Induction of Nuclear Translocation of Constitutive Androstane Receptor by Peroxisome Proliferator-activated Receptor α Synthetic Ligands in Mouse Liver

Received for publication, August 27, 2007, and in revised form, October 25, 2007 Published, JBC Papers in Press, October 25, 2007, DOI 10.1074/jbc.M707183200

Dongsheng Guo‡, Joy Sarkar‡, Kelly Suino-Powell†, Yong Xu‡, Kojiro Matsumoto‡, Yuzhi Jia‡, Songtao Yu‡, Sonal Khare‡, Kasturi Haldar‡, M. Sambasiva Rao‡, Jennifer E. Foreman†, Satdarshan P. S. Monga‡, Jeffrey M. Peters‡, H. Eric Xu‡, and Janardan K. Reddy†‡

From the †Department of Pathology, Northwestern University, Feinberg School of Medicine, Chicago, Illinois 60611-3008, ‡Laboratory of Structural Sciences, Van Andel Research Institute, Grand Rapids, Michigan 49503, *Center for Molecular Toxicology and Carcinogenesis, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, Pennsylvania 16802, and the †Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261

Peroxisome proliferators activate nuclear receptor peroxisome proliferator-activated receptor α (PPARα) and enhance the transcription of several genes in liver. We report here that synthetic PPARα ligands Wy-14,643, ciprofibrate, clofibrate, and others induce the nuclear translocation of constitutive androstane receptor (CAR) in mouse liver cells in vivo. Adenoviral-enhanced green fluorescent protein-CAR expression demonstrated that PPARα synthetic ligands drive CAR into the hepatocyte nucleus in a PPARα- and PPARβ-independent manner. This translocation is dependent on the transcription coactivator PPAR-binding protein but independent of coactivators PRIP and SRC-1. PPARα ligand-induced nuclear translocation of CAR is not associated with induction of Cyp2b10 mRNA in mouse liver. PPARα ligands interfered with coactivator recruitment to the CAR ligand binding domain and reduced the constitutive transactivation of CAR. Both Wy-14,643 and ciprofibrate occupied the ligand binding pocket of CAR and adapted a binding mode similar to that of the CAR inverse agonist androstenediol. These observations, therefore, provide information for the first time to indicate that PPARα ligands not only serve as PPARα agonists but possibly act as CAR antagonists.

Several structurally diverse synthetic compounds, designated as peroxisome proliferators, induce a set of highly predictable pleiotropic responses consisting of hepatomegaly, peroxisome proliferation in hepatocytes, selective increase in the transcription of genes encoding the fatty acid oxidation enzymes in liver, and the development of hepatocellular carcinomas in rats and mice (1–4). A receptor-based mechanism for the induction of these pleiotropic responses was first proposed forming the basis for the identification of peroxisome proliferator-activated receptor (PPAR) isoforms belonging to the nuclear hormone receptor superfamily (1, 5–7). PPARα (NR1C1) was first identified as a receptor that is activated by peroxisome proliferators, and its role in peroxisome proliferator-induced changes has been confirmed by generating mice with PPARα gene disruption (5, 8). The other two members of PPAR subfamily, designated as PPARβ/δ (NR1C2) and PPARγ (NR1C3), are also modulated by synthetic agents and participate in energy (lipid) metabolism (1, 6, 7, 9). Accordingly, all three members of the PPAR subfamily of nuclear receptors play essential roles in sensing certain xenobiotic substances including drugs, environmental chemical pollutants, and nutritional elements (7). PPARs heterodimerize with the 9-cis-retinoic acid receptor α (RXRα), and the PPAR-RXR heterodimers bind to peroxisome proliferator response elements (PPREs) consisting of a direct repeat of the consensus half-site motif (AGGNGCA) spaced by a single nucleotide (DR1) (1, 7, 11). Upon ligand binding, PPARs undergo conformational changes that facilitate the rapid dissociation of corepressor molecules to enable the recruitment of coactivator molecules such as CBP (cAMP-response element-binding protein (CREB)-binding protein)/p300, p160/SRC-1 family members, PBP (also known as TRAP220/DRIP205/MED1), PPAR-interacting protein (PRIP) (also known as ASC2/RAP250/TRBP/NRC), and others to initiate transcriptional activity of specific target gene (12–19). In this regard the transcriptional activation of PPAR-specific target genes may depend upon the availability of the ligand in a given cell/tissue, abundance of receptor and its heterodimerization partner RXR, nature of PPREs, and availability of transcription cofactors among others (1, 19–22).

§ This work was supported by the National Institutes of Health Grants GM23750 (to J. K. R.) and CA104578 (to J. K. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

1 To whom correspondence should be addressed: Dept. of Pathology, Northwestern University, Feinberg School of Medicine, 303 East Chicago Ave., Chicago, IL 60611-3008. Tel.: 312-503-7948; Fax: 312-503-8249; E-mail: jkreddy@northwestern.edu.

* This work was supported by the National Institutes of Health Grants GM23750 (to J. K. R.) and CA104578 (to J. K. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; PBP, PPAR-binding protein; PRIP, PPAR-interacting protein; PBREM, phenobarbital-response enhancer module; HD, 3-enooyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; EGFp, enhanced green fluorescent protein; TCPDBP, 1,4-bis[3-(3,5-dichloropyridin-2-yl)benzene; APAP, acetaminophen; LBD, ligand binding domain; RXR, 9-cis-retinoic acid receptor; AOX, acyl-CoA oxidase; CAR, constitutive androstane receptor; DAPI, 4’,6-diamidino-2-phenylindole; GST, glutathione S-transferase; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[3-chloroamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Like PPARα, constitutive androstane receptor (CAR) (NR113) is also a xenobiotic sensing receptor that is capable of being modulated by structurally diverse chemicals such as 1,4-bis-2-((3,5-dichloropyridyloxy)]benzene (TCPOBOP) and phenobarbital but is not known to be responsive to PPARα ligands (23–29). CAR is sequestered in the cytoplasm of liver parenchymal cells in a complex with accessory proteins, including CCRP (CAR cytoplasmic retention protein) and Hsp90 (heat shock protein 90) (30–32). When activated by an agonist that binds to the ligand binding domain of CAR, the receptor translocates to the nucleus where it heterodimerizes with RXRs (23–26, 33). In response to indirect activators such as phenobarbital, the cytoplasmic CAR complex recruits protein phosphatase 2A before translocation into the nucleus (32). In either event, nuclear translocation of CAR is an essential step for CAR activation by xenobiotics and the induction of target genes (23, 34). CAR-RXR heterodimers bind to the specific DNA responsive elements found in the target gene promoter resulting in the transcriptional enhancement of gene expression (26). As with other activated nuclear receptors, CAR activation also results in the recruitment of coactivators such as p160/SRC-1 family members, ASC-2/PRIP, and PBP (10, 35–37).

We now present evidence to show that synthetic PPARα ligands (peroxisome proliferators) induce the nuclear translocation of CAR in liver cells in vivo in addition to their well known property of activating PPARα (1, 2, 5, 10, 38, 39). PPARα ligand-induced nuclear translocation of CAR does not result in the enhancement of transcription of CAR target genes, in particular Cyp2b10 (23, 26–30). The PPARα ligand-induced nuclear translocation of CAR can occur in the absence of PPARα but requires the presence of coactivator PBP. These studies also show that pretreatment with PPARα ligands does not enhance acetaminophen hepatotoxicity in the mouse. Molecular modeling revealed that PPARα ligands fit well in CAR ligand binding pocket analogous to that of CAR antagonist androstenol (40, 41). These results indicate that PPARα ligands function as agonists of PPARα and inverse agonists (antagonists) of CAR. This dual function of PPARα ligands suggests possible cross-talk by which xenobiotics may exert their effects.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The construction of recombinant adenovirus EGFP-CAR (Ad/EGFP-CAR) was described elsewhere (42), pPBRU-2C1 luciferase vector (43) was a generous gift from Dr. Byron Kemper. PBP fragment (PPB-(440–740) which includes both PBP nuclear receptor binding LXXLL (L, leucine; X, any amino acid) signature motifs was constructed as described previously (44).

**Mouse and Treatment**—Wild-type (C57BL/6J), PPARα−/−, PPARβ−/−, AOX−/−, SRC-1−/−, PBPΔLIV, and PRIPΔLIV mice 4–6 weeks of age and weighing 20–25g were used in this study (8, 45–50). Mice were maintained on a 12-h light/dark cycle and had *ad libitum* access to water and pelleted chow. Mice were injected intravenously via the tail vein with 4 × 1011 recombinant adenovirus particles in a volume of 200 μl and killed 5 days after infection. Mice were given Wy-14,643 (60, 125, or 250 mg/kg of body weight), ciprofibrate (250 mg/kg of body weight), clofibrate (350 mg/kg of body weight), gemfibrozil (350 mg/kg of body weight), fenofibrate (350 mg/kg of body weight), artemisinin (200 mg/kg of body weight), or androstenol (250 mg/kg of body weight) by intraperitoneal injection and killed 3 h later (42, 50). For assessing the time-course of nuclear translocation of CAR and the persistence of CAR retention in the nucleus, mice were killed at 3, 6, 12, and 24 h after Wy-14,643 (250 mg/kg of body weight) injection. Small pieces of liver were placed in Tissue-Tek O.C.T. Compound (Sakura), frozen in a mixture of dry ice and 2-propanol, and sectioned (~8 μm thick) using a cryostatic ultramicrotome. Frozen sections were then fixed in 4% paraformaldehyde and examined using a fluorescence microscope. DAPI staining was used to visualize nuclear DNA. Groups of 4–6 mice received Wy-14,643 (0.125% weight/weight) or ciprofibrate (0.025% wt/wt) in a powdered diet for 2 weeks (48). For light microscopy, liver sections were fixed in 10% neutral buffered formalin or 4% paraformaldehyde, processed for embedding in paraffin, sectioned, and stained with either hematoxylin and eosin or processed for immunohistochemical localization of CAR by using antibody against CAR (M-150, SC-13065; lot 12404, Santa Cruz Biotechnology) (36). All animal procedures used in this study were reviewed and pre-approved by the Institutional Review Boards for Animal Research.

**Cell Lines and Culture**—HepG2 and Hep3B cells were purchased from ATCC. All cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Clontech, Palo Alto, CA) and incubated at 37 °C in a humidified atmosphere containing 95% air and 5% carbon dioxide. HepG2 and Hep3B cells (2 × 105) were plated and infected with Ad/EGFP-CAR at a multiplicity of infection of 100. Forty-eight hours later, cells were examined using an inverted fluorescence microscope.

**Northern and Immunoblot Procedures**—Total RNA isolated from liver using TRIzol reagent (Invitrogen) was glyoxylated, electrophoresed on 0.8% agarose gel, and transferred to nylon membrane for hybridization at 65 °C in rapid hybridization buffer (GE Healthcare) using 32P-labeled cDNA probes (39, 42, 46, 50). Equal loading of total RNA in the gel was verified by measuring the intensity of 18 S and 28 S rRNA bands stained with Fast RNA Stain (HealthGene Corp.) or by using glyceraldehyde-3-phosphate dehydrogenase northern blotting. Liver extracts were subjected to 4–20% SDS-PAGE, transferred to nitrocellulose membranes, and then immunoblotted using antibodies against fatty acyl-CoA oxidase 1, peroxisomal l-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme, and peroxisomal thiolase (all from Professor Takashi Hashimoto).

**GST Pulldown Assays**—[35S]Methionine-labeled CAR was synthesized by *in vitro* translation using the Tnt coupled transcription-translation system (Promega). GST-PBP-(440–740) fusion protein was expressed in *Escherichia coli* BL21 and purified by binding to glutathione-Sepharose (Amersham Biosciences). Immobilized GST-PBP-(440–740) fusion protein and [35S]methionine-labeled CAR and RXR were used in GST pulldown assays using CAR ligand TCPOBOP in the presence or absence of PPARα ligand Wy-14,643 or ciprofibrate. Bound proteins were resolved on SDS-PAGE and autoradiographed.
PPARα Ligand-induced Nuclear Translocation of CAR

Chromatin Immunoprecipitation Assays—Liver chromatin immunoprecipitation assays were performed as described (36, 48). Liver nuclei were purified and fixed for 30 min in 1% formaldehyde to cross-link the DNA-binding proteins to cognate cis-acting elements. Nuclear homogenates were sonicated to shear the chromosomal DNA to an average length of ~1000 bp (48). The primers used for amplification of the phenobarbital response unit of the mouse Cyp2b10 promoter region were 5’-CTCCAGTGAATTAGGAAGA-3’ and 5’-AAGTATTTGTGCCAGTTCGT-3’ (36, 48).

Transactivation Assay—HepG2 cells were plated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum without antibiotics in 24-well plates and cultured for 24 h before transfection with pEGFP-CAR, PBUR2C1-Luc, and pCMV-RL (expresses Renilla luciferase). Transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cyp2b10 phenobarbital response unit (PBUR) luciferase vector (PBUR2C1-Luc) (51), CAR expression vector and CAR ligand TCPOBOP, CAR antagonist androstenol, and PPARα ligand Wy-14,643 were used in these assays. Cell extracts were analyzed 24 h after transfection for luciferase activity. pEGFP-CAR permitted us to estimate transfection efficiency.

Electrophoretic Mobility Shift Assay—To examine the DNA binding dynamics of CAR, RXR, and PPARα on the HD-PPRE and PBREM, electrophoretic mobility shift analysis was performed with in vitro synthesized proteins and γ-32P-labeled HD-PPRE and PBREM double-stranded oligonucleotides (36). CAR, RXR, and PPARα were synthesized in vitro using the TnT-coupled transcription-translation system (Promega). Oligonucleotides corresponding to the HD-PPRE (5’-CTT TGA CCT ATT GAA CTA TT-3’ and its complement) or PBREM (5’-TCT GTA CTT TCC TGA CCT TG-3’ and its complement) were annealed and labeled with the T4 polynucleotide kinase and [γ-32P]dATP. Combinations of CAR-RXR or CAR-PPARα reticulocyte lysate were incubated in binding buffer containing 10 mM Tris, pH 8.0, 30 mM KCl, 5 mM MgCl2, 5 μg of bovine serum albumin, and 5% glycerol with 0.2 mM dithiothreitol, and 0.25 μg of poly(dI-dC) for 30 min at room temperature with 2 pmol of [γ-32P]dATP-labeled HD-PPRE or PBREM. Reaction mixtures were further loaded on 5% native PAGE containing 5% glycerol in 0.5× Tris borate-EDTA buffer in the cold room. Gels were dried and autoradiographed.

Alanine Aminotransferase Measurement—Wild-type mice were given a single intraperitoneal injection of acetaminophen (APAP) at 250 or 700 mg/kg of body weight to induce hepatic necrosis (36). To assess the effect of pretreatment with CAR activator phenobarbital or with PPARα ligand Wy-14,643 on APAP-induced hepatotoxicity, groups of mice were pretreated for 3 days with phenobarbital (100 mg/kg, by intraperitoneal injection) or Wy-14,643 (250 mg/kg, by gavage) or both. They were then given a single dose of APAP (250 mg/kg) by intraperitoneal injection 24 h before killing. To assess the effect of antimalarial agent artemisinin on APAP-induced hepatotoxicity, mice were pretreated with this compound for 3 days at an intraperitoneal dose of 60 mg/kg and then given APAP (250 mg/kg) intraperitoneally and killed 24 h later. Blood collected from inferior vena cava was used for the determination of serum alanine aminotransferase (ALT) activity by using an ALT assay kit (Sigma).

Coactivator Binding Assay—The effect of PPARα ligands on coactivator binding to CAR ligand binding domain (LBD) was determined by Alpha Screen assay using a hexahistidine detection kit from PerkinElmer Life Sciences. CAR-LBD protein was prepared as a Hist6-GST fusion protein. The experiment was conducted with ~20 nM CAR-LBD, ~20 nM biotinylated Tf2 (SynBioSci), in the presence of 5 μg/ml donor and acceptor beads in a buffer containing 50 mM MOPS, 50 mM NaF, 50 μM CHAPS, and 0.1 mg/ml bovine serum albumin, all adjusted to a pH of 7.4. Data were derived from three repeated experiments with S.E. typically less than 10% of the measurements.

Molecular Docking—The initial structure (code 1XLS) of CAR-LBD was retrieved from the Protein Data Bank. The ICM program (ICM, Version 3.4.8b, 2006; Molsoft LLC, San Diego, CA) was used for protein and ligand preparation. The residues after the tail of Helix 10 (Asn-334—Ser-358) were deleted for simulating inactive conformation. The resultant structure was converted into ICM project. The molecular structures of Wy-14,643 and ciprofibrate were constructed in ICM interface and were optimized with MMFF94 force field (52). These two small molecules were docked into the ligand binding pocket with default parameter implemented in ICM.

RESULTS

PPARα Ligands Induce Nuclear Translocation of Endogenous CAR in Mouse Liver—Immunohistochemical staining of liver sections for CAR revealed that the PPARα agonists Wy-14,643 and ciprofibrate induced the translocation of endogenous CAR into mouse hepatocyte nucleus in vivo (supplemental Fig. 1, A–D). Translocation of native CAR occurred in the presence (supplemental Fig. 1A–C) and absence (supplemental Fig. 1D) of PPARα.

Exogenous EGFP-CAR Translocates to the Nucleus in Response to PPARα Agonists—Recently, we utilized adeno-virally driven EGFP-CAR vector for the expression of CAR in liver in vivo and in hepatocytes in vitro (42, 50). This method offers an advantage in that the cytoplasmic to nuclear shift of EGFP-CAR can be visualized without the limitations of immunohistochemical staining using CAR antibodies. We have shown that the exogenously expressed recombinant CAR is visualized prominently in hepatic parenchymal cell cytoplasm.

In the absence of CAR activator phenobarbital or TCPOBOP, the EGFP-CAR was localized in the cytoplasm (42, 50). In the present study we examined whether synthetic PPARα ligands Wy-14,643 and ciprofibrate induce nuclear translocation of adeno-virally driven EGFP-CAR in mouse liver cells in vivo (Fig. 1, A–C). In the absence of PPARα ligand, the abundantly expressed EGFP-CAR was seen primarily in hepatocyte cytoplasm (Fig. 1A). In contrast, dramatic translocation of cytoplasmic EGFP-CAR into hepatocyte nucleus occurred in response to both Wy-14,643 and ciprofibrate (Fig. 1, B and C). All of the cytoplasmic fluorescence shifted to the nucleus in a relatively rapid manner, and by 3 h post-injection all hepatocyte nuclei displayed intense fluorescence (Fig. 1, B and C). These hepatocytes revealed no detectable cytoplasmic fluorescence 3 h after Wy-14,643 or ciprofibrate injection, implying that the over-
PPARα Ligand-induced Nuclear Translocation of CAR

FIGURE 1. Expression and nuclear translocation of EGFP-CAR in wild-type mouse liver. A–C, PPARα−/− mice injected intravenously (tail vein) with Ad/EGFP-CAR were killed 5 days after infection. A, livers of mice not treated with a PPARα agonist. B and C, livers of mice given a single dose of PPARα agonist Wy-14,643 (250 mg/kg of body weight) (B) or ciprofibrate (250 mg/kg) (C) intraperitoneally 3 h before killing. To ascertain the persistence of CAR nuclear translocation, mice were also killed after 6 h (D), 12 h (E), and 24 h (F) Wy-14,643 treatment. Frozen sections of liver were analyzed for EGFP fluorescence and DAPI staining. The EGFP fluorescence (left panel), DAPI-staining (middle panel), and the merged images (right panel) are shown. In the absence of either Wy-14,643 or ciprofibrate, EGFP-CAR is localized almost exclusively in hepatocyte cytoplasm (A), whereas treatment with PPARα ligands Wy-14,643 (B, D, E, and F) and ciprofibrate (C) caused nuclear translocation of CAR. Nuclear fluorescence persisted for up to 24 h after Wy-14,643. Boxed areas represent selected groups of matching cells.

FIGURE 2. PPARα ligand-mediated nuclear translocation of EGFP-CAR in liver is independent of the presence of PPARα and AOX, but dependent on PBP. A–C, PPARα−/− mice were given Ad/EGFP-CAR by tail vein injection and killed 5 days later after a single dose of Wy-14,643 (B) or ciprofibrate (C) 3 h earlier as above. A, in the absence of PPARα ligands, EGFP-CAR reveals intense cytoplasmic fluorescence in hepatocytes. B and C, in the presence of PPARα ligands Wy-14,643 (B) or ciprofibrate (C), EGFP-CAR translocates to the hepatocyte nucleus in PPARα−/− mice, implying that the ability of these agents to drive CAR into nucleus is not dependent upon the presence of PPARα. AOX null mice (D and E) and PBP−/− (F and G) were given Ad/EGFP-CAR and killed 5 days later. Some mice were given a single dose of Wy-14,643, administered to some mice 3 h before killing to ascertain CAR translocation. No spontaneous translocation of CAR into hepatocyte nucleus occurred in the AOX−/− mice, which exhibit sustained PPARα activation due to unmetabolized natural PPARα ligands, suggesting activation of PPAR and translocation of CAR are not interlinked. However, administration of Wy-14,643 to AOX null mice resulted in the translocation of CAR into hepatocyte nucleus (E). F and G, transcription coactivator PBP is required for PPARα ligand Wy-14,643-mediated nuclear translocation of EGFP-CAR. Wy-14,643 failed to cause CAR translocation in PBP null hepatocytes (G). The EGFP fluorescence (left panel), DAPI-staining (middle panel), and the merged images (right panel) are shown. CAR translocation into the nucleus occur in the absence of AOX but did not occur in the absence of coactivator PBP.

abundantly expressed EGFP-CAR is efficiently driven to the nucleus by PPARα ligands (Fig. 1, B and C). The translocation of CAR into nucleus caused by PPARα ligands is reminiscent of nuclear translocation induced by CAR activator phenobarbital or TCPOBOP (26, 42). The translocated CAR nuclear fluorescence mediated by PPARα ligand was also seen in the liver of mice killed at 6, 12, and 24 h after Wy-14,643 injection suggesting retention of CAR in the nucleus (Fig. 1, D–F). Translocation of CAR into the nucleus was also induced by clofibrate, gemfibrozil, and fenofibrate, the more commonly used PPARα agonists (supplemental Fig. 2).

PPARα Ligand-induced Nuclear Translocation of CAR Is Independent of PPARα and PPARβ/δ—Wy-14,643 and ciprofibrate are PPARα activators, and the PPARα/RXR heterodimers bind to PPREs in target genes to elicit transcription (9). To examine whether the PPARα ligand-induced nuclear translocation of CAR is dependent upon the presence and activation of PPARα, we used PPARα gene disrupted mice (8). Translocation of exogenously expressed CAR into hepatocyte nucleus occurred in PPARα−/− mouse hepatocytes with Wy-14,643 and ciprofibrate (Fig. 2, A–C), indicating that PPARα ligand-induced CAR nuclear translocation is independent of PPARα. Wy-14,643 treatment also caused translocation of endogenous CAR into the nucleus (supplemental Fig. 1D). These observations suggest that PPARα does not serve as a chaperone for CAR and that it is not needed for possible retention of CAR in the nucleus. The doses of PPARα agonists used in this study were chosen for the maximal induction of fatty acid β-oxidation system enzymes in liver after a single injection (39). This might be of some concern because PPAR agonists in general are isotype-selective but not isotype-specific (6, 7, 53). Therefore, it is possible that PPARβ/δ, which is also expressed in liver, might be activated at those doses. We addressed this issue by testing CAR nuclear translocation in PPARβ/δ null mouse liver (46) by PPARα in agonists Wy-14,643 and ciprofibrate. Nuclear translocation of EGFP-CAR occurred in null mouse hepatocytes indicating that PPARβ/δ, like PPARα, is not responsible for CAR translocation (not illustrated).
PPARα Ligand-induced Nuclear Translocation of CAR

We examined whether sustained activation of PPARα by natural ligands, as noted in fatty acyl-CoA-deficient (AOX−/−) mouse liver (45, 47), leads to the translocation of exogenous CAR into the nucleus. We did not detect CAR nuclear translocation, implying that the concentrations of natural ligands capable of activating PPARα in a sustained manner fail to cause CAR nuclear translocation (Fig. 2D). On the other hand, PPARα synthetic ligand, Wy-14,643, efficiently induced CAR nuclear translocation in fatty acyl-CoA oxidase (AOX) null hepatocytes (Fig. 2E).

**PPARα Ligand-induced Nuclear Translocation of CAR Requires Transcription Coactivator PBP but Not PRIP and SRC-1—**The binding of specific ligand to a nuclear receptor results in the recruitment of several nuclear receptor coactivator complexes to the receptor bound DNA elements in target gene promoter (10, 12, 19, 21, 22). These coactivator complexes are necessary for priming the target gene for transcription, but there is limited information regarding the gene-specific function of a given coactivator (36, 48, 50). Previously, we demonstrated that phenobarbital-induced nuclear translocation of CAR does not occur in mouse hepatocytes deficient in the transcription coactivator PBP but occurs in the absence of PRIP (36, 42, 50). We now examined the role of coactivator PBP in PPARα ligand-induced translocation of CAR into the nucleus using mice with conditional disruption of PBP gene (PBPΔliv) in hepatocytes (48). PBP gene-disrupted hepatocytes revealed abundant expression of EGFP-CAR in the cytoplasm after Ad/EGFP-CAR injection (Fig. 2F). PPARα ligand administration did not alter the cytoplasmic localization of the recombinant CAR in these PBPΔliv hepatocytes, and no EGFP-CAR fluorescence appeared in hepatocyte nucleus (Fig. 2G). DAPI staining and merger of images confirmed the failure of CAR to translocate into the nucleus in the absence of PBP. We also determined the role of two other transcription coactivators, SRC-1 and PRIP, in the PPARα ligand-induced nuclear translocation of CAR (Fig. 3, A–D). Liver cells deficient in SRC-1 (SRC-1−/−) (49) or PRIP (PRIPΔliv) (50) revealed cytoplasmic fluorescence after Ad/EGFP-CAR in the absence of treatment with a PPARα ligand (Fig. 3, A and C). In contrast, intense nuclear fluorescence was seen in these SRC-1−/− and PRIPΔliv hepatocytes 3 h after Wy-14,643 treatment, implying that neither PRIP nor SRC-1 is required for PPARα ligand-mediated CAR nuclear translocation (Fig. 3, B and D). We further determined the ability of androstenol, a known CAR antagonist (40), and artemisinin, an antimalarial sesquiterpene lactone endoperoxide that is capable of up-regulating Cyp2b10 (54, 55), to induce nuclear translocation of CAR using exogenous Ad/EGFP-CAR expression approach (42). Both androstenol and artemisinin induced nuclear translocation of EGFP-CAR in mouse liver as compared with wild-type mouse livers (data not shown) after Wy-14,643 treatment, but no such increase was noted with Wy-14,643 and ciprofibrate (Fig. 4A). Artemisinin-mediated CAR nuclear translocation appeared independent of the presence of coactivator SRC-1 (Fig. 3G). These observations with genetically altered mouse lineages (PBP, PRIP, and SRC-1 null livers) clearly establish that PBP is required for nuclear translocation of CAR but not PRIP and SRC-1.

**HepG2 and Hep3B Cells Reveal Spontaneous CAR Nuclear Translocation—**To determine whether nuclear translocation of CAR can be studied in human cells, we utilized two established human hepatocellular carcinoma cell lines, HepG2 and Hep3B. These two cell lines exogenous EGFP-CAR translocated to the nucleus spontaneously (supplemental Fig. 3, A and B).

**PPARα Ligand-induced Nuclear Translocation of CAR Is Not Associated with Transcriptional Activation of CAR Target Genes—**Transcription of CAR target genes such as Cyp2b10 in liver by phenobarbital-like agonists requires the translocation of cytoplasmic CAR into the nucleus (26, 32, 56, 57). We ascertained whether the nuclear translocation of CAR resulting from the administration of PPARα ligands leads to the induction of relevant Cyp genes in mouse liver. As expected, phenobarbital-induced CAR nuclear translocation is associated with a robust increase in Cyp2b10 mRNA content in the livers of wild-type (Fig. 4A) and PPARα−/− mice (data not shown). In contrast, the mRNA concentration of this CAR target gene was not altered in PPARα−/− and PPARα−/− mouse livers (data not shown) after PPARα ligand treatment despite the nuclear localization of CAR. A slight increase in Cyp3a11 mRNA level occurred in the mouse liver after phenobarbital administration, but no such increase was noted with Wy-14,643 and ciprofibrate (Fig. 4A). Phenobarbital-mediated Cyp3a11 induction appeared slightly greater in PPARα−/− mouse liver as compared with wild-type PPARα−/− mice.
animals. Wy-14,643 treatment resulted in a slight reduction in Cyp3a11 in wild-type mouse liver (Fig. 4A). We also examined the changes in mRNA levels of PPARα-regulated microsomal \( \omega \)-oxidation system (Cyp4a1 and Cyp4a3) and peroxisomal fatty acid \( \beta \)-oxidation system (AOX, \( \beta \)-bifunctional enzyme, and peroxisomal thiolase) genes to show that PPARα ligands increase the level of expression of these genes in wild-type mouse liver and not in PPARα gene-disrupted mouse liver (1, 8, 38). Phenobarbital treatment did not alter the expression of these PPARα-regulated genes (data not shown). Immunoblot analysis confirmed the increase in the amount of all three peroxisomal \( \beta \)-oxidation system genes in wild-type mouse liver (data not shown). No increase in fatty acid \( \beta \)-oxidation genes was seen in phenobarbital-treated wild-type mouse. As expected, these PPARα-regulated genes were not induced in PPARα null mouse livers (data not shown).

**Pretreatment with PPARα Ligands Does Not Enhance Acetaminophen Hepatotoxicity**—CAR activators phenobarbital and TCPOBOP enhance the conversion of hepatotoxic drug acetaminophen into its toxic intermediate metabolite, \( N \)-acetyl-\( \beta \)-benzoquinoneimine (23, 27, 56, 57). Accordingly, pretreatment of mice with CAR agonists enhances the hepatotoxic effects of acetaminophen as these agonists influence the translocation of CAR into the nucleus to transcriptionally activate genes that play a role in acetaminophen metabolism (27, 56, 57). Although translocation of CAR into hepatocyte nuclei was induced by PPARα ligands, there was no accompanying induction of CAR target genes such as Cyp2b10 to accelerate the conversion of acetaminophen into a toxic intermediate. Nonetheless, it appeared necessary to determine whether the translocation of CAR resulting from PPARα ligand administration enhances the hepatotoxic effect of acetaminophen. Nuclear translocation of CAR caused by PPARα ligands fails to increase acetaminophen hepatotoxicity (supplemental Fig. 4). Acetaminophen at a dose of 250 mg/kg of body weight failed to induce centrilobular hepatocyte necrosis but at a higher dose (700 mg/kg) produced substantial centrilobular necrosis (supplemental Fig. 4, A and B). Pretreatment of wild-type mice with CAR agonist phenobarbital resulted in massive hepatic necrosis even with the low dose acetaminophen (250 mg/kg) (supplemental Fig. 4, C and D). On the other hand, pretreatment with a PPARα ligand Wy-14,643 failed to increase acetaminophen hepatotoxicity in wild-type mice (supplemental Fig. 4, E and F). Of interest is that acetaminophen at 250 mg/kg of body weight did not induce hepatic necrosis in mice given both phenobarbital and Wy-14,643, implying a possible protective role for PPARα ligand in the prevention of acetaminophen-induced hepatic necrosis (supplemental Fig. 4G). Essentially similar results were obtained with PPARα/− mice (supplemental Fig. 4, H–J). Wild-type mice given a PPARα agonist exhibited resistance to acetaminophen hepatotoxicity, implying that these agonists may exert both positive and negative influence on the induction of drug metabolizing enzymes (58). Mice pretreated with artemisinin showed hepatic necrosis after acetaminophen injection, which is consistent with the ability of this compound to induce CAR target genes (supplemental Fig. 4, K and L; Figs. 4A and 5). As shown in Fig. 4, both phenobarbital and artemisinin induced Cyp2b10, Cyp3a11, and Cyp1a2, whereas PPARα ligands and androstenol were ineffectual. We examined whether PPARα ligands abrogate CAR agonist-induced Cyp2b10 level (Fig. 4B). Reduction in the levels of Cyp2b10, Cyp3a11, and Cyp1a2 mRNA induction is evident when CAR agonist is administered along with Wy-14,643 or ciprofibrate (Fig. 4B). Androstenol reduced the basal levels of Cyp2b10 mRNA in liver and nearly abrogated the phenobarbital-mediated induction (data not shown) (59). Furthermore, PPARα agonists caused a reduction in the induction of Cyp genes resulting from phenobarbital treatment (Fig. 4B). Because both Wy-14,643 and androstenol failed to induce CAR target genes despite their ability to induce CAR nuclear translocation, it became necessary to examine whether the CAR antagonist androstenol might also function as PPARα ligand similar to Wy-14,643. As illustrated in Fig. 4C, androstenol did not induce PPARα target genes responsible for peroxisomal fatty acid \( \beta \)-oxidation (39). When administered in combination with Wy-14,643, androstenol caused a modest reduction in the
induction by Wy-14,643 (Fig. 4C). For these co-administration studies, we used Wy-14,643 at 250 mg/kg of body weight, a dose that induces maximal induction of PPARα target genes (Fig. 4D). The mRNA levels of all three peroxisomal β-oxidation system genes were up-regulated in liver after PPARα ligand treatment but not after androstenol, suggesting that this compound does not function as PPARα ligand.

Serum alanine aminotransferase activity, used as a marker of hepatocyte necrosis, was high in mice given acetaminophen after phenobarbital pretreatment or artemisinin but not in mice pretreated with Wy-14,643 (Fig. 5). No increase in serum alanine level was observed in mice pretreated with both phenobarbital and Wy-14,643 given acetaminophen (Fig. 5). Accordingly, these observations clearly establish that nuclear translocation of CAR is not always reflective of the induction of drug metabolizing enzymes and hepatotoxicity.

Coactivator Binding to CAR Ligand Binding Domain Is Reduced by PPARα Ligands—The failure of PPARα ligands to induce CAR target gene activation despite their ability to translocate the receptor into the nucleus suggested that these chemicals might be functioning as CAR inverse agonists (antagonists). Therefore, we determined the effect of PPARα ligands on coactivator binding to CAR ligand binding domain (Fig. 6A). For this purpose we used an Amplified Luminescence Proximity Homogeneous Assay (AlphaScreen, PerkinElmer Life Sciences) to measure the effect of ligands on the CAR recruitment of coactivator peptide containing the TIF2/SRC-2 third LXXLL motif (51). In the absence of any ligands, CAR exhibits a high basal interaction with the TIF2 coactivator motif, which is consistent with the constitutive activity of CAR (51). The addition of TCPOBOP, a CAR agonist, further increased the TIF2-CAR interaction (Fig. 6A). In contrast, the addition of androstenol, a CAR antagonist, decreased the TIF2-CAR interaction. The addition of various concentrations of the PPARα compounds ciprofibrate and Wy-14,643 shows a progressive reduction of CAR-TIF2 interactions, with an IC₅₀ values of 100–500 μM, suggesting that both compounds acted as CAR antagonists to inhibit coactivator recruitment by CAR. Furthermore, GST pulldown assays using the PBP fragment that contains both LXXLL motifs (PBP-(440–740)) of this protein revealed that both compounds acted as CAR antagonists of PBP recruitment by CAR. The addition of PPARα ligands diminished TCPOBOP-mediated PBP-CAR interaction. C–E, transactivation using HepG2 cells. HepG2 cells transfected with pEGFP-CAR revealed similar transfection efficiency (>25%) in the absence (C) and presence (D) of CAR ligand. E, transactivation was measured using PBR2G-CAR and pCMV-RL, which expresses Renilla luciferase. Values for firefly luciferase were normalized. F, chromatin immunoprecipitation assays to show recruitment of CAR and PBP to the phenobarbital response unit in wild-type mouse liver. Wy-14,643, ciprofibrate (Cipro), and androstenol (An) decreased the binding of CAR to the phenobarbital response unit. No detectable PBP recruitment was found in the presence of Wy-14,643, ciprofibrate, and androstenol.

**FIGURE 6.** PPARα ligands on CAR function. A, PPARα ligands Wy-14,643 and ciprofibrate reduce coactivator TIF2/SRC-2 binding to CAR. The effect of PPARα ligands on coactivator binding to CAR LBD was determined by an AlphaScreen assay using a hexahistidine detection kit from PerkinElmer Life Sciences. CAR ligand TCPOBOP enhanced TIF2/SRC-2 binding to CAR, but the addition of androstenol, a CAR antagonist, decreased the TIF2-CAR interaction. The addition of various concentrations of PPARα ligands also caused progressive reduction in TIF2-CAR interaction. Data were derived from three repeated experiments with S.E., typically less than 10% of the measurements. B, GST pulldown assay to assess CAR interaction with coactivator peptide PBP-(440–740) containing both LXXLL motifs. Interaction of in vitro-translated [35S]-labeled CAR with bacterially expressed GST-PBP-(440–740) or GST alone in the presence of Wy-14,643 (a) or ciprofibrate (b) and/or TCPOBOP, as indicated. C, CAR ligand TCPOBOP (TC) enhanced PBP-CAR interaction, but PPARα ligands failed to facilitate this interaction. The addition of Wy-14,643 or ciprofibrate diminished the TCPOBOP-mediated PBP-CAR interaction. C–E, transactivation using HepG2 cells. HepG2 cells transfected with pEGFP-CAR revealed similar transfection efficiency (>25%) in the absence (C) and presence (D) of CAR ligand. E, transactivation was measured using PBR2G-CAR and pCMV-RL, which expresses Renilla luciferase. Values for firefly luciferase were normalized. F, chromatin immunoprecipitation assays to show recruitment of CAR and PBP to the phenobarbital response unit in wild-type mouse liver. Wy-14,643, ciprofibrate (Cipro), and androstenol (An) decreased the binding of CAR to the phenobarbital response unit. No detectable PBP recruitment was found in the presence of Wy-14,643, ciprofibrate, and androstenol.
PPARα Ligand-induced Nuclear Translocation of CAR

CAR binding to this coactivator (Fig. 6B). Supplemental Fig. S5 shows the binding of PPARα and CAR to their heterodimerization partner RXR in the presence and absence of CAR and PPARα ligands. PPARα binding to RXR was not influenced by CAR ligand TCPOBOP but enhanced in the presence of PPARα ligand Wy-14,643 (supplemental Fig. S5A). A slight reduction in the binding of CAR to RXR was discerned in the presence of PPARα ligand Wy-14,643 at 2 mM concentration (supplemental Fig. S5B). A gel shift assay showed that CAR/RXR heterodimer bound to both HD-PPRE (supplemental Fig. S5C) (60) and PBREM (supplemental Fig. S5D). PPARα/RXR heterodimer bound to PPRE but not to PBREM (supplemental Fig. S5, C and D).

Finally, transactivation assays were performed using HepG2 cells transfected with pEGFP-CAR. pEGFP-CAR expression enabled the assessment of the degree of transfection (Fig. 6, C–E). Transfection efficiency exceeded >25% in the HepG2 cells. pEGFP-CAR fluorescence was predominantly limited to HepG2 cell nucleus in the absence of CAR ligand TCPOBOP. It is known that HepG2 cells lack the capability to retain CAR in the cytoplasm resulting in the spontaneous accumulation of this receptor in the nucleus (30). As shown above, exogenous EGFP-CAR also translocates to the nucleus in these cells (supplemental Fig. S3). CAR inverse agonist androstenol reduced CAR transactivation activity in the presence and absence of CAR ligand TCPOBOP (Fig. 6E). Similar inhibitory effect was observed with PPARα ligand Wy-14,643 as it failed to transactivate phenobarbital responsive unit in the transiently transfected HepG2 cells (Fig. 6E).

These two compounds with best score conformation were well fitted in the ligand binding pocket of CAR (Fig. 7, A and B). Both Wy-14,643 and ciprofibrate adapted a binding mode similar to that of androstenol, a CAR antagonist (Fig. 7C). Precious crystal structures of CAR bound to androstenol and TCPOBOP reveal the molecular mechanisms of antagonist and agonist for these two respective compounds (41, 61). TCPOBOP serves as a CAR agonist through the direct contacts by the C-ring of TCPOBOP to the CAR AF2 helix, the linker helix, and AF2 helix (61). These interactions are required to stabilize the CAR AF2 in the active configuration (Fig. 7D). Androstenol is smaller than TCPOBOP and does not occupy the C-ring space of TCPOBOP. The binding of androstenol leaves a void between the androstenol and helix 10 that is normally occupied by the C-ring of TCPOBOP, thus inducing the collapse of helix 10 that leads to inactive conformation of CAR. Superimposition of Wy-14,643 and ciprofibrate with TCPOBOP showed that neither Wy-14,643 nor ciprofibrate occupies space of the C-ring of TCPOBOP (41, 62). It is likely that both Wy-14,643 and ciprofibrate induce collapse of helix 10 that leads to antagonism of CAR in a manner similar to the action of androstenol.

DISCUSSION

In the absence of activation by an agonist, the constitutively active nuclear receptor CAR is confined to the cytoplasm in liver cells in a complex with Hsp90 and co-chaperone CAR cytoplasmic retention protein (CCRP) (23, 32, 42, 63). In response to CAR agonist such as TCPOBOP, which binds to the ligand binding domain of the receptor, this complex recruits

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays using liver chromatin from wild-type mice demonstrating the recruitment of CAR to the CAR target gene Cyp2b10 promoter region was increased in response to TCPOBOP but decreased after Wy-14,643, ciprofibrate, and androstenol. In the absence of TCPOBOP, coactivator PBP did not show any binding to the Cyp2b10 promoter region, but PBP was recruited in livers treated with TCPOBOP (Fig. 6F). No appreciable recruitment of PBP was noted in response to Wy-14,643, ciprofibrate, and androstenol, implying that under in vivo conditions Wy-14,643, ciprofibrate, and antrostenol do not play a role in PBP recruitment to Cyp2b10 promoter region, possibly accounting for their inability to induce this gene.

Molecular Modeling—Molecular modeling suggested that Wy-14,643 and ciprofibrate occupied the ligand binding pocket of CAR (Fig. 7).
protein phosphatase 2A, and this step enables the translocation of CAR into the nucleus (32). Nuclear translocation of CAR is an initial step of its activation by xenobiotics (25, 26, 30, 32). CAR activity is also modulated by indirect activators such as phenobarbital, which do not bind directly to the ligand binding domain of the receptor but increase the receptor activity by facilitating its nuclear translocation (23, 56, 63). The ability of CAR to activate target gene transcription can be blocked by inhibitors of Ca\(^{2+}\)/calmodulin kinase, although CAR nuclear translocation is not blocked (32, 34). Within the nucleus, CAR heterodimerizes with RXR, and the CAR-RXR heterodimers recruit p160/SRC-1 and other coactivator proteins (23, 26, 37, 49). The affinity of CAR-RXR heterodimers for coactivators is enhanced by an agonist (TCPOBOP) and an indirect activator (phenobarbital) resulting in the enhancement of enzyme system expression (41). The binding of inverse agonists to CAR and the resultant failure to recruit and retain coactivators reduce the constitutive activity of CAR (23, 56, 63). Finally, CAR activity can be down-regulated by yet to be identified classical agonists that do not possess intrinsic inhibitory activity in the absence of a bound agonist (23). In this regard, the inverse agonists are generally referred to as CAR antagonists.

Overall, relatively few CAR modulators are known despite the relative abundance of both natural and synthetic chemicals currently in use (23). Using the adeno-virally driven EGFP-CAR expression system, we recently demonstrated that exogenous CAR translocates into the nucleus of wild-type mouse hepatocytes after treatment with a direct agonist and an indirect activator of CAR under both in vivo and in vitro conditions (42, 50). This relatively simple system permits the visualization of cytoplasmic to nuclear translocation of exogenously expressed CAR within the hepatocyte (42). The availability of Ad/EGFP-CAR virus as well as genetically altered mice with disrupted PPAR\(\alpha\) genes permitted us to screen for compounds that facilitate cytoplasmic to nuclear translocation of CAR (8, 42, 45–50).

We now describe for the first time that PPAR\(\alpha\) ligands Wy-14,643 and ciprofibrate induce nuclear translocation of CAR in PPAR\(\alpha\)\(^{+/+}\) and PPAR\(\alpha\)\(^{-/-}\) mouse liver cells implying that CAR translocation/nuclear retention function of these agents is independent of PPAR\(\alpha\) participation. Because CAR nuclear location is needed for transcriptional enhancement of CAR regulated genes in liver (26, 56, 64), it was necessary to determine whether PPAR\(\alpha\) ligand–induced CAR translocation leads to functional activation of CAR target genes in liver. Our results showed that CAR translocation induced by PPAR\(\alpha\) ligands is not accompanied by transcriptional enhancement of CAR target genes, mainly Cyp2b10, in liver, suggesting that PPAR\(\alpha\) ligands function as CAR antagonists. Consistent with this observation is the finding that pretreatment of mice with PPAR\(\alpha\) ligands failed to enhance acetaminophen hepatotoxicity, further confirming the inability of these agents to transcriptionally activate CAR despite their ability to induce nuclear translocation of this receptor.

Previously we demonstrated that TCPOBOP and phenobarbital-inducible CAR nuclear translocation failed to occur in PBP-deficient (PBP\(^{ΔA14}\)) hepatocytes (36, 42). Furthermore, PBP gene deletion in liver abrogated acetaminophen hepatotoxicity, attributed in part to the failure of CAR nuclear translocation vis à vis the absence of CAR target gene induction (36). On the other hand, the absence of coactivator PRIP and SRC-1 failed to interfere with the nuclear translocation of CAR caused by CAR activators, implying that CAR activation is dependent upon recruitment and assembly of selective coactivators and that PBP is essential for CAR translocation and/or retention in the nucleus induced by CAR activators (Figs. 2 and 3). PBP contains two LXXLL nuclear receptor binding motifs necessary for interaction with the AF2 region of the nuclear receptor, and it is possible that nuclear retention of translocated CAR requires PBP as an anchor or scaffold (42). The data presented in this paper now show that PPAR\(\alpha\) ligand–induced nuclear translocation/retention of CAR is also dependent on coactivator PBP but not on PRIP and SRC-1 (Figs. 2 and 3). This is similar to that encountered with CAR agonists and indirect activators and that PBP seems essential for CAR translocation (42, 50).

The mechanism by which PPAR\(\alpha\) ligands induce nuclear translocation of CAR remains to be elucidated. The shift of cytoplasmic CAR into the nucleus may involve two steps, (i) transport into the nucleus and (ii) retention within the nucleus. It is possible that the PPAR\(\alpha\) ligands enhance nuclear translocation (import) of CAR by a PBP-dependent mechanism. The available data show that neither PPAR\(\alpha\) ligands nor CAR ligands induce CAR nuclear translocation in the absence of coactivator PBP, suggesting a role for this coactivator in shutting the cytoplasmic CAR into the nucleus (42, 50). Alternately, CAR bound to an agonist or antagonist requires the presence of PBP in the nucleus to strengthen the CAR-RXR heterodimer binding to specific DNA elements (36, 42). Although CAR agonists augment the assembly of coactivator complexes to the liganded nuclear receptor, CAR antagonists have been shown to hinder the coactivator recruitment (23, 37, 56). It is possible that PPAR\(\alpha\) ligands bind to CAR also inhibit coactivator assembly. Evidence shows that the absence of PBP in cells diminishes the recruitment or association of other cofactors necessary for transcription (36, 48). PBP and CAR interact robustly through the two LXXLL motifs of PBP, and CAR ligands enhance this interaction (36, 42). GST pulldown and coactivator binding assays reveal that PPAR\(\alpha\) ligands markedly diminish the CAR–coactivator interaction in vitro (Fig. 6, A and B). Based on these observations, it is reasonable to conclude that PBP is required for the import of agonist- or antagonist-bound CAR but not for nuclear retention. In the nucleus CAR functions differently, based on the conformational change induced by an agonist or antagonist, which determines the coactivator assembly necessary for transcription.

Our studies with PPAR\(\alpha\) ligands establish that CAR nuclear translocation is not reflective of transcriptional activation. Interestingly, the well known CAR inverse agonist (antagonist)
androstenediol also induces CAR nuclear translocation, although it is not a positive regulator of CAR (40). On the other hand, we demonstrate that antimalarial drug artemisinin induces translocation of CAR into the nucleus, which is consistent with the earlier biochemical findings (55). Pretreatment with artemisinin enhanced acetaminophen toxicity, raising the possibility that artemisinin treatment might predispose to hepatotoxic injury by xenobiotics in certain individuals. The data show that both agonists and antagonists of CAR induce nuclear translocation, but the function of this receptor depends on the ability of these ligands to interact with the ligand binding domain of the receptor and recruit coactivator proteins for the transcription to proceed (37, 41, 42). Binding of ligand TCPOBOP stabilizes the active-state conformation of helix 10/AF2, and this change favors coactivator binding. On the other hand, the binding of the inverse agonist disrupts the interactions of H12/AF2 with the ligand binding domain and interferes with coactivator assembly (41, 63). Molecular modeling observations reveal that PPARα ligands mimic the occupancy of androstenediol in CAR ligand binding pocket (41, 61). These results strongly suggest that PPARα ligands function as CAR antagonists (inverse agonists). Further studies are needed to establish the ability of PPARα ligands to displace radiolabeled androstenediol from CAR.

In summary, these studies reveal a potential cross-talk between PPARα and CAR in that PPARα ligands function as CAR antagonists. Previous studies have shown that cross-talk between PPARα and thyroid hormone receptor results from their common interaction with the heterodimeric partner RXR, resulting in mutual competition (65). The results reported here point to competition between PPARα and CAR occurring at the level of ligand. Thus, PPARα ligands influence two receptor signaling pathways similar to that shown recently with retinoic acid receptor (RAR) ligand retinoic acid, which is guided to RAR and PPARβ/δ to exert opposing effects (66). The biological significance of the PPARα agonists to serve as inverse agonists of CAR is not clear, but it is possible that inhibiting CAR function serves as a protective measure in PPARα ligand-induced pleiotropic responses. Fibric acids, which are PPARα ligands, have a greater affinity for PPARα than fibrate esters, whereas the fibrate esters were shown to exhibit greater affinity for nuclear receptor LXR (liver X receptor) (67). Further studies would be of interest to determine whether PPARα ligands exert enhanced responses in mice deficient in CAR in that the absence of CAR might increase the ability of these compounds to interact with PPARα.

Acknowledgments—We thank Drs. Frank Gonzalez for the PPARα knock-out mice, Byron Kemper for providing us the phenobarbital response unit plasmid, and Masahiko Negishi for critically reading this manuscript and for helpful discussions. We thank Dr. Reddy’s Laboratory, Hyderabad, for the generous gift of Wy-14,643 and Drs. Balachandra Diwan, Amedeo Columbano, and Stephen Safe for the TCPOBOP.

PENARTα Ligand-induced Nuclear Translocation of CAR

REFERENCES
1. Reddy, J. K. (2004) Am. J. Pathol. 164, 2305–2321
2. Reddy, J. K., and Hashimoto, T. (2001) Annu. Rev. Nutr. 21, 193–230
3. Reddy, J. K., and Krishnakanthan, T. P. (1975) Science 190, 787–789
4. Reddy, J. K., Azarnoff, D. L., and Hignite, C. (1980) Nature 283, 397–398
5. Isselam, I., and Green, S. (1990) Nature 347, 645–650
6. Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., and Wahl, W. (1992) Cell 68, 879–887
7. Michalik, L., Auwerx, J., Burger, J. P., Chatterjee, V. K., Glass, C. K., Gonzalez, F. I., Grimball, P. A., Kadawaki, T., Lazar, M. A., O’Rahilly, S., Palmer, C. N. A., Plutsky, J., Reddy, J. K., Spiegelman, B. M., Staels, B., and Wahl, W. (2006) Pharmacol. Rev. 58, 726–741
8. Lee, S. T. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez-Salgueiro, P. M., Westphal, H., and Gonzalez, F. J. (1995) Mol. Cell. Biol. 15, 3012–3022
9. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Cell 79, 1147–1156
10. Reddy, J. K., Guo, D., Jia, Y., Yu, S., and Rao, M. S. (2006) Advances in Developmental Biology (Taneja, R., ed), Vol. 16, pp. 389–420, Elsevier, San Diego, CA
11. Klevwer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) Nature 358, 771–774
12. Lonard, D. M., and O’Malley, B. W. (2006) Cell 125, 411–414
13. Rosenfeld, M. G., Lunyak, V. V., and Glass, C. K. (2006) Genes Dev. 20, 1405–1428
14. Roeder, R. G. (2005) FEBS Lett. 579, 909–915
15. Spiegelman, B. M., and Heinrich, R. (2004) Cell 119, 157–167
16. Fondell, J. D., Ge, H., and Roeder, R. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8329–8333
17. Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) Nature 398, 824–828
18. Ryu, S., Zhou, S., Ladurner, A. G., and Tijian, R. (1999) Nature 397, 446–450
19. Yu, S., and Reddy, J. K. (2007) Biochim. Biophys. Acta. 1771, 936–951
20. Bookout, A. L., Jeong, Y., Downes, M., Yu, R. T., Evans, R. M., and Mangelsdorf, D. J. (2006) Cell 126, 789–799
21. Hermanson, O., Glass, C. K., and Rosenfeld, M. G. (2002) Trends Endocrinol. Metab. 13, 55–60
22. McKenna, N. J., and O’Malley, B. W. (2002) Cell 108, 465–474
23. Chang, T. K. H., and Waxman, D. J. (2006) Drug Metab. Rev. 38, 51–73
24. Columbano, A., Ledda-Columbano, G. M., Pibri, M., Concas, D., Reddy, J. K., and Rao, M. S. (2001) Hapatology 34, 262–266
25. Honakoski, P., and Negishi, M. (1998) J. Biochem. Mol. Toxicol. 12, 3–9
26. Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, L., Washburn, K., and Negishi, M. (1999) Mol. Cell. Biol. 19, 6318–6322
27. Tazemli, I., Pissios, P., Schetz, E. G., and Moore, D. D. (2000) Mol. Cell. Biol. 20, 2951–2958
28. Wei, P., Zhang, J., Egan-Halley, M., Liang, S., and Moore, D. D. (2000) Nature 407, 920–923
29. Zhang, J., Huang, W., Chua, S. S., Wei, P., and Moore, D. D. (2002) Science 298, 422–424
30. Kobayashi, K., Sueyoshi, T., Inoue, K., Morre, R., and Negishi, M. (2003) Mol. Pharmacol. 64, 1069–1075
31. Koike, C., Moore, R., and Negishi, M. (2005) FEBS Lett. 579, 6733–6736
PPARα Ligand-induced Nuclear Translocation of CAR

Yoshinari, K., Kobayashi, K., Moore, R., Kawamoto, T., and Negishi, M. (2003) FEBS Lett. 548, 17–20

Hosseinpour, F., Moore, R., Negishi, M., and Suyoshi, T. (2006) Mol. Pharmacol. 69, 1095–1102

Kondo, Y., Suzuki, M., Miyazaki, Y., Matsuzaki, M., Nakamura, T., Kurose, K., Sawada, J.-I., and Inouye, Y. (2007) Biochim. Biophys. Acta 1773, 934–944

Xia, J., Liao, L., Sarkar, J., Matsumoto, K., Reddy, J. K., and Kemper, B. (2005) Arch. Biochem. Biophys., in press

Jia, Y., Guo, G. L., Surapureddi, S., Sarkar, J., Qi, C., Guo, D., Xia, J., Kashireddi, P., Yu, S., Cho, Y. W., Rao, M. S., Kemper, B., Ge, K., Gonzalez, F. J., and Reddy, J. K. (2007) Proc. Natl. Acad. Sci. U. S. A. 102, 12531–12536

Wright, E., Vincent, J., and Fernandez, E. J. (2004) J. Biol. Chem. 279, 24427–24434

Qi, C., Zhu, Y., Pan, J., Yeldandi, A. V., Rao, M. S., Maeda, N., Subbarao, V., Pulikuri, S., Hashimoto, T., and Reddy, J. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1585–1590

Sarkar, J., Qi, C., Guo, D., Ahmed, M. R., Jia, Y., Usuda, N., Rao, M. S., and Reddy, J. K. (2007) Gene Expr. 13, 255–269

Min, G., Kemper, J. K., and Kemper, B. (2002) J. Biol. Chem. 277, 26356–26363

Halgren, E. A. (1999) J. Comput. Chem. 20, 720–729

Desvergne, B., and Wahli, W. (1999) Endocr. Rev. 20, 649–688

Klayman, D. L. (1985) Science 228, 1049–1055

Simonsson, U. S. H., Lindell, M., Raffalli-Mathieu, F., Lannerbro, A., Honkakoski, P., and Lang, A. (2006) Eur. J. Clin. Invest. 36, 647–653

Swales, K., and Negishi, M. (2004) Mol. Endocrinol. 18, 1589–1598

Huang, W., Zhang, J., Washington, M., Liu, J., Parrant, J. M., Lazano, G., and Moore, D. D. (2005) Mol. Endocrinol. 19, 1646–1653

Manautou, J. E., Hoivik, D. J., Tveit, A., Hart, S. G., Khairallah, E. A., and Cohn, S. D. (1994) Toxicol. Appl. Pharmacol. 129, 252–263

Yamada, H., Yamaguchi, T., and Oguri, K. (2000) Biochem. Biophys. Res. Commun. 277, 66–71

Kassam, A., Winrow, C. J., Fernandez-Rachubinski, F., Capone, J. P., and Rachubinski, R. A. (2000) J. Biol. Chem. 275, 4345–4350

Suino, K., Peng, L., Reynolds, R., Li, Y., Cha, J. Y., Repa, J. J., Kliwer, S. A., and Xu, H. E. (2004) Mol. Cell 16, 907–917

Guo, D., Sarkar, J., Ahmed, M. R., Viswakarma, N., Jia, Y., Yu, S., Rao, M. S., and Reddy, J. K. (2006) Biochem. Biophys. Res. Commun. 347, 485–495

Xia, J., and Kemper, B. (2005) J. Biol. Chem. 280, 7285–7293

Zhu, Y., Qi, C., Jain, S., Rao, M. S., and Reddy, J. K. (1997) J. Biol. Chem. 272, 25500–25506

Fan, C.-Y., Pan, J., Chu, R., Lee, D., Kluckman, K. D., Usuda, N., Singh, L., Yeldandi, A. V., Rao, M. S., Maeda, N., and Reddy, J. K. (1996) J. Biol. Chem. 271, 24698–24710

Peters, J. M., Lee, S. S., Li, W., Ward, J. M., Gasviloa, O., Everett, C., Reitman, M. L., Hudson, L. D., and Gonzalez, F. J. (2000) Mol. Cell. Biol. 20, 5119–5128

Fan, C.-Y., Pan, J., Usuda, N., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (1998) J. Biol. Chem. 273, 15639–15645

Jia, Y., Qi, C., Kashireddi, P., Surapureddi, S., Zhu, Y., Rao, M. S., Rothi, D. L., Chambon, P., Gonzalez, F. J., and Reddy, J. K. (2004) J. Biol. Chem. 279, 24427–24434