Inhibitory Cross-talk between Estrogen Receptor (ER) and Constitutively Activated Androstane Receptor (CAR)

CAR INHIBITS ER-MEDIATED SIGNALING PATHWAY BY SQUELCHING p160 COACTIVATORS

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Gyesik Min‡§, Hwajin Kim‡, Yangjin Bae, Larry Petz, and Jongsook Kim Kemper¶
From the Department of Molecular and Integrative Physiology, University of Illinois, Urbana, Illinois 61801

Estrogen receptor (ER) activity can be modulated by the action of other nuclear receptors. To study whether ER activity is altered by orphan nuclear receptors that mediate the cellular response to xenobiotics, cross-talk between ER and constitutive androstane receptor (CAR), steroid and xenobiotic receptor, or peroxisome proliferator-activated receptor γ was examined in HepG2 cells. Of these receptors, CAR substantially inhibited ER-mediated transcriptional activity of the vitellogenin B1 promoter as well as a synthetic estrogen responsive element (ERE)-containing promoter. Treatment with an agonist of CAR, 1,4-bis-(2-(3,5-dichloropyridoxy)yl)benzene, potentiated CAR-mediated transcriptional repression. In contrast, an antagonist of CAR, androstenol, alleviated the repression effect. Although CAR interacted with the ER in solution, CAR did not interact with the ER bound to the ERE. CAR/retinoid X receptor bound to the ERE but with much lower affinity than ER. Incremental amounts of CAR elicited a progressive reduction of the ER activity induced by the p160 coactivator glucocorticoid receptor interacting protein 1 (GRIP-1). In turn, increasing amounts of GRIP-1 progressively reversed the depression of ER activity by CAR. An agonist or antagonist of CAR potentiated or alleviated, respectively, the CAR-mediated repression of the GRIP-1-enhanced ER activity, which is consistent with the ability of these ligands to increase or decrease, respectively, the interaction of CAR with GRIP-1. A CAR mutant that did not interact with GRIP-1 did not inhibit ER-mediated transactivation. Our data demonstrate that xenobiotic nuclear receptor CAR antagonizes ER-mediated transcriptional activity by squelching limiting amounts of p160 coactivator and imply that xenobiotics may influence ER function of female reproductive physiology, cell differentiation, tumorigenesis, and lipid metabolism.

The hormone estrogen mediates diverse biological effects in the cell. Estrogen plays a fundamental role in development and maintenance of female reproductive organs and is involved in the initiation and progression of tumors in these organs. In addition, estrogen has been implicated in the control of gene regulation unrelated to cell growth and reproduction, such as lipid metabolism in the liver (1, 2). Estrogen action is mediated by the nuclear receptor, estrogen receptor (ER),† which is a ligand-dependent transcription factor and consists of distinct modular domains with distinct biological functions (3, 4). Ligand-bound ER either binds to the ERE or interacts indirectly to the DNA by tethering to other transcriptional factors in estrogen-responsive target genes (5). Ligand binding induces a conformational change of the ER and recruits differential sets of coactivators or corepressors that determine biological activity by altering the magnitude of the transcriptional responses according to the physiological needs (6).

There is extensive evidence that ER-mediated transcriptional activity is modulated by actions of other nuclear receptors and transcription factors. Thyroid hormone receptor and PPARG have been shown to bind the ERE with high affinity and inhibit gene expression, perhaps by competing with the ER for binding to the ERE (7, 8). The xenobiotic nuclear receptor, aromatic hydrocarbon receptor, has also been shown to modulate ER activity in human breast and hepatic cell lines (9, 10). Progesterone receptor isoforms A and B have been shown to act as potent repressors for ER activity by interfering with the interaction of ER with the transcriptional machinery (11). Rather than inhibiting ER activity, polypeptide growth factors such as epidermal growth factor and insulin-like growth factor have been shown to stimulate ER-mediated transcription in an E2-independent manner (12).

Previous studies established that CAR influences steroid homeostasis through transcriptional regulation of CYP2B genes, which are steroid hydroxylases (13). Unlike classical nuclear receptors, transcriptional activity of CAR is ligand-independent (14). This constitutive activity can be inhibited by the testosterone metabolites, androstenol and androstanol (15). These androstanes antagonize ligand-independent transcription activation by decreasing the interaction of CAR with coactivators, such as GRIP-1 (16) or SRC-1 (15). CAR is sequestered in the cytoplasm, and treatment with agonists, such as TCPOBOP (17) and phenobarbital, results in the translocation of CAR into the nucleus (18), where it binds to its cognate recognition sites as a heterodimer with RXR. Some agonists of CAR also enhance transcriptional activity by promoting the interaction of CAR with the coactivator SRC-1 or GRIP-1 (15, 19).

† The abbreviations used are: ER, estrogen receptor; ERE, estrogen responsive element; CAR, constitutively activated androstane receptor; GRIP, glucocorticoid receptor interacting protein; SRC, steroid hormone receptor coactivator; TCPOBOP, 1,4-bis-(2-(3,5-dichloropyridoxyl))benzene; RXR, retinoid X receptor; SXR, steroid and xenobiotic sensor receptor; PPARG, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; GST, glutathione S-transferase.

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‡ These two authors contributed equally to this study.
§ Present address: Dept. of Microbiological Engineering, Jinju National University, Gyeongsangnam-Do Jinju-City, Chilam-Dong 150, Korea.
¶ To whom correspondence should be addressed. Tel.: 217-333-6317; Fax: 217x-333-1133; E-mail: jongsook@uiuc.edu.
Inhibitory Cross-talk between ER and CAR

In this study, we investigated the role of the xenobiotic orphan nuclear receptors, CAR, SXR, and PPARγ, in modulating ER activity. The liver is the major organ that metabolizes steroids and one of the target organs for estrogen action in the body. ERα as well as these liver-enriched orphan receptors and their heterodimeric partner RXR are all expressed in the liver (20). Recent studies showed that the p160 coactivators, such as SRC-1, GRIP-1, and SRC-3, are expressed in the liver (21, 22) and play a role in transcriptional activation mediated by the orphan receptors, CAR and SXR (16, 23). Therefore, we examined whether biological cross-talk between the ER and these xenobiotic nuclear receptors occurred in the hepatic HepG2 cell line.

We found that CAR significantly inhibited ER-mediated transactivation of the vitellogenin B1 promoter, as well as a synthetic ERE-driven promoter by a mechanism in which CAR squelches limiting amounts of p160 coactivators, such as SRC-1 and GRIP-1, which are essential for ER action.

**EXPERIMENTAL PROCEDURES**

**Materials and Plasmids—** The ligands moxestrol, rifampicin, and 9-cis-retinoic acid were purchased from Sigma. The synthetic ligand for PPARγ, rosiglitazone (BRL49653), was obtained from Glaxo-SmithKline. The mammalian expression plasmid pCDNA3CAR and the bacterial expression plasmids pETCAR and pGEX2TK-CAR have been described previously (16, 24). A CAR mutant in which 8 amino acids were deleted from the C terminus of the mouse wild-type CAR (25) was generated by PCR-mediated mutagenesis. The deletion mutation of the C terminus of the CAR vector was confirmed by sequencing, and the mammalian expression plasmid, pCDNA3 CAR mutant, was generated by substituting the EcoNI/EcoRI fragment of the mutant CAR vector for the corresponding fragment in the wild-type CAR vector. Purification of the GST-CAR or GST-CAR mutant, a BamHI/EcoRI fragment containing the CAR cDNA was inserted into pGEX2TK (Pharmacia Corp.) digested with the same enzymes. The mammalian expression plasmids pCMXSR and pCMXXRα were obtained from R. Evans, mammalian expression plasmids for PPARγ were from C. K. Glass and V. K. Chandra, and the GST-BRLBD containing the E, D, and F domains of the receptor were from B. Katzenellenbogen, and plasmids CMVRe 4ERE-tk-luciferase, and vitellogenin-luciferase were from D. Shapiro. Coactivator expression plasmids for SRC-1 and GRIP-1 were obtained from B. W. O’Mally and M. R. Stallcup, respectively.

**Cell Culture and Transfection—** HepG2 cells were maintained and transfected using LipofectAMINE 2000 ( Invitrogen) as described (16, 24). For transfection, the cells were seeded in 24-well plates, and 200 ng of ATC4 (4ERE-tk-luciferase) or 500 ng of vitellogenin-luciferase vector, 10 ng of pRL-SV40 for measuring the transfection efficiency, and varying amounts of expression plasmids for ER, CAR, CAR mutant, SXR, and PPARγ were added to each well. In some experiments, 10 ng of moxestrol, 5 μM of TCPOBOP, 4 μM of androstenediol, 10 μM of rifampicin, and 1 μM of BRL49653, which are ligands for the receptors, ER, CAR, SXR, and PPARγ, respectively, were added. The cells were incubated for 16–24 h after transfection, fresh medium containing the ligands was added, and the cells were incubated an additional 24 h. For coactivator analyses, the indicated amounts of expression plasmids for SRC-1 or GRIP-1 were added to the DNA mixture and processed as described above. Dual luciferase activities were measured as described by the manufacturer (Promega). Firefly luciferase activities were normalized to the Renilla luciferase values for each sample.

**GST Pull-down Assay—** The GST fusion proteins GST-E RLBD, GST- CAR, GST-CAR mutant, and GST-GRIP-1 were expressed in Escherichia coli BL21 (DE3 (pLyS)) and purified by binding to glutathione-Sepharose (Pharmacia Corp.) as described previously (24). 35S-Labeled proteins were synthesized by in vitro transcription and translation (TNT kit; Promega) according to the manufacturer’s instructions. The labeled proteins were preincubated by incubation with GST-bound glutathione-Sepharose at 4°C for 30 min. One μg of GST or GST fusion protein was incubated at 4°C for 2 h with 4 μl of the preclarified reticulocyte lysate containing the labeled proteins in 100 μl of binding buffer (25 mM Hepes-KOH, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml transester serum albumin, 10% glycerol, and 0.25% Nonidet P-40) in the presence of protease inhibitors. When appropriate, 100 ng E2, 100 nM TCPOBOP, and 100 μM androstenediol were added during the incubation. After the incubation, the samples were extensively washed and eluted with 20 μl reduced glutathione, the eluted proteins were separated by SDS-PAGE, and radioactivity was visualized by autoradiography.

**Gel Mobility Shift Assay and Antibody Supershift Assay—** Six-histidine tagged CAR (pET28CAR) and FLAG-tagged RXR were purified as described (24). Purified FLAG-tagged ERα protein, a generous gift from A. Nardulli, was expressed and purified from Sf9 cells infected with recombinant baculovirus provided by J. Kadonaga and W. L. Kraus. Gel mobility shift assays were carried out as described (24, 26). Briefly, the DNA probe was a 32P-labeled oligonucleotide containing the consensus ERE (AGGTCA-TGACCT) and 5,000–10,000 cpm were added to each reaction. Various amounts of ER or CAR/RXR were added to the reaction buffer (30 mM KCl, 15 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10% glycerol, 4 μM dithiothreitol, and 50 μM ZnCl2) in the presence of nonspecific carrier DNA such as poly(dI-dC) or salmon sperm DNA in a final volume of 20 μl. After incubation at room temperature, the resulting protein-DNA complex was fractionated on a nondenaturing acrylamide gel. For antibody supershift assays, 0.4–2 μg of antibodies were added after the incubation, and the reactions were incubated for an additional 5 min and processed as described above. Polyclonal antibody for CAR was made in a rabbit against purified mouse His-tagged CAR at the Immunological Center at the University of Illinois at Urbana Champaign as described (24). Antibodies for the ERα and RXR were purchased from Santa Cruz Biotechnology.

**RESULTS**

**CAR Antagonizes ER-mediated Transcriptional Activity—** To investigate whether functional cross-talk occurs between the ER and the xenobiotic orphan nuclear receptors, CAR, SXR, and PPARγ, transient transfection experiments were performed in HepG2 cells. A human ER mammalian expression plasmid was cotransfected with expression plasmids of the orphan nuclear receptor and a synthetic 4ERE-tk-luciferase reporter plasmid. We used moxestrol as a ligand for the ER in HepG2 cells because moxestrol is resistant to metabolism in hepatic cells. As shown in Fig. 1A, treatment with 10 nM moxestrol induced the ER-mediated 4ERE-tk luciferase activity over 500-fold when ER and CAR expression plasmids were cotransfected. TCPOBOP, an agonist for CAR, did not increase luciferase activity and thus is not a ligand for ERs in this assay system, nor does CAR activate the ER-containing promoter. Treatment with TCPOBOP potently inhibited the moxestrol dependent ER-mediated transactivation over 70% (Fig. 1A). In contrast, activation of SXR by rifampicin (10 μM) only modestly inhibited ER-mediated transactivation by 25% (Fig. 1B), and activation of PPARγ by BRL49653 (1 μM) did not affect ER-mediated transcriptional activity (Fig. 1C). Our results show that, of these xenobiotic receptors, CAR significantly inhibits luciferase reporter activity induced by moxestrol-activated ER.

To determine whether CAR represses ER-mediated transactivation in a dose-dependent manner, increasing amounts of CAR expression plasmid were cotransfected. Transfection of increasing amounts of the expression plasmid for CAR led to a progressive reduction of the promoter activity induced by the moxestrol-activated ER (Fig. 2, lanes 2–5). Intriguingly, an agonist ligand of CAR, TCPOBOP, substantially potentiated CAR-mediated repression of ER action (lanes 6–9). Consistent with this result, an antagonist of CAR, androstenediol, alleviated the CAR-mediated repression of the ER transcriptional activity (lanes 10–12). These results demonstrate that the orphan nuclear receptor CAR substantially inhibits activation of the 4ERE-containing luciferase reporter activity induced by moxestrol.
Inhibitory Cross-talk between ER and CAR

Fig. 1. Functional cross-talk between the ER and xenobiotic orphan nuclear receptors, CAR, SXR, and PPARγ. HepG2 cells were transfected with 250 ng of 4ERE-tk-luciferase vector, 10 ng of pRLSV40 for the internal control for transfection efficiency, and 2 ng of CMV-ERα to modulate CAR-mediated ER transcriptional activity. The values for firefly luciferase were normalized by dividing by the Renilla luciferase values. The standard errors of the mean were calculated from 12 independent determinations.

Fig. 2. Agonistic or antagonistic ligands of CAR, TCPOBOP, or androstenol, respectively, modulate CAR-mediated ER transcriptional repression. HepG2 cells were cotransfected with 250 ng of 4ERE-tk-luciferase, 10 ng of pRLSV40, 2 ng of CMV-ERα, and increasing amounts (10, 50, and 250 ng) of pCDNA3-CAR. Transfected cells were treated with vehicle (ETOH), 5 μM TCPOBOP (TCP), or 4 μM androstenol (AS) for 20 h as indicated. The cells were harvested for dual luciferase assays. The values for firefly luciferase were normalized by dividing by the Renilla luciferase values. The standard errors of the mean are indicated for six independent determinations. MOX, moxestrol.

Inhibitory Cross-talk between ER and CAR

Because the reporter 4ERE-tk-luciferase is a synthetic ERE-containing reporter plasmid, we also carried out similar experiments using a reporter that contains the Xenopus vitellogenin B1 promoter (27). The vitellogenin gene is a well studied estrogen-responsive gene expressed in hepatic cells. Luciferase reporter plasmid, vitellogenin-luciferase, contains a 610-bp fragment from the 5′-flanking region of the Xenopus vitellogenin B1 genomic clone and contains a functional ERE in this 610-bp fragment (28). HepG2 cells were cotransfected with vitellogenin-luciferase reporter and ER expression plasmids either in the presence or absence of CAR expression plasmids. Moxestrol led to a dramatic increase in ER-mediated transactivation of the vitellogenin-luciferase reporter activity (Fig. 3, lanes 1, 2, 5, and 6). Treatment of TCPOBOP alone did not induce ER-mediated transactivation (lanes 3 and 7) regardless of the presence of endogenous or exogenously transfected CAR. The combined treatment of moxestrol and TCPBOBOP repressed the moxestrol-dependent ER-mediated transactivation of the vitellogenin B1 promoter (lanes 2, 4, 6, and 8). An agonistic ligand for the CAR, TCPOBOP, potentiated the inhibitory effect mediated by either endogenous CAR or transfected CAR. When CAR was exogenously transfected, the CAR-mediated transcriptional repression of ER transactivation was more pronounced. In contrast, increasing amounts of transfected SXR or PPARγ expression plasmid did not elicit detectable changes in the ER-mediated transcriptional activity on the vitellogenin B1 promoter (data not shown). These results demonstrate that CAR suppresses the ER-mediated transcriptional activity in the ERE-containing vitellogenin B1 promoter as well as synthetic ERE promoter in HepG2 cells.

CAR Interacts with ERα in Vitro—To study the mechanism by which CAR could antagonize ER-mediated transactivation, GST pull-down experiments were carried out to examine whether ER physically interacts with CAR in vitro. The GST-ER LBD, which contains the D, E, and F domains of the ERα, was incubated with 35S-labeled CAR in the presence of ethanol, E2, TCPOBOP, or combined treatment of E2 and TCPOBOP. A strong interaction was observed between the GST-ER LBD and 35S-labeled CAR (Fig. 4A). Likewise, interaction between 35S-labeled full-length ER and GST-CAR was observed (Fig. 4B). Addition of ligands for the ER or CAR had little effect on the interaction (Fig. 4). These results demonstrate that CAR interacts with ERα in vitro in a ligand-independent manner. These GST pull-down assays suggest the possibility that CAR may inhibit the ER-mediated signaling pathway by interacting with ERα to form a functionally inactive...
Inhibitory Cross-talk between ER and CAR

CAR inhibits ER-mediated transcriptional activity of the vitellogenin promoter. HepG2 cells were transfected with 500 ng of a vector containing vitellogenin promoter fused to the luciferase reporter gene (vitellogenin-luciferase), 10 ng of pRLSV40, or 2 ng of pCDNA3-CAR. The ligands were added for 24 h after transfection as indicated. The values for firefly luciferase were normalized by dividing by the Renilla luciferase values. The standard errors of the mean were calculated from nine independent determinations from three transfection experiments. MOX, moxestrol; TCP, TCPOBOP.

**FIG. 3**. CAR inhibits ER-mediated transcriptional activity of the vitellogenin promoter. The nuclear receptors, thyroid hormone receptor, or PPARα has been shown to repress the ER activity by binding to the ERE site in Eβ-responsive genes (7, 8, 28). Likewise, CAR/RXR could potentially interfere with ER transcriptional activity by inhibiting ER binding to the ERE. To examine this possibility, gel mobility shift assays were carried out using purified recombinant proteins from E. coli or Sf9 insect cells. When 32P-labeled oligonucleotides containing the consensus ERE sequence were incubated with purified human ERα, one major protein-DNA complex was formed (Fig. 5A, lane 1). This major protein-DNA complex was supershifted by the ER-specific antibody (lane 3). The addition of purified CAR/RXR to this binding reaction resulted in an additional more rapidly migrating protein-DNA complex (lane 2). Antibody for ERα did not supershift this band (lane 3), but the band disappeared when antibody for CAR or RXR was added to the reaction (lanes 4 and 6). Preimmune serum from the rabbit in which polyclonal antibody was raised against CAR was used as a negative control for experiment, and the addition of preimmune serum did not supershift the band (lane 5). These experiments demonstrate that purified CAR/RXR proteins can bind to the ERE sequence. To further examine whether CAR/RXR expressed in transfected HepG2 cells can also bind to the ERE site, the gel mobility shift assay was carried out using HepG2 nuclear extracts. CAR/RXR from the transfected HepG2 cells was able to bind to the ERE, and the complex was antibody supershifted by antibody of CAR/RXR, confirming that CAR/RXR can bind to the consensus ERE in vitro (data not shown).

CAR in 100-fold Excess Does Not Inhibit ER Binding to the ERE and Does Not Interact with the ER Bound to the ERE—To determine whether CAR/RXR could inhibit ER binding to the ERE sequence, increasing amounts of CAR/RXR were added to the in vitro binding reaction containing a constant amount of ER. Incremental addition of CAR/RXR (100–500 fmol) did not inhibit ER binding (5 fmol of ER) to the ERE site (Fig. 5B, lanes 1–3). Conversely, increasing amounts of ER (2.5–20 fmol) added to the binding reaction containing constant amounts of CAR (500 fmol) elicited a progressive decrease in CAR/RXR binding (Fig. 5B, lanes 4–7). These results suggest that although CAR/RXR can bind to the ERE, this heterodimer binds to the ERE with much lower affinity than that of ER homodimers, and thus, CAR/RXR does not inhibit ER binding to the ERE site even when in great excess compared with ER. Therefore, it is unlikely that competition for binding to the ERE by CAR/RXR can explain the inhibition of ER action by CAR in the cell.

Our results from the GST pull-down experiments showed that CAR, but not RXR (data not shown), interacted with the ER in vitro. Although CAR could interact with the ER in solution, to mediate transcriptional repression of the ER activity by interacting with the ER, CAR must interact with the ER dimers in the ERE context. ER dimerization and/or binding of the ER to the ERE could either mask the ER interacting domain for the CAR or induce conformational change in the ER, so that interaction of ER with CAR could be altered. The addition of increasing amounts of CAR did not induce a protein-DNA complex with a slower mobility compared with that of ER in the native gel (data not shown). In turn, when increasing amounts of ER were added to the CAR/RXR binding reaction, a higher molecular weight protein-DNA complex was not formed (Fig. 5B). These results indicate that CAR did not interact with ER in the ERE context, although CAR strongly interacted with ER in the GST pull-down assay. These results suggest that CAR may inhibit the ER-mediated transactivation by mechanisms other than inhibiting the binding of the ER to DNA or by the formation of inactive protein-DNA complex. Thus, squelching the p160 coactivators, which are common essential coactivators for ER action as well as CAR action, is a remaining possibility.

**CAR Inhibits the ER-mediated Transactivation by Squelching the p160 Coactivators**—Both CAR and ER are activated by p160 coactivators, so that competing for these proteins could explain mutual inhibition. To analyze whether CAR squelches the p160 coactivator for the ER transcriptional activity in the cells, competition transfection experiments were performed. When GRIP-1 was cotransfected, the ER-mediated transcriptional activity was increased 2–3-fold (Fig. 6, lanes 3 and 4). When increasing amounts of CAR were transfected, GRIP-1-mediated transcriptional activation of the ER was progressively decreased (Fig. 6, lanes 5–7). Intriguingly, the reduction of GRIP-1-induced transcriptional enhancement was modulated by CAR ligands. The repression of GRIP-1-induced transactivation by CAR was increased by TCPOBOP, whereas it was modestly decreased by androstenediol (compare lanes 10–12 and 15–17). TCPOBOP-bound CAR efficiently blocked the GRIP-1-induced transcriptional activity mediated by ER, which suggests that competition for GRIP-1 may underlie the CAR-mediated inhibition.

To test this possibility, increasing amounts of GRIP-1 were cotransfected in the presence of agonist or antagonist of the CAR. Repression of the ER-mediated transactivation by CAR was gradually reversed by increasing amounts of GRIP-1 (Fig. 7). Consistent with a squelching mechanism, transfection of GRIP-1 to the androstenediol-treated cells derepressed the ER transcriptional activity more efficiently than the TCPOBOP-treated cells (compare lanes 3–8 and 9–14). Thus, increased or decreased interaction of GRIP-1 with CAR in the presence of TCPOBOP or androstenediol, respectively, correlates with increased or decreased inhibition of ER-mediated transactivation. Similar results were obtained with SRC-1 as well as

![Graph](http://www.jbc.org/)

**Fig. 3.** CAR inhibits ER-mediated transcriptional activity of the vitellogenin promoter. HepG2 cells were transfected with 500 ng of a vector containing vitellogenin promoter fused to the luciferase reporter gene (vitellogenin-luciferase), 10 ng of pRLSV40, or 2 ng of pCDNA3-CAR. The ligands were added for 24 h after transfection as indicated. The values for firefly luciferase were normalized by dividing by the Renilla luciferase values. The standard errors of the mean were calculated from nine independent determinations from three transfection experiments. MOX, moxestrol; TCP, TCPOBOP.
GRIP-1 (data not shown). Our transfection data imply that CAR interacts with p160 coactivators, such as GRIP-1 or SRC-1, in a ligand-modulating manner and thus antagonizes the ER-mediated transcriptional activity by competitively inhibiting the interaction of ER with p160 coactivators.

A CAR Mutant in Which the Interaction with GRIP-1 Was Abolished Did Not Block the ER Transcriptional Activity—To further test whether CAR inhibits the ER-mediated transactivation by squelching the p160 coactivator, a mutation was introduced into the CAR AF-2 region. Proper conformation of the C terminus of AF2 domain in nuclear receptors is essential for the transactivation function of nuclear receptors (29, 30). We deleted 8 amino acids from the C terminus of the CAR as reported previously (25) (Fig. 8A). As with the wild type CAR,
car, 500 ng). The ligands were added for 20 h as indicated: 10 nM of moxestrol, and increasing doses of pSG5-GRIP-1 (0, 5, 20, 100, and 4 μM of androstenol. The standard errors of the mean were calculated from six independent determinations. MOX, moxestrol; TCP, TCPOBOP; AS, androstenol.

![Figure 6](http://www.jbc.org/)

**Fig. 6.** CAR inhibits GRIP-1-enhanced ER transcriptional activity. HepG2 cells were cotransfected with 250 ng of 4ERE-tk-luciferase, 10 ng of pRLSV40, 2 ng of CMVERα, 50 ng of pSG5-GRIP-1, and increasing amounts of CAR (0, 10, 50, and 250 ng). The ligands were added for 20 h as indicated: 10 nM of moxestrol, 5 μM of TCPOBOP, and 4 μM of androstenol. The standard errors of the mean are indicated for six independent determinations. MOX, moxestrol; TCP, TCPOBOP; AS, androstenol.

![Figure 7](http://www.jbc.org/)

**Fig. 7.** GRIP-1 reverses the CAR-mediated inhibition of ER transactivation. HepG2 cells were cotransfected with 250 ng of 4ERE-Etk-luciferase, 10 ng of pRLSV40, 2 ng of CMV-ERα, 50 ng of pCDNA3-CAR, and increasing doses of pSG5-GRIP-1 (0, 5, 20, 100, and 500 ng). The ligands were added for 20 h as indicated: 10 nM of moxestrol, 5 μM of TCPOBOP, and 4 μM of androstenol. The standard errors of the mean are indicated for six independent determinations. MOX, moxestrol; TCP, TCPOBOP; AS, androstenol.

this mutant CAR heterodimerized with RXR and bound to the CAR/RXR binding site; however, transcriptional ability was completely abolished (25). This mutant CAR has also been reported to be expressed at levels similar to wild type (25). We first determined whether this mutant CAR is able to interact with the p160 coactivators. When GST-GRIP-1 was incubated with 35S-labeled wild type CAR or GST-CAR with 35S-GRIP-1, specific interaction occurred, and ligands of CAR modulated the interaction (Fig. 8, B and C), consistent with the result previously published (16). In contrast, when the GST-GRIP-1 was incubated with the mutant CAR or the GST mutant CAR with 35S-labeled GRIP-1, the interaction was completely abolished (Fig. 8, B and C).

To determine whether this mutant CAR represses the ER-mediated transactivation, competition cotransfection assays were carried out. Wild type CAR (10 ng) repressed the ER transactivation with or without exogenous GRIP-1 expression (Fig. 8D, lanes 1, 2, 5, and 6), consistent with the results presented above. In contrast, 50 or 250 ng of the transfected mutant CAR expression plasmid did not repress the ER activity (Fig. 8D, lanes 1, 3, 4, 5, 7, and 8). These studies show that a transcriptionally active CAR, which can interact with GRIP-1, sequesters the GRIP-1 to repress ER-mediated transactivation.

**DISCUSSION**

The liver is a major target organ for estrogen action, so that the liver-abundant xenobiotic orphan nuclear receptors, CAR, or SXR, have the potential to modulate steroid hormone homeostasis (20). On one level, these nuclear receptors may regulate estrogen action by virtue of their induction of the cytochrome P-450 drug metabolizing enzymes that alter hepatic metabolism of estrogens. On a second level, the receptors may more directly affect estrogen action by cross-regulation. These orphan receptors, for example, have been reported to interact with the p160 coactivator, SRC-1, which is also an essential transcriptional coactivator for ER action, so that the regulatory pathways of the two receptor systems have components in common (17, 23). These observations led us to a hypothesis that the xenobiotic orphan nuclear receptors may influence the ER-mediated signaling pathway in hepatic cells. Our results demonstrated that SXR very modestly repressed the ER-mediated transactivation, whereas the PPARy did not repress the ER-mediated transactivation in transfected human hepatic HepG2 cells. In contrast, cotransfection of increasing amounts of a CAR expression plasmid resulted in a substantial reduction of ER transactivation. Even without treatment with an agonistic ligand for CAR, TCPOBOP, CAR inhibited ER transcriptional activity, consistent with the constitutive activity of CAR (14). The inhibition of ER-mediated transactivation by CAR was increased by a CAR agonist, TCPOBOP (17), and was decreased by a CAR antagonist, androstenol. These results demonstrate that there is regulatory cross-talk between CAR and the ER. The modulation of the effect by CAR ligands indicates that the cross-talk is related to the transcriptional activity of CAR.

**CAR-mediated Inhibitory Mechanism of Transcriptional Activity of the ER—**Three potential mechanisms by which CAR could repress ER-mediated transcriptional activity in the HepG2 cells are: 1) formation of an inactive complex with ER by interacting directly with the ER, 2) competition for ER binding to the ERE, and 3) sequestering a coactivator of ER action, such as GRIP-1 or SRC-1. To determine whether CAR-mediated transcriptional repression of ER activity results from the inhibition of ER binding to the ERE site and/or the formation of the inactive heterodimer with ER by blocking the dimerization interface or coactivator interacting domain, we analyzed in vitro protein-DNA interaction studies using GST pull-down assays and gel mobility shift assay. Although CAR could interact with the ER in solution in GST pull-down assays, we were not able to detect an interaction of CAR with the ER when the ER was bound to the ERE. The first possibility of direct binding of CAR to the ER, therefore, is unlikely to explain the cross-regulation.

CAR is promiscuous in binding to a variety of nuclear receptor-binding sites (17, 31), so it was not too surprising that we found that transfected CAR/RXR from HepG2 nuclear extracts, as well as purified CAR/RXR, was able to bind to the consensus ERE. However, the binding affinity of CAR for the ERE was much less than that of the ER, so that a several hundred-fold excess of CAR did not affect binding of the ER to the ERE in vitro, whereas ER competed efficiently for CAR binding. Further, CAR ligands did not affect the binding of CAR to the ERE.
In contrast to their effects on inhibition of ER action (data not shown). It seems unlikely, therefore, that competition for binding to the ERE can explain the inhibition of ER action by CAR. The third possibility, the squelching of coactivators, seems the most likely possibility.

ER works with many other non-DNA-binding proteins in the regulation of gene expression. These coregulators play crucial roles in the ER action in the cells. They influence the magnitude of transcriptional activation or repression and alter the dose-responsive profiles to the ligands depending on the natures of the ligand (6). The involvement of coregulators, coactivators such as SRC-1, GRIP-1, and corepressors, such as SMRT (silencing mediator for retinoid and thyroid hormone receptor), nuclear receptor corepressor-1, in transcriptional regulation by the ER is now well established (32, 33). Therefore, we tested the possibility that CAR interferes with the ER transactivation by squelching the ER coactivators, GRIP-1 and SRC-1. As shown recently (16), the interaction of CAR with GRIP-1 and the transcriptional activity of CAR are increased and decreased by CAR agonists and antagonists, respectively. Similar modulation of the CAR-mediated inhibition of ER action by CAR ligands is consistent with competition for binding of these p160 coactivators between CAR and ER, resulting in squelching. Reversal of the CAR effect by increasing amounts of GRIP-1 further supports this possibility. The strongest evidence for this hypothesis is the loss in inhibition of ER action by CAR if the C-terminal 8 amino acids of CAR are deleted. This mutant retains the ability to bind to DNA (25) and is
Inhibitory Cross-talk between ER and CAR

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Inhibitory Cross-talk between Estrogen Receptor (ER) and Constitutively Activated Androstane Receptor (CAR): CAR INHIBITS ER-MEDIATED SIGNALING PATHWAY BY SQUELCHING p160 COACTIVATORS

Gyesik Min, Hwajin Kim, Yangjin Bae, Larry Petz and Jongsook Kim Kemper

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