The Ratio of Constitutive Androstane Receptor to Pregnane X Receptor Determines the Activity of Guggulsterone against the Cyp2b10 Promoter

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ABSTRACT

Guggulsterone is the active ingredient in gugulipid, an organic extract of the Commiphora mukul plant. Gugulipid has been used for nearly 3000 years in Ayurvedic medicine, mainly as a treatment for arthritis. Herbal practitioners currently use gugulipid therapy in conditions as diverse as rheumatism, coronary artery disease, arthritis, hyperlipidemia, acne, and obesity. The active ingredient in gugulipid is guggulsterone, a plant sterol compound recently identified as a pregnane X receptor (PXR; NR1I2) ligand. We show herein that guggulsterone treatment represses the expression of cytochrome P450 2b10 (Cyp2b10) gene expression by inhibiting constitutive androstane receptor (CAR; NR1I3) activity in hepatocytes lacking functional PXR (PXR-knockout). We also show that PXR-CAR cross-talk determines the net activity of guggulsterone treatment toward Cyp2b10 gene expression. Using mammalian two-hybrid assays, we show that treatment with guggulsterone differentially affects protein cofactor recruitment to these two nuclear receptors. These data identify guggulsterone as an inverse agonist of the nuclear receptor CAR. When viewed together with the data showing that PXR and CAR expression is highly variable in different ethnic populations and that CAR expression is under the control of a circadian rhythm, our data provide important insight into the molecular mechanism of interindividual variability of drug metabolism. These data, together with the recent resolution of the crystal structures of PXR and CAR, will likely aid in the rational design of more specific CAR inverse agonists that are currently viewed as potential antiobesity drugs.

Gugulipid is an herbal remedy derived from the gum resin of the Commiphora mukul tree. Gugulipid has been used in Ayurvedic medicine mainly to treat arthritis and inflammation. A number of clinical trials performed in India determined that gugulipid treatment produces favorable lipid profiles in patients with high cholesterol (Agarwal et al., 1986; Gopal et al., 1986; Nityanand et al., 1989). This finding has led to the widespread import and use of gugulipid extract in humans as a cholesterol-lowering agent sold in nutrition centers without a prescription in western societies. A recent human clinical trial has raised questions regarding the efficacy of gugulipid extract in lowering serum cholesterol (Sappary et al., 2003). Moreover, our laboratory has recently demonstrated that the use of gugulipid induces the expression and activity of drug-metabolizing cytochrome P450 genes through activation of the nuclear receptor protein PXR in cells (Brobst et al., 2004). The notion that gugulipid produces herb-drug interactions in humans is further supported by the results of a small, well controlled study that revealed significantly reduced (P < 0.01) peak plasma concentrations of either diltiazem or propranolol in volunteer patients cofed 1 g of gugulipid in comparison with those that received one of these drugs in combination with a placebo (Dalvi et al., 1994).

The active ingredients in gugulipid are the ketosteroids cis- and trans-4,17(20)-pregnadiene-3,16-dione, also known as E- and Z-guggulsterone, respectively (Tripathi et al., 1984; Tripathi et al., 1988; Beg et al., 1996; Verma et al., 1998). Recent experiments in mice lacking the farnesoid X receptor suggest that guggulsterones lower hepatic cholesterol levels in rodents by antagonizing the activity of this nuclear receptor (Urizar et al., 2002). Other research suggests that guggulsterone is a selective farnesoid X receptor modulator that differentially regulates the expression of a subset of target genes (Cui et al., 2003). Additional research has demonstrated that guggulsterone interacts with multiple nuclear receptor superfamilies, including the estrogen, mineralocorticoid, progesterone, and androgen receptors (Brobst...
et al., 2004; Burris et al., 2005). Together, these data indicate that the molecular basis of guggulipid’s biological activity is more complex than previously recognized.

PXR is a xenobiotic sensor that is activated by a large number of structurally diverse compounds (for review, see Kliewer, 2003). CAR was originally identified as xenobiotic sensor that, together with PXR, regulates drug-inducible expression of CYP2B and CYP3A genes in liver (Honkakoski et al., 1998). A number of studies have shown that PXR and CAR share ligands and target genes (Moore et al., 2000; Maglighi et al., 2002; Wei et al., 2002). Analysis of the human orthologues of these two cytochrome P450 genes reveals that CAR and PXR compete for the same or overlapping enhancer sequences located in the promoters of these two genes (Xie et al., 2000; Goodwin et al., 2001). More recently, CAR has been distinguished from PXR in that CAR seems to be activated in response to metabolic and nutritional stress, whereas PXR is not (Maglighi et al., 2004). Moreover, CAR plays a role in the regulation of the expression of genes involved in the metabolism of fatty acids and glucose homeostasis, whereas PXR does not (Ueda et al., 2002). The finding that activation of CAR in mice lacking PXR (PXR-KO) produces much higher levels of induction of shared target genes in liver suggests that the presence of PXR might affect the functional regulation of shared CAR-PXR-target genes in vivo (Staudinger et al., 2003).

We show herein that that both cis- and trans-guggulsterone function as efficacious CAR inverse agonists. Our data reveal that guggulsterone represses Cyp2b10 expression in PXR-KO hepatocytes but not in wild-type hepatocytes. We demonstrate that PXR-CAR cross-talk determines the net effect of guggulsterone treatment toward the Cyp2b10 promoter. Moreover, we show that in the presence of guggulsterone, the ratio of PXR to CAR determines the net activity of these two receptors by regulating the amount of the steroid receptor coactivator protein-1 (SRC-1) associated with either PXR or CAR. Together, our studies underscore the complexity of regulation of PXR- and CAR-shared target gene networks and provide important information on the potential mechanism of interindvidual variability in drug metabolism. These data together with the recently solved crystal structures of PXR and CAR (Watkins et al., 2001; Shan et al., 2004; Suino et al., 2004; Xu et al., 2004) will likely aid in the rational design of more specific CAR inverse agonists, which due to their likely ability to modulate serum thyroid hormone levels are viewed as potential antiobesity drugs (Maglighi et al., 2004; Qatanani et al., 2005).

Materials and Methods

Animal Care. Generation of the PXR knockout mice was described previously (Staudinger et al., 2001). All rodents were maintained on standard laboratory chow and were allowed food and water ad libitum. The studies reported herein have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Plasmids and Chemicals. The full-length mouse CAR and mouse PXR mammalian expression vectors were described previously. VP16-mPXR was described previously (Ding and Staudinger, 2005a,b). Cyp2b10-Luc was described previously (Rivera-Rivera et al., 2003). VP16-mCAR encodes full-length mouse CAR fused to VP16 (BD Biosciences, Palo Alto, CA). GAL-SRC-1 was a generous gift from Dr. Barry Forman (The Beckman Research Institute, Duarte, CA). The SV-β-Gal plasmids are commercially available (Invitrogen, Carlsbad, CA). All compounds were purchased from Sigma-Aldrich (St. Louis, MO) except guggulsterone (Steraloids, Newport, RI) and were dissolved as 1000× stocks in DMSO.

Cell Culture and Transient Transfection of CV-1 Cells. CV-1 cells were plated on 96-well plates as described previously (Ding and Staudinger, 2005a). For the Cyp2b10-LUC reporter gene assays, each well was transfected with 20 ng of reporter gene, 5 ng of nuclear receptor expression vector(s) unless otherwise indicated, 40 ng of SV-β-Gal, and was added with pBlueScript to 110 ng of total DNA per well. The mammalian two-hybrid system analysis was performed as described previously (Ding and Staudinger, 2005b). Transfection mixtures contained 20 ng of pFR-LUC, 20 ng of GAL-SRC-1, 10 ng of VP16-mPXR and/or VP16-CAR (unless otherwise indicated), 40 ng of pSV-β-Gal, and was added with pBlueScript to 110 ng of total DNA per well. Twenty-four hours post-transfection, cells were drug-treated for 24 h. The luciferase and β-galactosidase activities were determined using the Dual-Light kit per the manufacturer’s instructions (Applied Biosystems, Foster City, CA).

Northern Blot Analysis. Mouse hepatocytes were isolated using a two-step perfusion. Briefly, the liver was cleared with wash solution (150 ml of wash solution is made by mixing 15 ml of 10× Hanks’ balanced salt solution (Invitrogen), 150 μl of 0.5 M EGTA, 825 μl of 1.0 M glucose (dextrose), and 134 ml of H2O) at a flow rate of 16 ml/min for 4 min at 37°C. The liver was digested for 8 min at a flow rate of 8 ml/min at 37°C with digestion solution (150 ml of digestion solution is made by mixing 15 ml of 10× HBSS, 225 μl of 1.0 M CaCl2, 825 μl of 1.0 M glucose, 70 to 75 mg of collagenase type I (200–300 units/mg; Invitrogen), dissolved in 20 ml of digest medium that does not contain collagenase, and then filter-sterilized using a 0.45-μm filter) and 134 ml of H2O). Then, digested liver was put in 10 to 15 ml of digest solution in a sterile Petri dish and cut into small pieces. Hepatocytes were dissociated by pipetting using a 5-ml pipet. The suspension was filtered through a 100-μm cell strainer (BD Biosciences). The cells were pelleted at 93g for 5 min at 4°C and washed twice in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 nM dexamethasone, 1 μM insulin, 2 mM l-glutamine, and antibiotics. Cell viability was determined using trypan blue staining. Generally, >90% viability was achieved.

Mouse hepatocytes were plated in six-well collagen-coated plates at 8 × 10² cells/well in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 nM dexamethasone, 1 μM insulin, 2 mM l-glutamine, and antibiotics. Cell viability was determined using trypan blue staining. Generally, >90% viability was achieved. Mouse hepatocytes were plated in six-well collagen-coated plates at 8 × 10² cells/well in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 nM dexamethasone, 1 μM insulin, 2 mM l-glutamine, and antibiotics. Cell viability was determined using trypan blue staining. Generally, >90% viability was achieved.

Real-Time Quantitative PCR. One microgram of DNase I-treated RNA was reverse transcribed using random primers following the manufacturers’ instructions (Promega, Madison, WI). Equal amounts of cDNA were used in real-time quantitative PCR reactions by using the Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). Reactions included 200 nM fluorogenic probe and 300 nM primers specific for 18S or Cyp2b10. The fluorogenic probe and primer sets were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). BioSearch Technologies (Novato, CA) synthesized the fluorogenic probes. The sequences (5’ to 3’) for the primers and probes are as follows: Cyp2b10, forward
primer (GACTTGGGATGGGAAAGAG), fluorogenic probe (FAM-TAGTGAGGAAACCTGCAGA-TCC-BHQ1), reverse primer (CCAAAC-ACAATGGAGCAGAT); and 18S, forward primer (CCATGAGATGGCTCATAA), fluorogenic probe (FAM-CGATTTGGGATGGGAAAGAG), reverse primer (GGTTCACCTACGGAAACCTT). Cycling conditions were 95°C for 2 min followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, 68°C for 15 s using the Cepheid Smart Cycler system. Fold-induction was calculated as described previously (Staudinger et al., 2003).

Results

Repression of Cyp2b10 Promoter by Guggulsterone.

To investigate the regulation of Cyp2b10 promoter activity by guggulsterones, we performed a series of reporter gene assays in CV-1 cells. Cells were transiently transfected with the expression vector that encodes mouse CAR together with CAR-responsive luciferase reporter gene Cyp2b10-Luc. This reporter gene contains the phenobarbital response element enhancer sequence that was previously shown to be required for activation of Cyp2b10 gene expression by CAR (Rivera-Rivera et al., 2003). Both cis- and trans-guggulsterone repressed the expression of Cyp2b10-luc in the presence of CAR with an efficacy similar to that achieved with androstanol, a prototypical inverse agonist of mouse CAR, and similar results were obtained when the experiment was performed in the presence of the known CAR agonist 1,4-bis(2-(3,5-dichloropyridyloxy))benzene (TCPBOB) (Fig. 1A). Full concentration-response analysis of cis-guggulsterone revealed IC50 values of approximately 900 nM and 8.4 μM in the absence and presence of 250 nM TCPBOB, respectively (Fig. 1B). Moreover, full concentration-response analysis of TCPBOB demonstrated that addition of 10 μM cis-guggulsterone increased the EC50 value of TCPBOB against CAR by approximately 1 order of magnitude (21–200 nM) (Fig. 1C).

Cellular PXR-to-CAR Ratio Regulates the Expression of Cyp2b10 after Treatment with Guggulsterone.

To determine the effect of guggulsterone on Cyp2b10 gene expression in liver cells, we treated primary mouse hepatocytes and performed Northern blot analysis. Androstanol was used as a positive control for CAR antagonism. Because our previous studies show that guggulsterone is a PXR agonist and to rule out the involvement of PXR, we first performed our experiments in PXR-KO mouse hepatocytes. Both cis- and trans-guggulsterone treatments repressed Cyp2b10 gene expression levels in PXR-KO mouse hepatocytes (Fig. 2A). Surprisingly, treatment with androstanol did not produce repression of Cyp2b10 expression, indicating that the biological activity of androstanol might be more complicated in cultured primary hepatocytes than generally believed. To determine the biological activity of guggulsterones in a normal cellular environment, we also performed similar experiments in wild-type cultured mouse hepatocytes. Unlike in PXR-KO mouse hepatocytes, treatment with cis- and trans-guggulsterone modestly induced the expression levels of Cyp2b10 in WT mouse hepatocytes, and a similar effect was observed after treatment with androstanol (Fig. 2B). To quantitatively determine the effect of guggulsterone on the Cyp2b10 gene expression and to confirm that the repression of Cyp2b10 gene expression by guggulsterone in PXR-KO mouse hepatocytes was mediated by CAR, we also cotreated PXR-KO mouse hepatocytes with guggulsterone and the prototypical rodent CAR agonist TCPBOB and performed real-

Fig. 1. Guggulsterones repress CAR activity in Cyp2b10-Luc reporter gene assays. A, CV-1 cells transfected with the expression vector for mouse CAR and the Cyp2b10-Luc reporter gene. Cells were treated with vehicle (Veh, 0.1% DMSO) or 10 μM cis-guggulsterone (Cis); trans-guggulsterone (Trans); or androstanol (An), both in the absence and presence of 250 nM TCPBOB. B, CV-1 cells transfected as in A and treated with increasing concentrations of cis-guggulsterone both in the absence and presence of 250 nM TCPBOB. C, transfected CV-1 cells treated with increasing concentrations of TCPBOB both in the absence and presence of 10 μM cis-guggulsterone. All cells were treated for 24 h. The data represent the mean of replicates ± S.E.M. (n = 4) and are normalized against β-galactosidase activity and expressed as fold induction over vehicle control.
Guggulipid extract has been used in Ayurvedic medicine to treat various diseases ranging from dyslipidemia to inflammation for thousands of years and is also gaining popularity in Western societies for their putative cholesterol-lowering properties. The stereoisomers and ketosteroids cis- and trans-guggulsterone, respectively, are the main ingredients in guggulipid. Besides lipid-lowering effects, guggulsterone also has been shown to have thyroid-stimulating activity in rats (Tripathi et al., 1984, 1988). Although the mechanism is not clear, it is suggested to be different from that of TSH and is probably not mediated through the pituitary.

**Discussion**

Guggulsterone treatment represses Cyp2b10 gene expression in PXR-KO mouse hepatocytes but not in wild-type mouse hepatocytes. A, PXR-KO cultured primary mouse hepatocytes treated with vehicle (Veh, 0.1% DMSO) or 10 μM cis-guggulsterone (Cis); trans-guggulsterone (Trans); or androstanol (An). B, wild-type primary mouse hepatocytes transfected with fixed amount of CAR expression vector (5 ng) and increasing amounts of PXR expression vector (0.25–5 ng), or a fixed amount of PXR expression vector (5 ng) and increasing amounts of CAR expression vector (0.25–5 ng) and treated with 10 μM cis-guggulsterone (Fig. 3B). When the PXR-to-CAR ratio was high, guggulsterones transactivated the Cyp2b10-Luc promoter, but when the PXR-to-CAR ratio was low, guggulsterones transrepressed the Cyp2b10-Luc promoter.

**Guggulsterone Differentially Regulates the Association of SRC-1 with CAR and PXR.** The interaction between steroid receptor coactivator proteins and nuclear receptors enhances nuclear receptor activity in cells. Because we observed differential effects of guggulsterones on the expression of Cyp2b10 in different cellular environments, we sought to determine whether guggulsterones differentially modulated the strength of CAR-SRC-1 and PXR-SRC-1 protein-protein interaction using the mammalian two-hybrid system. CV-1 cells were transfected with the expression vector encoding the receptor interacting domain from the nuclear coactivator protein SRC-1 fused to GAL4 DNA-binding domain together with expression vectors encoding either VP16-tagged mouse CAR or VP16-tagged mouse PXR together with the GAL4-responsive luciferase reporter gene pFR-LUC. Similar to androstanol, treatment with guggulsterone recruited VP16-tagged PXR to GAL-SRC1, but displaced VP16-CAR from GAL-SRC1 (Fig. 4A). Interestingly, in the presence of both VP16-CAR and VP16-PXR, treatment of cis-guggulsterone modulated the reporter activity in a PXR-CAR ratio-dependent manner (Fig. 4B). When cells were transfected with excessive amounts of VP16-CAR, the reporter gene activity was repressed by the treatment with cis-guggulsterone, indicating that SRC-1 dissociation from CAR is predominant over its recruitment to PXR. However, as more VP16-mPXR was titrated into this system, the response of the pFR-Luc reporter gene to cis-guggulsterone treatment moved from negative to positive in a dose-dependent manner, demonstrating that recruitment of VP16-PXR to GAL-SRC1 became predominant. Interestingly, the presence of higher levels of VP16-PXR produced an increase in basal reporter gene activity.

**Materials and Methods**

Real-time quantitative PCR analysis was performed as described under Materials and Methods. The data are normalized to 18S levels and are expressed as average values (n = 3) ± S.E.M.

**Fig. 2.** Guggulsterone treatment represses Cyp2b10 gene expression in PXR-KO mouse hepatocytes but not in wild-type mouse hepatocytes. A, PXR-KO cultured primary mouse hepatocytes treated with vehicle (Veh, 0.1% DMSO) or 10 μM cis-guggulsterone (Cis); trans-guggulsterone (Trans); or androstanol (An). B, wild-type primary mouse hepatocytes treated as in A. All cells were treated for 24 h before RNA isolation. Total RNA (10 μg) was used for Northern blot analysis. The blots were probed sequentially with 32P-labeled fragments of Cyp2b10 and 18S. C, PXR-KO cultured primary mouse hepatocytes treated with vehicle, 10 μM cis-guggulsterone, 10 μM TCPOBOP, or both for 24 h. RNA was subjected to real-time quantitative PCR analysis to determine the relative expression levels of Cyp2b10 as described under Materials and Methods. The data are normalized to 18S levels and are expressed as average values (n = 3) ± S.E.M.
Like its cousin PXR, CAR was originally believed to be a xenobiotic sensor that regulates the expression of xenobiotic responsive genes after xenobiotic insults. However, recent work suggests that, unlike PXR, CAR plays an important role in energy homeostasis (Ueda et al., 2002; Maglich et al., 2004). Treatment with the synthetic CAR ligand TCPOBOP produces lower serum levels of thyroid hormones in wild-type mice but not in CAR knockout mice (Maglich et al., 2004). Fasting for 24 h also produces decreases in serum levels of both triiodothyronine and T4 in wild-type mice but not in CAR-KO mice. The decrease in thyroid hormone levels in wild-type mice is associated with induction of UDP glucuronosyltransferase 1a1, Sultn, Sult1a1, and Sult2a1 gene expression (Maglich et al., 2004; Qatanani et al., 2005). These genes encode thyroid hormone-metabolizing enzymes, and induction of these genes by fasting and CAR ligands is totally absent in CAR knockout mice. More importantly, CAR-knockout mice lost over twice as much weight as their wild-type littermates when both were placed on a 40% caloric restriction diet for 12 weeks (Maglich et al., 2004). In light of these data, our results suggest that some of the thyroid-stimulating and lipid-burning activity of guggulsterone observed in rats might be mediated through antagonism of CAR.

Although Wei et al. (2002) clearly demonstrated the repressive effect of androstanol on the induction Cyp2b10 gene expression by TCPOBOP in vivo in mice, it remains unknown how androstanol treatment affects the basal level of Cyp2b10 in vivo. Although we did not observe a significant repressive effect of androstanol on the induction of Cyp2b10 by TCPOBOP in cultured primary mouse hepatocytes (data not shown), guggulsterone did repress Cyp2b10 gene expression in the absence as well as presence of TCPOBOP (Fig. 2). There are several potential reasons underlying this discrepancy. First, in our cultured mouse hepatocyte system, all compounds were used at 10 μM, which are likely very different from the concentrations achieved in vivo during experiments performed by Wei et al. (2002). It is possible that the
repression of the induction of Cyp2b10 by androstanol requires a relatively low concentration of TCPOBOP and a high concentration of androstanol. Such possibility is currently under investigation in our laboratory. Second, although androstanol and guggulsterone have similar activities against PXR and CAR, the regulation of Cyp2b10 gene expression by these two compounds might involve distinct mechanisms. Finally, it is possible that some important hepatic factors are missing in cultured hepatocytes, and these factors are necessary to mediate the repressive effect of androstanol on Cyp2b10 gene expression but are not required for guggulsterone to repress Cyp2b10 gene expression.

Although the observed repression of Cyp2b10 expression in PXR-KO mouse hepatocytes produced by guggulsterone treatment is consistent with their function as inverse agonists of CAR, guggulsterone did not repress Cyp2b10 expression in wild-type mouse hepatocytes. This is likely due to the activation of PXR. This observation is also consistent with the effects of the known inverse CAR agonist androstanol in our culture system.

Because PXR and CAR gene expression exhibits a high degree of interindividual variability (Lamba et al., 2004a,b), and because CAR expression exhibits circadian rhythms (Kanno et al., 2004), we decided to investigate how the ratio of PXR to CAR affects the biological activity of guggulsterone on the Cyp2b10 promoter. Our reporter gene studies clearly
demonstrate that the ratio of PXR to CAR determines the effect of guggulsterone treatment on the expression of the Cyp2b10 promoter. The differential modulation of the effect of guggulsterones on Cyp2b10 promoter by the ratio of PXR to CAR is illustrated in the model in Fig. 5. When PXR expression is predominant compared with CAR, the net effect of guggulsterones on Cyp2b10 promoter is positive. On the other hand, when CAR expression is dominant compared with PXR, the net effect is negative. Therefore, compounds or drugs with guggulsterone-like activity can induce very different response in different people or even in the same person at different times of the day.

There are at least two classes of ligands that regulate the activity of CAR through two distinct mechanisms. The first class of CAR ligand is represented by phenobarbital, which activates CAR through a cytoplasm-to-nucleus translocation mechanism and does not involve direct binding to CAR (Kawamoto et al., 1999). On the other hand, TCPOBOP and androstanol represent a second class of CAR modulators that regulate the activity of CAR by modulating CAR-cofactor interactions and involve direct binding of these compounds to CAR (Forman et al., 1998; Tzameli et al., 2000). Our mammalian two-hybrid data reveal that guggulsterone treatment reduces CAR-SRC-1 interaction, which suggests that guggulsterone modulates CAR activity, likely by direct binding and displacing the SRC1 coactivator protein in a manner similar to androstanol.

During the preparation of this manuscript, the crystallographic structure of CAR was published (Shan et al., 2004; Suino et al., 2004; Xu et al., 2004). Our studies, together with the crystal structures of PXR and CAR, provide important information on the regulation of PXR and CAR by direct ligands and therefore help in the rational design of specific CAR ligands. Such compounds are currently viewed as having a high potential as antiobesity drugs due to their likely ability to modulate serum thyroid hormone levels.

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