IDENTIFICATION OF HMG-CoA REDUCTASE INHIBITORS AS ACTIVATORS FOR HUMAN, MOUSE AND RAT CONSTITUTIVE ANDROSTANE RECEPTOR

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ABSTRACT:

Constitutive active (or androstan) receptor (CAR, NR1I3), a member of the nuclear receptor family, is a major regulator for induction of cytochrome P450 2B (CYP2B) genes by phenobarbital. Phenobarbital-like inducer, 1,4-bis[2-(3,5-dichloroaryl)oximino]benzene is a potent mouse CAR ligand that has been used to study CAR target genes in mice but does not activate human CAR (hCAR) or rat CAR (rCAR). Although 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) was reported to be an hCAR agonistic ligand, activation of hCAR by CITCO in cell-based reporter assay was weak. Therefore, we performed a screening of 50 drugs and chemicals using cell-based reporter assays to identify activators of hCAR. Among them, HMG-CoA reductase inhibitors (cerivastatin, simvastatin, fluvastatin, and atorvastatin) enhanced the hCAR-mediated transcriptional activation of phenobarbital-responsive enhancer module reporter gene by up to 3-fold. Similar activation by HMG-CoA reductase inhibitors was also observed with mouse and rat CARs. On the other hand, pravastatin did not activate hCAR at the concentrations tested (up to 30 μM). The extent of activation by the HMG-CoA reductase inhibitors was stronger than that by CITCO. Cerivastatin, simvastatin, fluvastatin, and atorvastatin induced CYP2B6 mRNA in stable hCAR-expressed FLC7 cells but not in original FLC7 cells. Therefore, we concluded that CAR mediates the effects of HMG-CoA reductase inhibitors on the induction of CYP2B genes, although HMG-CoA reductase inhibitors also activate pregnane X receptor. HMG-CoA reductase inhibitors such as cerivastatin would be useful to study for elucidating molecular and cellular mechanisms of hCAR.

Constitutive active (or androstan) receptor (CAR, NR1I3), a member of the nuclear receptor family, is a major regulator of hepatic cytochrome P450 2B1 (CYP2B1) in rats (Muangmoonchai et al., 2001), CYP2B10 in mice (Honkakoski and Negishi, 1997; Kawamoto et al., 1999), and CYP2B6 in humans (Sueyoshi et al., 1999). Treatment of primary hepatocytes with phenobarbital results in the translocation of CAR into the nucleus, where CAR binds to phenobarbital-responsive enhancer module (PBREM) located upstream of the CYP2B gene as a heterodimer with the retinoid X receptor-α (RXRα, NR2B1) and activates transcription of CYP2B genes (Sueyoshi and Negishi, 2001). 1,4-bis[2-(3,5-Dichloroaryl)oximino]benzene (TCPOBOP) is a potent mouse CAR (mCAR) ligand that has been used to study CAR target genes in mice but does not activate human CAR (hCAR) or rat CAR (rCAR) (Poland et al., 1981; Moore et al., 2000; Tzameli et al., 2000). Phenobarbital translocates hCAR, mCAR, and rCAR into the nucleus and induces CYP2B; however, it is not known whether PB acts as an agonistic ligand for CAR since no direct binding of PB to CAR has been observed (Sueyoshi and Negishi, 2001). 5α-Androstan-3α-ol and clotrimazole bind to hCAR, whereas these compounds are antagonist ligands of hCAR (Forman et al., 1998; Moore et al., 2000). Recently, 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) and phenytoin have been reported to be hCAR agonist ligands (Maglich et al., 2003; Wang et al., 2004). However, the activation of hCAR by CITCO or phenytoin in a cell-based reporter assay was negligible. Therefore, the molecular and cellular mechanisms of the hCAR-mediated transcription activation remain poorly understood at the present time.

On the other hand, pregnane X receptor (PXR, NR1I2), a member of the nuclear receptor family, mediates induction of CYP3A gene transcription (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). In the presence of a ligand, PXR binds to proximal PXR response element (prPXRE) and distal xenobiotic-responsive enhancer module (XREM) in regulatory regions of the CYP3A4 gene as a heterodimer with RXRα. Unlike CAR, PXR can be activated by numerous compounds that are known CYP3A4

ABBREVIATIONS: CAR, constitutive active (or androstan) receptor; PBREM, phenobarbital-responsive enhancer module; hCAR, human CAR; mCAR, mouse CAR; prPXRE, proximal PXR response element; PXR, pregnane X receptor; rCAR, rat CAR; XREM, xenobiotic-responsive enhancer module; RXRα, retinoid X receptor-α; TCPOBOP, 1,4-bis[2-(3,5-dichloroaryl)oximino]benzene; PB, phenobarbital; CITCO, 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; HPXR, human PXR; SR12813, (2-(3,5-di-tert-butyl-4-hydroxy-phenyl)-1-(diethoxy-phosphoryl)-vinyl)-l-phosphonic acid diethyl ester; OATP-C, organic anion-transporting peptide-C.
inducers, including drugs, steroids, and environmental chemicals, in cell-based reporter assays (Jones et al., 2000; Moore et al., 2000). Thus, selective chemical tools (e.g., rifampicin) are available to study the function of PXR in humans.

Previous studies have demonstrated that 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, which are in widespread clinical use for the treatment of hypercholesterolemia, increase the expression of P450s. Kocarek and Reddy (1996) demonstrated that treatment of primary cultures of rat hepatocytes with lovastatin, simvastatin, or fluvastatin increased the levels of CYP2B and CYP3A mRNA and immunoreactive protein. In contrast, pravastatin had little or no effect on the amount of any of the P450s examined in rat hepatocyte cultures. In addition, fluvastatin activated luciferase expression in primary cultures of rat hepatocytes that were transfected with the CYP2B1 reporter plasmid in the presence of cotransfected plasmid expressing mCAR or rCAR (Kocarek and Mercer-Haines, 2002). After the discovery that PXR mediates the effects of many CYP3A-inducing agents, El-Sankary et al. (2001) demonstrated that simvastatin or lovastatin, but not pravastatin, activated transcription from a CYP3A4 reporter plasmid in transiently transfected HepG2 cells. Furthermore, treatment of primary cultures of human hepatocytes with lovastatin, simvastatin, fluvastatin, or atorvastatin increased the levels of CYP3A4 and CYP2B6 mRNA and immunoreactive protein, but treatment with pravastatin did not (Kocarek et al., 2002). Although some HMG-CoA reductase inhibitors could activate human PXR and increase the amounts of CYP3A4 mRNA and immunoreactive protein as described above, it is not known whether activation of hCAR is involved in the induction of transcription of the CYP2B6 gene by treatment with HMG-CoA reductase inhibitors.

Therefore, we performed a screening of 50 drugs, including HMG-CoA reductase inhibitors, by using cell-based reporter assays to identify hCAR activators. Among them, HMG-CoA reductase inhibitors (cerivastatin, simvastatin, fluvastatin, and atorvastatin) enhanced the hCAR-mediated transcriptional activation of the PBREM-reporter gene. They also enhanced mCAR- and rCAR-mediated activation.

Materials and Methods

Chemicals. 5α-Androstane-3α-ol (3α-androstanol) was obtained from Steraloids (Newport, RI). Atorvastatin, cerivastatin, fluvastatin, pravastatin, and simvastatin were kindly provided by Sankyo Co. Ltd. (Tokyo, Japan). Lovastatin and atorvastatin were kindly provided by Sankyo Co. Ltd. (Tokyo, Japan). Atorvastatin, cerivastatin, simvastatin, and fluvastatin were generously supplied by BIOMOL Research Laboratories (Plymouth Meeting, PA). TCPOBOP was synthesized as described by Kende et al. (1985). CITCO was kindly provided by Sankyo Co. Ltd. (Tokyo, Japan). Primers (5'-CACTATGAGGGACTTCGG-3' and 5'-TGACGTCATGGCCAGTAGGGAAGATG-3') were synthesized as described by Kende et al. (1985). CYP2B6 cDNA was a gift from Dr. Tatsuo Yoshinari (Nihon University, Tokyo, Japan). cDNA for human CYP2B6 was obtained from American Type Culture Collection (Bethesda, MD). An expression plasmid of human CYP2B6 (pGL3-PBREM) was kindly provided by Dr. Koichi Fukumura (University of Tokyo, Tokyo, Japan).

PCR from human genomic DNA as a template and was cloned into pGL3-XREM/prPXRE. A human CYP3A4 luciferase reporter plasmid containing XREM and prPXRE (pGL3-XREM/prPXRE) was generated as described previously (Kobayashi et al., 2004). Intron 3 of CYP3A4 was amplified as an internal control.

mRNA Levels. A stable hCAR-transfected FLCL cell line (FLCL-hCAR) was cotransfected with pTARGET-hCAR expression vector and selected by neomycin resistance. To measure the CYP2B6 mRNA level, cDNA, prepared from total RNA of FLCL-hCAR cells and FLCL cells treated with a 30-fold concentration of each HMG-CoA reductase inhibitor, was subjected to quantitative real-time PCR with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The mRNA levels of CYP2B6 were determined by using Human Assays-On-Demand (Applied Biosystems), a gene expression product for CYP2B6. The mRNA levels were normalized against 18S rRNA determined by Predesigned TaqMan Assay Reagents for 18S rRNA (Applied Biosystems). To measure CYP2B6 mRNA levels in HepG2 cells and FLCL cells, total RNA was prepared from nontreated cells using TRIzol reagent (Invitrogen). Each cDNA (0.5 μl in 25 μl of reaction mixture) was synthesized from 2 μg of RNA was subjected to 40-cycle amplification of CYP2B6 mRNA. The primers (5'-CACTATGAGGGACTTCGG-GATGG-3' and 5'-GCCATGATGTGGTGGCTAATGG-3') were designed from the reported sequences (GenBank accession number NM_000767) across intron 3. PCRs were performed using AmpliTaq Gold (Applied Biosystems) at 95°C for 1 min, 60°C for 30 s, and 72°C for 30 s. The amplified DNA (151 base pairs) was separated on a 3% agarose gel and visualized by staining with ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified as an internal control.

Statistics. Results are expressed as mean ± S.D. Differences between two groups were analyzed using a two-sample t test.

Results

Identification of hCAR Activators in a Cell-Based Reporter Gene Assay Screening. Fifty drugs, including five HMG-CoA reductase inhibitors, were screened in cell-based reporter gene assays using hCAR and PBREM-reporter gene construct. These drugs consisted of anticonvulsants, antidepressants, antiarhythmic drugs, NSAIDs, etc., in addition to HMG-CoA reductase inhibitors, which are hydrophilic and mainly metabolized via P450. Therefore, we considered that these drugs penetrated the cell membrane and were screened in cell-based reporter gene assay. As shown in Fig. 1A, compounds that increased hCAR-mediated transcriptional activity at the concentration of 10 μM were cerivastatin (2.8-fold), simvastatin (1.9-fold), atorvastatin (1.8-fold), and fluvastatin (1.7-fold). Temazepam and thiordizine decreased hCAR-mediated transcriptional activity by >50%. The effects of the other 44 chemicals on the hCAR-mediated transcrip-
Simvastatin, fluvastatin, and atorvastatin also showed significant activation of the reporter gene at the concentration of 30 μM. The manner of activation of hCAR-mediated transcription by the four HMG-CoA reductase inhibitors was dose-dependent. Maximum induction of hCAR-mediated transcriptional activity by the four HMG-CoA reductase inhibitors at the concentrations tested was 2- to 4-fold. On the other hand, pravastatin did not show dose-dependent activation of hCAR at the concentrations tested (up to 30 μM). In addition, we tried treating the cells with HMG-CoA reductase inhibitors in the absence of exogenous mevalonate, whereas the results were essentially the same as those obtained under standard treatment conditions (data not shown).

Repressive Effects of Androstanol on HMG-CoA Reductase Inhibitor-Induced hCAR-, mCAR- and rCAR-Mediated Transactivation. In agreement with previous reports (Sueyoshi et al., 1999), expression of the transfected PBREM-reporter gene was activated by cotransfection of the hCAR expression vector in FLC7 cells (Fig. 2A). 3α-Androstanol repressed this hCAR-mediated activation by 45%. 3α-Androstanol also repressed the increase in PBREM-luciferase activity induced by HMG-CoA reductase inhibitors (30 μM). When not only hCAR but also mCAR or rCAR was cotransfected, increased in PBREM-luciferase activity induced by HMG-CoA reductase inhibitors and repression by 3α-androstanol were observed (Fig. 2, B and C). On the other hand, distinct activation profiles by the potent PB-type inducer TCPOBOP were observed when the hCAR, mCAR, or rCAR expression vector was cotransfected with a PBREM-luciferase reporter. TCPOBOP elicited a significant induction of the reporter in the presence of mCAR, but induction by TCPOBOP at an equivalent concentration was not evident when hCAR or rCAR was cotransfected.

Potency of Cerivastatin for CAR Activation Compared with That of CITCO. As shown in Fig. 3, hCAR has been shown to transactivate the PBREM-reporter gene in the absence of any exogenous ligands. In addition, CITCO, a hCAR-specific ligand, enhanced the hCAR-mediated transcriptional activation of PBREM-reporter (by ~1.3-fold) at a concentration of 10 μM, as reported by Jinno et al. (2004). On the other hand, the effect of an HMG-CoA reductase inhibitor, cerivastatin, at the concentration of 10 μM on hCAR-mediated transcriptional activity was much greater than that of CITCO.

hCAR-Mediated Induction of CYP2B6 mRNA in Human Hepatoma Cell Line. To assess the hCAR-mediated induction of CYP2B6 mRNA by HMG-CoA reductase inhibitors, HMG-CoA reductase inhibitors were treated with hCAR-expressing cells derived from FLC7 cells (FLC7-hCAR). Unlike HepG2 cells, FLC7 cells constitutively express CYP2B6 mRNA (Fig. 4A). However, no inductive effect of HMG-CoA reductase inhibitors on CYP2B6 mRNA levels was observed in FLC7 cells. In contrast, CYP2B6 mRNA levels in FLC7-hCAR were increased by cerivastatin, simvastatin, fluvastatin, and atorvastatin but not by pravastatin (Fig. 4B).

Effects of HMG-CoA Reductase Inhibitors on hPXR-Mediated Transcriptional Activities. To compare the effects of HMG-CoA reductase inhibitors on transactivation of hCAR with those of human PXR, a human PXR expression vector was cotransfected with the PBREM-reporter gene, and a reporter gene assay was performed. Except for atorvastatin and pravastatin, the HMG-CoA reductase inhibitors significantly enhanced the expression of the PBREM-reporter gene (Fig. 5A). The extent of the activation at the concentration of 30 μM was about 3-fold. On the other hand, when XREM/ prPXRE-reporter gene construct was used, atorvastatin (12.2-fold), in addition to cerivastatin (29.4-fold), simvastatin (26.6-fold), and fluvastatin (39.8-fold) also enhanced the expression of the XREM/ prPXRE-reporter gene (Fig. 5B).

Discussion

The present study showed that HMG-CoA reductase inhibitors, cerivastatin, simvastatin, fluvastatin, and atorvastatin, were effective in activating hCAR in a cell-based reporter gene assay using human hepatocellular carcinoma FLC7 cells. In addition, the increase in the level of CYP2B6 mRNA induced by these HMG-CoA reductase inhibitors was shown to be regulated by hCAR. These findings were...
supported by the following results. Since the expression of CAR and PXR in FLC7 cells is negligible (data not shown), no effect of treatment with HMG-CoA reductase inhibitors on CYP2B6 mRNA level was observed in FLC7 cells. In contrast, significant induction of CYP2B6 mRNA by treatment with HMG-CoA reductase inhibitors was detected in stable hCAR-expressing FLC7 cells (termed FLC7-hCAR; Fig. 4B). CITCO has been reported to be a selective hCAR

Fig. 2. Repressive effects of 3α-androstanol on hCAR (A)-, mCAR (B)-, and rCAR (C)-mediated transactivation by HMG-CoA reductase inhibitors. Construct pGL3-PBREM (200 ng/well) was transiently transfected with pTARGET-hCAR, pTARGET-mCAR, or pCR3-rCAR (each 20 ng/well) into FLC7 cells. Cells were treated with 0.2% DMSO (vehicle), a 30 μM concentration of each of the HMG-CoA reductase inhibitors, or 250 nM TCPBOP in the presence or absence of 10 μM 3α-androstanol for 24 h. Data are expressed as means ± S.D. of at least three experiments. **, p < 0.01, and *, p < 0.05 compared with vehicle control. †††, p < 0.001, ††, p < 0.01, and †, p < 0.05 compared with data in the absence of 3α-androstanol.

Fig. 3. Comparison of cerivastatin (CRV) and CITCO for hCAR-, mCAR-, and rCAR-mediated transactivation of PBREM-luciferase gene. Construct pGL3-PBREM (200 ng/well) was transiently transfected with or without pTARGET-hCAR, pTARGET-mCAR, or pCR3-rCAR (each 20 ng/well) into FLC7 cells. Cells were treated with 0.2% DMSO (vehicle), 10 μM CITCO, or 10 μM cerivastatin for 24 h.

Fig. 4. A, reverse transcription-PCR analysis of CYP2B6 mRNA in HepG2 cells and FLC7 cells. Total RNAs were prepared from two different cell types, HepG2 cells and FLC7 cells, and were subjected to reverse transcription-PCR and 3% agarose gel electrophoresis. DNA bands were visualized by ethidium bromide. B, hCAR-mediated induction of CYP2B6 mRNA in FLC7 cells. Cells (FLC7-hCAR cells) were treated with 0.1% DMSO (vehicle) or with a 30 μM concentration of each of the HMG-CoA reductase inhibitors for 24 h. Total RNA was extracted as described under Materials and Methods and was subjected to real-time PCR. Relative mRNA abundance is normalized for 18S rRNA. **, p < 0.01, and *, p < 0.05 compared with vehicle control.
ligand and to activate hCAR (Maglich et al., 2003). However, the extent of transactivation by CITCO in a reporter gene assay was weak (Fig. 3), although induction by CITCO of CYP2B6 in vivo was strong. On the other hand, phenobarbital and phenytoin, which induce CYP2B6 via hCAR (Sueyoshi et al., 1999; Wang et al., 2004), do not enhance hCAR-mediated transcriptional activity in a cell-based reporter gene assay. The lack of an effective activator of hCAR in a cell-based reporter gene assay makes the characterization of hCAR difficult. The present study suggested that HMG-CoA reductase inhibitors might provide us with useful tools to study the function of CAR in humans.

In this study, FL7 cells were used for the reporter gene assay. Our preliminary study showed that the mRNA levels of CAR in FL7 cells were much lower, but the heterodimerization partner RXRα is expressed in sufficient amounts. In addition, the activation of mCAR by TCPOBOP and the deactivation of hCAR and mCAR by androstanol observed in FL7 cells (Fig. 2) are in agreement with previously reported results in HepG2 cells (Moore et al., 2000). Therefore, it is considered that the assay system using FL7 cells is a valid model for examination of CAR-mediated activation or deactivation.

TCPOBOP is a potent mCAR ligand that has been used for identification of CAR target genes but does not activate hCAR or rCAR in a cell-based reporter gene assay (Fig. 2). In contrast, phenobarbital activates CAR of any species, but phenobarbital does not bind to the receptor. It is well known that CAR is translocated from the cytoplasm to the nucleus after treatment with phenobarbital (Kawamoto et al., 1999). Although it has not been determined whether HMG-CoA reductase inhibitors directly bind to CAR, HMG-CoA reductase inhibitors could activate hCAR, rCAR, and mCAR at least in cell-based reporter gene assay (Fig. 2). The availability of activators for hCAR, rCAR, and mCAR will enable direct comparison of their similarities and differences in the transactivation mechanism.

Currently available information on the role of hCAR in the regulation of human CYP2B6 gene expression is limited. Accumulated evidence suggests that the human CYP2B6 gene is also regulated in a PXR-dependent manner. The majority of known PXR activators are capable of potently inducing CYP2B6 in primary human hepatocyte cultures, including those that activate hPXR only (rifampicin, hyperforin, SR12813), those that activate both hPXR and hCAR (phenobarbital), or those that selectively activate hCAR (phenytoin). The present study showed that cerivastatin, simvastatin, fluvastatin, and atorvastatin enhanced the PBREM-reporter gene mediated by hCAR (Fig. 1B) and the XREM/prPXRE-reporter gene mediated by hPXR (Fig. 5B). Furthermore, Kocarek et al. (2002) reported that simvastatin, fluvastatin, and atorvastatin increased the amount of CYP2B6 and CYP3A mRNA in primary human hepatocytes. Therefore, it was thought that cerivastatin, simvastatin, fluvastatin, and atorvastatin are capable of activating both hCAR and hPXR and of inducing CYP2B6 in primary human hepatocyte cultures.

Among the HMG-CoA reductase inhibitors studied, only pravastatin did not enhance the hCAR-mediated expression of the PBREM-reporter gene (Fig. 1B). Due to the hydrophilic nature of pravastatin, the amount of cellular uptake by diffusion is small. In human hepatocytes, organic anion-transporting peptide-C (OATP-C) plays a predominant role in the uptake of some HMG-CoA reductase inhibitors such as pravastatin (Nakai et al., 2001). However, HepG2 cells, a frequently used cell model of human hepatocytes, have been immuno-histochemically demonstrated to express no OATP-C (Nakai et al., 2001). Therefore, an OATP-C expression vector was cotransfected in FL7 cells with hCAR and a reporter vector as a preliminary study. However, hCAR-mediated transcriptional activity was not induced by pravastatin, even in the presence of OATP-C (data not shown). Kocarek et al. (2002) reported that treatment with pravastatin had no effect on the mRNA level of either CYP2B6 or CYP3A4 in human primary hepatocytes, although pravastatin treatment did produce the expected increase in HMG-CoA reductase mRNA level. Therefore, it appears that pravastatin was not an activator of hCAR in either human primary hepatocytes or human hepatoma cells.

The present screening in cell-based reporter gene assays using hCAR and PBREM-reporter gene construct indicated that temazepam and thiadiazine decreased hCAR-mediated transcriptional activity (Fig. 1A). 3α-Androstanol and antmycotic clotrimazole have been reported to deactivate hCAR through direct interactions with the ligand-binding domain of hCAR (Moore et al., 2000). Although further studies to clarify dose-responsibility and direct interaction with hCAR of temazepam and thiadiazine are needed, these drugs may also be deactivators or antagonists of hCAR.

In conclusion, cerivastatin, simvastatin, fluvastatin, and atorvastatin enhanced hCAR-mediated transcriptional activity in FL7 cells and induced the expression of CYP2B6 mRNA in hCAR-expressing FL7 cells. The activation of hCAR by these HMG-CoA reductase inhibitors in a cell-based reporter gene assay is more effective than that by a specific hCAR ligand, CITCO, although these HMG-CoA reductase inhibitors also activated hPXR. Therefore, HMG-CoA reductase inhibitors are thought to be useful tools to study the function of hCAR in a cell-based reporter gene assay using FL7 cells.
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