ER8 DNA binding elements, as does CAR (6), does not bind to the DR1 motif in CYP7A1, but instead competes with HNF-4 for binding to the coactivator PGC-1α (22). Therefore, these two related xenobiotic nuclear receptors both inhibit HNF-4 transactivation and share the ability to compete with HNF-4 for interaction with coactivators such as PGC-1α. However, even though PXR and CAR can bind to many of the same DNA binding sites, only CAR can bind to the HNF-4 DR1 motif and inhibit binding of HNF-4 to DNA. PXR and CAR, therefore, have overlapping but distinct mechanisms for inhibition of HNF-4 transactivation.

CAR/RXR binds promiscuously to multiple DNA binding motifs including the estrogen receptor response element (40), but it has not been shown previously to bind to the DR1 motif. CAR/RXR binds to the DR1 motif in the CYP7A1, CYP8B1, and PEPCK promoters with an affinity similar to that of HNF-4 because HNF-4 binding to DNA was inhibited 50% by approximately equimolar concentrations of CAR. This contrasts with the >100-fold excess of CAR/RXR needed to compete for binding of the estrogen receptor to its binding site (40). In principle, CAR could bind to a site near the DR1 site and sterically inhibit HNF-4 binding, but unlabeled oligonucleotides containing a mutated DR1 site did not compete for binding of CAR to the CYP7A1 promoter.

FIGURE 7. PB treatment causes recruitment of CAR and dissociation of endogenous HNF-4 and coactivators at the Cyp7a1 and PEPCK promoters in mouse liver. A–C, mice were treated with PB or saline (C) for 90 min and the livers were collected for ChIP assays. Chromatin was isolated and pre-cleared as described under “Experimental Procedures.” Pre-cleared chromatin was immunoprecipitated by antibodies against CAR, HNF-4, PGC-1α, GRIP-1, SRC-1, p300, or normal rabbit or goat serum (NS). Precipitated chromatin was extensively washed and DNA isolated from the input chromatin before precipitation (Input) or from the precipitated chromatin was analyzed by semi-quantitative PCR using primer sets specific for Cyp7a1, PEPCK, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as indicated by the arrows in the schematic diagrams of the genes. A representative result from multiple ChIP assays is shown. D and E, average amounts of amplified promoter sequence from two to six independent ChIP assays are shown. The band intensity was measured by densitometry and the amount of promoter sequence precipitated by each antibody was normalized by dividing by the amount of promoter sequence in the input DNA sample, and the value in the control group for each factor were assigned as a value of 1. The S.E. is indicated and differences between control and PB-treated samples were analyzed by the Student’s t test. *, indicates p < 0.05, and **, p < 0.01.

FIGURE 8. Model of the dual mechanisms of CAR inhibition of HNF-4 transactivation. The data indicating that CAR inhibits HNF transactivation by competing for binding both to DNA and coactivators are consistent with the following model: HNF-4 activates transcription by binding as a homodimer to a DR1 motif in the promoters of the CYP7A1, CYP8B1, and PEPCK genes and recruiting coactivator complexes. CAR activation by PB or TCPOBOP induces CAR nuclear localization in animal liver. A, CAR/RXR competes with HNF-4 for binding to the DR1 motif in the HNF-4 target promoters, but does not recruit coactivators because of the context of the promoter. B, CAR also competes with HNF-4 for binding to common coactivators, such as GRIP-1 and PGC-1α. Both actions contribute to suppression of HNF-4-mediated transactivation of key genes involved in hepatic lipid and glucose metabolism.
whereas wild type DR1 oligonucleotides did, which provides strong evidence that CAR binds to the DR1 site. ChIP assays in mouse liver demonstrated that binding of endogenous HNF-4 to the promoters of Cyp7a1, Cyp8b1, and Pepck genes were decreased and that of CAR was increased, suggesting that the similar affinities of the two factors for the DR1 site observed in vitro with purified DNA was also reflected in binding to the sites in a chromatin context.

One intriguing observation from this study is that although CAR/RXR competes with HNF-4 for DNA binding and, thus, binds to the DR1 motif in the native promoters of Cyp7a1, Cyp8b1, and Pepck genes, these genes are suppressed, not activated. Thus, it is unlikely that CAR recruits coactivator complexes to these promoters, which was also indicated by our ChIP studies in mouse liver. It is, therefore, possible that the context of the promoter is important so that HNF-4, cooperatively with other activators at these promoters, recruits coactivators complexes. CAR/RXR, on the other hand, may not contribute to cooperative recruitment of coactivators in the context of the CYP7A1, CYP8B1, and PEPCK genes, which are suppressed, but does so in the context of the CYP2B promoter/enhancers, which are induced (15, 39, 43). A second possibility is that HNF-4 acts as an architectural factor such that it binds to closed chromatin and opens up the chromatin structure at the promoter region to permit binding of other activation factors, as has been described for HNF-3 (45). By competing for HNF-4 binding, CAR/RXR may prevent the changes in chromatin structure and consequent activation of these HNF-4 target genes.

In addition to HNF-4, other nuclear receptors, peroxisome proliferator-activated receptor α, chicken ovalbumin upstream promoter-transcription factor, and retinoic acid receptor, are abundantly expressed in liver and also utilize the DR1 motif as a consensus DNA binding element (46, 47). These nuclear receptors play critical roles in the regulation of diverse biological activities including lipid and glucose metabolism, gluconeogenesis, and differentiation (8, 28, 48). Because CAR/RXR binds to the DR1 motif, CAR could compete with these nuclear receptors for DNA binding and, thus, influence these diverse signaling pathways. Additional studies defining the relative amounts of these nuclear receptors, concentrations of their ligands, and concentrations of coactivators and corepressors in the hepatic nucleus are required to determine whether inhibitory cross-talk between CAR and these other nuclear receptors is physiologically relevant.

Recent DNA microarray studies showed that Pepck, Cpt, and Eci genes were down-regulated by PB treatment in wild type mice but not in CAR null mice (10, 11), suggesting that PB-activated CAR inhibits expression of these genes. Pepck is a key gene in hepatic gluconeogenesis and Cpt and Eci are involved in fatty acid β-oxidation. Consistent with the DNA array analysis, treatment with PB or TCPOBOP significantly reduced expression of the hepatic gluconeogenic enzymes Pepck and Glc-6-6-P, consistent with studies showing decreased glucose production (49, 50). Decreased glucose production may in part explain the decreased glucose levels and increased insulin sensitivity observed in diabetic patients treated with PB (49, 50). Many of these genes involved in gluconeogenesis and fatty acid metabolism are induced in fasted animals by pathways involving Foxo-1, HNF-4, and PGC-1α (33, 34, 51). If CAR suppresses HNF-4 transactivation, then, activation of CAR in the fasted animal should suppress expression of these genes. Consistent with this idea, the expression of these genes was induced by fasting, and TCPOBOP treatment reduced their expression only in fasted animals. These results are consistent with a more general role for CAR-mediated suppression of HNF-4 transactivation in glucose and fatty acid metabolism in addition to drug and bile acid metabolism.

Interestingly, in fasted animals, mRNA levels of CAR were elevated more than 3-fold. The relatively greater effect of TCPOBOP in the fastest compared with fed animals may be in part due to the essential role of the HNF-4/Foxo-1/PGC-1α pathway in the activation of gluconeogenesis and fatty acid β-oxidation in the fasted animal, but the increased concentrations of CAR in the fasted animal may also contribute to increased suppression of these genes. The mechanisms by which CAR mRNA levels are elevated during fasting is not known, but AMP signaling or the transcriptional regulators PGC-1α and Foxo-1 may be involved. Levels of PGC-1α and Foxo-1 are known to be elevated in fasted animals and might induce CAR gene expression (33, 34, 51). AMP kinase is a key sensor for cellular energy changes and constitutively active mutants of this enzyme mimic the PB induction of CYP2B genes, whereas expression of a dominant negative form inhibits the induction (52). Thus, activation of AMP kinase in fasted animals may potentiate PB-mediated nuclear accumulation of CAR and increase the interaction of CAR with coactivators such as GRIP-1 and PGC-1α.

Our studies indicate that CAR can function not only in inducing enzymes that metabolize xenobiotic compounds, but also in the suppression of enzymes involved in bile acid production, gluconeogenesis, and fatty acid β-oxidation in the liver. The studies on CYP7A1, CYP8B1, and PEPCK indicate that CAR suppresses expression of these genes by competing for binding of HNF-4 to its DNA binding site and to coactivators. HNF-4 positively regulates the expression of many of the genes involved in these pathways so that suppression of HNF-4 transactivation by CAR could be a general mechanism for inhibiting these metabolic pathways.

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