Identification of Human CYP2C8 as a Retinoid-Related Orphan Nuclear Receptor Target Gene

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ABSTRACT

Retinoid-related orphan nuclear receptors (RORs) α and γ (NR1F1, −3) are highly expressed in liver, adipose tissue, thymus, and brain and are involved in many physiological processes, such as circadian rhythm and immune function. Enzymes in the cytochrome P450 2C subfamily metabolize many clinically important drugs and endogenous compounds, such as the anticancer drug paclitaxel and arachidonic acid, and are highly expressed in liver. Here, we present the first evidence that RORs regulate the transcription of human CYP2C8. Overexpression of RORα and RORγ in HepG2 cells significantly enhanced the activity of the CYP2C8 promoter but not that of the CYP2C9 or CYP2C19 promoters. Computer analyses, promoter deletion studies, gel shift assays, and mutational analysis identified an essential ROR-responsive element at −2045 base pairs in the CYP2C8 promoter that mediates ROR transactivation. Adenoviral overexpression of RORα and −γ significantly induced endogenous CYP2C8 transcripts in both HepG2 cells and human primary hepatocytes. Knockdown of endogenous RORα and −γ expression in HepG2 cells by RNA interference decreased the expression of endogenous CYP2C8 mRNA by ~50%. These data indicate that RORs transcriptionally upregulate CYP2C8 in human liver and, therefore, may be important modulators of the metabolism of drugs and physiologically active endogenous compounds by this enzyme in liver and possibly extrahepatic tissues where RORs are expressed.

The human CYP2C subfamily consists of four genes: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. CYP2C18 does not seem to be expressed at the protein level. The other three CYP2C enzymes metabolize approximately 20% of currently used clinical drugs, including the antidiabetic drugs tolbutamide and rosiglitazone, the anticoagulant drug warfarin, the anticancer drug paclitaxel, and numerous nonsteroidal anti-inflammatory drugs, such as ibuprofen (Goldstein, 2001). They are also involved in the oxidative metabolism of endogenous compounds, such as arachidonic acid and retinoic acid.

It is well known that there is marked variability in the metabolism of CYP2C substrates in human populations because of the occurrence of genetic polymorphisms in the CYP2C genes. The expression of CYP2C enzymes also has been reported to be induced by various drugs, including rifampicin, hyperforin (the active constituent in St. John’s Wort), phenobarbital, and dexamethasone (Raucy et al., 2002; Madan et al., 2003; Komoroski et al., 2004). This induction further amplifies the individual variability in drug metabolism in human populations and may lead to a change in the half-life of drugs and then eventually result in drug tolerance or therapeutic failure.

A number of nuclear receptors have been discovered to play important roles in the mediation of transactivation of P450 enzymes. In many cases, these nuclear receptors bind ligands, which may be involved in initiating translocation of the receptor to the nucleus, where the receptor binds to responsive elements within gene promoters and recruits co-activators to affect chromatin structure and increase the transcription of target genes (Handschin and Meyer, 2003). Studies from our laboratory and other laboratories have revealed that several nuclear receptors that mediate drug-induced transactivation of CYP2C9, -2C8, and -2C19 genes include the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the glucocorticoid receptor (Gerbal-Chaloin et al., 2002; Chen et al., 2003, 2004; Fergus-
son et al., 2005). On the other hand, the constitutive regulation of the CYP2C genes is believed to be regulated in liver by receptors that include the hepatic nuclear factor (HNF) 4α, HNF3γ, and CCAAT/enhancer-binding protein (Jover et al., 1998, 2001; Bort et al., 2004; Chen et al., 2005). HNF4α also seems to cross-talk with CAR/PXR, which binds to more distant CAR/PXR-responsive elements to achieve synergistic activation with CAR and increase PXR-mediated induction by rifampicin (Chen et al., 2005).

Another family of receptors, the retinoic acid receptor-related orphan receptors, consists of three members, RORα, RORβ, and RORγ (Jenett and Joo, 2006). Several isoforms with varying lengths of the N terminus occur because of alternative promoter usage. They bind as a monomer to RORE-responsive elements (ROREs) consisting of a 6-bp AT-rich sequence preceding the half-core PuGGTCGA motif and act as either transcription activators or repressors, depending on which cofactors are recruited. Studies from ROR knockout mice have revealed that these receptors exhibit critical roles in a number of physiological and pathological processes, including cerebellar ataxia, inflammation, atherosclerosis, immune response, and circadian rhythm (Jetten and Joo, 2006). RORα seems to cross-talk with CAR/PXR, which binds to more P450 enzymes (Kang et al., 2007). Wada et al. (2008) further demonstrated that RORα could bind to the murine Cyp7b1 promoter to directly transactivate this gene.

In humans, RORα1, -α4, and -γ1 are the forms expressed in liver, but only a few hepatic genes, including apolipoprotein C-III (RORα1) (Jenett and Joo, 2006), apolipoprotein A5 (RORα1 and -α4) (Genoux et al., 2005), and hepatic synthesis of the plasma protein B-fibrinogen (Chauvet et al., 2005) have been identified as RORα1 and -α4 targets. Because recent knockout studies indicated several murine P450 genes may be targets for ROR regulation in liver (Kang et al., 2007), we hypothesized that RORs might also be transcriptional modulators of human P450 genes, contributing to the regulation of gene expression in liver and various extrahepatic tissues. In this study, we investigated the regulation of human CYP2C8, -2C9, and -2C19 genes by RORα1, RORα4, and RORγ1. We provide experimental evidence showing that both RORα and RORγ1 up-regulate the transcription of CYP2C8 in HepG2 cells, human primary hepatocytes, and Caco-2 cells by activating the CYP2C8 promoter directly through binding an RORE in the promoter region. The observations suggest an important role for RORs in the regulation of CYP2C8 expression in human liver and, as a consequence, a role in drug metabolism.

**Materials and Methods**

**Promoter Constructs and Expression Plasmids.** The wild-type CYP2C9-2923, CYP2C8-2966, CYP2C8-2527, and CYP2C19-2.7kb/pGL3_Basic constructs were as described previously (Chen et al., 2003; Ferguson et al., 2005). Of the CYP2C8 promoter region, −2 and −1.5 kb were amplified by PCR and constructed into pGL3 Basic to yield 2C8-2k and −1.5k promoter constructs (Ferguson et al., 2005). CYP2C8-2966 was used as the template to produce two mutants (CYP2C8-2966/−2289m and CYP2C8-2966/−2045m) by using QuickChange Site-directed mutagenesis (Stratagene, La Jolla, CA) and a deletion construct, CYP2C9-2966/ΔBglII, by BglII digestion.

The forward primers utilized for mutagenesis are as follows (the hexamer half-sites are indicated by boldface capital letters and mutated nucleotides are underlined): distal RORE mutation, 5′-ctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
and RORα or GFP/β-Gal and RORγ1 (1000 particles/cell) for 1 to 4 days, followed by total RNA isolation with an RNeasy Mini kit (QIAGEN). Human primary hepatocytes purchased from CellzDirect/Invitrogen (Carlsbad, CA) were cultured in Williams’ medium E (Sigma-Aldrich) complemented with 1× ITS+ supplement (Sigma-Aldrich; containing 10 μg/ml insulin from bovine pancreas, 5.5 μg/ml human transferrin, 5 ng/ml sodium selenite, 0.5 ng/ml bovine serum albumin, and 4.7 μg/ml linoleic acid), 15 mM HEPES, 2 mM t-glutamine, 10 mM dexamethasone, and penicillin (50 U/ml)/streptomycin (50 μg/ml). Hepatocytes were infected by viruses for 60 h, and total RNA was isolated for quantitative RT-PCR to measure mRNA concentration for CYP2C8, murine and human RORα, murine and human RORγ, and TPB as the endogenous control.

**Quantitative RT-PCR.** Total RNA was extracted using an RNeasy Mini prep system (QIAGEN). RT-PCR analysis was performed in two steps by initial reaction with Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). PCR with Taqman Universal PCR Master Mix (Applied Biosystems) was then performed with gene-specific primers using relative quantitation methods (2-ΔΔCT) and measured on an Applied Biosystems 7900HT Sequence Detection System using Taqman probes for CYP2C8, RORα, RORγ, HNF4α, GAPDH, mRORα, mRORγ, and the endogenous control TBP.

**Western Blot Analysis.** To analyze the exogenous ROR protein expression in HepG2 cells, whole-cell lysates were prepared from HepG2 cells infected with control GFP virus or adenoviruses containing the different FLAG-tagged RORs in six-well plates by using radioimmunoprecipitation assay buffer (Pierce Chemical). Equal volumes of lysates were subjected to 4 to 12% NuPAGE gel electrophoresis and transferred to nitrocellulose membranes, and blocked with 5% nonfat milk blocking buffer. Horseradish peroxidase-conjugated monoclonal anti-FLAG antibody (Sigma-Aldrich) was used for immunoblotting at room temperature for 1 h, and then the membrane was washed six times. Detection was achieved using a Super Signal West Femto kit (Pierce Chemical, Rockford, IL).

**Reduction of Endogenous Gene Expression by Small Interference RNAs.** To reduce endogenous ROR expression in HepG2 cells, cells were seeded into a 24-well plate (10^5/well) and transfected the next day with small interference RNAs (siRNAs) against human RORα and RORγ (Dharmacon RNA Technologies, Lafayette, CO) at two doses of 50 and 100 nM following the instructions for DharmaFECT 4 transfection reagent. Nonspecific siRNA and siRNA against GAPDH were transfected at the same time as the negative and positive controls, respectively. Twenty-four hours after transfection, cells were harvested, and total RNAs were isolated. Quantitative RT-PCR was performed to analyze mRNA levels of human RORα, RORγ, GAPDH, and CYP2C8.

**Statistical Analysis.** Statistical analysis was performed in Systat Software version 3.5 (Systat Software, Inc., San Jose, CA) using either one- or two-way analysis of variance as appropriate; subsequent pair-wise comparisons were made using the Bonferroni t-test method.

**Results**

**RORs Specifically Transactivate the CYP2C8 Promoter in HepG2 Cells.** To examine whether RORs can activate the transcription of human CYP2C8 genes, expression plasmids for the murine nuclear receptors RORα and RORγ were cotransfected into HepG2 cells along with the luciferase promoter constructs CYP2C9-2923, CYP2C8-2966, CYP2C19-2.7k, or the empty luciferase vector pGL3 Basic. Among the CYP2C promoters, only the CYP2C8-2966 promoter was strongly activated by RORα and RORγ (13- and 9-fold, respectively, \( p < 0.001 \)), whereas the CYP2C9 and CYP2C19 promoters showed no response to exogenous RORs (Fig. 1A). The nonresponsiveness of the CYP2C9 and CYP2C19 promoters to ROR was confirmed using 12-kb upstream promoter constructs for these two genes in transfection assays in HepG2 cells (data not shown). To confirm the specificity of activation of the CYP2C8 promoter by RORs, we cotransfected HepG2 cells with CYP2C8 promoter constructs and expression plasmids containing two ROR mutants and wild-type RORα, RORγ, and RORγ2. The mutant RORα4C13A harbors a mutation at its DNA binding domain and does not bind to ROR-responsive elements in DNA, whereas in RORγ2ΔAF2, the AF2 domain is truncated and, therefore, unable to recruit coactivators. As shown in Fig. 1B, wild-type RORα, RORγ, and RORγ2 significantly elevated the activity of CYP2C8 promoter (\( p < 0.001 \)), whereas the two mutants had no significant effect on promoter activity. These data further confirm that the CYP2C8 promoter is transactivated by RORα, RORγ, and RORγ2. We then examined the effect of progressive deletions of the CYP2C8 promoter construct (Fig. 1C) on ROR-mediated transactivation to identify the region in the CYP2C8 promoter that mediates ROR activation. As shown in Fig. 1D, significant ROR activation of CYP2C8 was observed with the CYP2C8-2966 and CYP2C8-2572 constructs (\( p < 0.001 \)), but not with CYP2C8-2k and -1.5k constructs, suggesting that ROREs within the 500-bp fragment from -2.5 to -2 kb of the CYP2C8 promoter are responsible for this transactivation. We also created a construct in which the distal and proximal promoter regions were retained, but the middle region including this 500-bp fragment was deleted; no ROR activation was observed with the deletion construct. This result further supports the hypothesis that the localization of the functional RORE(s) is within the 500-bp promoter fragment identified above.

**Identification of Two Putative ROREs That Are Required for ROR Activation of the CYP2C8 Promoter.** With the sequence of the core motif for RORE, (AT)\(_n\)AGGTCA, we performed a computer search (Motif/Vector NTI) for putative ROREs within the 3 kb of the CYP2C8 promoter and found two putative ROREs at -2289 and -2045 bp, respectively. Both sites are localized within the 500-bp promoter region identified above. Therefore, gel shift assays were performed to determine whether these two putative ROREs bind ROR in vitro. As displayed in Fig. 2B, transcribed and translated in vitro RORα-RORγ4 products formed strong complexes with the \(^{32}P\)-labeled oligonucleotide probe CYP2C8-2045RORE (lane 15) and with the positive control, a known RORE previously characterized from the murine Purkinje cell protein-2 (m-pcp-2) gene (lane 3), whereas products transcribed and translated in vitro from the empty pCR3.1 vector (V) did not produce any specific bands (lanes 2, 6, and 14 for all radiolabeled probes). In vitro-synthesized ROR4 exhibited a much weaker affinity for the 2C8-2289RORE, despite the fact that 10 × reactive probe was added to the gel (Fig. 2B, lane 7). Preincubation with 5 × or 50 × excess of wild-type cold competitor ROREs effectively competed out the formation of all of these complexes (see lane 4 for m-pcp-2; lanes 8, 9, and 12 for 2C8-2289RORE; and lanes 16, 17, and 20 for 2C8-2045), but mutated cold ROREs were unable to compete for these complexes (lanes 10 and 11 for 2C8-2289RORE, lanes 18 and 19 for 2C8-2045RORE). In vitro-transcribed ROR1 also bound both the positive control m-pcp-2 probe and the 2C8-2045RORE (Fig. 2C, lanes 3 and 15). Again, the wild-type cold probes could effectively com-
Neither of the ROR mutants activated in human liver and HepG2 cells (Chauvet et al., 2002). Another splicing variant, ROR\_H9251 and lanes 22 and 23 for ROR13 and 14 for ROR\_H9253 (1). It's ability to compete for the binding to RORs (Fig. 3A, lanes 20-45m2) (shown in Fig. 2A) resulted in severe impairment in complex could be supershifted by antibody to ROR\_H9251. We also saw a supershift with ROR antibody to the positive mouse pcp control (Fig. 3A, lane 6). These results demonstrate the existence of ROR\_a in these complexes. Introduction of one mutation in the 2C8-2045ORE oligo (2C8-2045m2) (shown in Fig. 2A) resulted in severe impairment in its ability to compete for the binding to RORs (Fig. 3A, lanes 13 and 14 for ROR\_4 and lanes 22 and 23 for ROR\_1). Another splicing variant, ROR\_1, is known to be expressed well in human liver and HepG2 cells (Chauvet et al., 2002). In Fig. 3B, gel shift assays clearly show that ROR\_1 could also bind to both ROREs within the CYP2C8 promoter, although the proximal site again showed higher affinity. The complex could be supershifted by antibody to ROR\_a. Taken together, these data clearly show that both ROREs can interact with RORs but that the 2C8-2045ORE has a much stronger affinity for both ROR\_4 and ROR\_1 than the 2C8-2289ORE.

To further examine the binding of RORs to the 2C8-2045ORE, antibody against ROR\_a and goat IgG was included in the incubation mixture before the addition of hot probes. A supershifted complex with retarded mobility was observed with the specific ROR\_a antibody, as shown by the arrow (Fig. 3A, lane 16), but not with control IgG (lane 15). We also saw a supershift with ROR antibody to the positive mouse pcp control (Fig. 3A, lane 6). These results demonstrate the existence of ROR\_a in these complexes. Introduction of one mutation in the 2C8-2045ORE oligo (2C8-2045m2) (shown in Fig. 2A) resulted in severe impairment in its ability to compete for the binding to RORs (Fig. 3A, lanes 13 and 14 for ROR\_4 and lanes 22 and 23 for ROR\_1). Another splicing variant, ROR\_1, is known to be expressed well in human liver and HepG2 cells (Chauvet et al., 2002). In Fig. 3B, gel shift assays clearly show that ROR\_1 could also bind to both ROREs within the CYP2C8 promoter, although the proximal site again showed higher affinity. The complex could be supershifted by antibody to ROR\_a. Taken together, these data clearly show that both ROREs can interact with RORs but that the 2C8-2045ORE has a much stronger affinity for both ROR\_4 and ROR\_1 than the 2C8-2289ORE.

To functionally investigate the roles of these two putative ROREs in the activation of CYP2C8 by RORs, we analyzed the effect of mutations within the two putative ROREs (Fig. 4A) on ROR-mediated activation of the CYP2C8 promoter in HepG2 cells by transient transfection assays. As shown in Fig. 4B, mutation of the -2289ORE did not significantly affect the activation of CYP2C8, either by murine ROR\_4 or -\_1, whereas mutation of the -2045ORE almost totally abolished the transactivation of the CYP2C8 promoter by ROR\_4 and ROR\_1. In contrast to wild-type ROR, the ROR\_4 mutant did not significantly increase the activity of the CYP2C8 promoter (p < 0.01), whereas the ROR\_1 mutant had much less activity than wild-type ROR\_1. Because ROR\_1 selectively transactivates the ApoIII promoter in human colon carcinoma Caco-2 cells (but not in HepG2 cells), we compared the transactivation of the CYP2C8 promoter by human ROR\_1 in HepG2 cells and Caco-2 cells. As shown in Fig. 4, C and D, the activity of the CYP2C8 promoter was increased similarly by ROR\_1 (p < 0.001) in both cell lines, and the mutation of the proximal RORE, but not the distal one, completely abolished this activation. Similar activation of the CYP2C8 promoter by ROR\_4 and ROR\_1 was also observed in Caco-2 cells (data not shown). These data are consistent with those from electrophoretic mobility shift assay and further indicate that the activation of the CYP2C8...
proteins were overproduced in wild-type ROR and -As confirmed in Western blots (Fig. 5, C and D), both the CYP2C8 overexpress wild-type mROR protein in HepG2 cells.

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**Endogenous CYP2C8 Was Elevated by Exogenous RORs in HepG2 Cells and in Human Primary Hepatocytes.** We introduced exogenous murine ROR proteins into HepG2 cells to investigate their effects on the expression of endogenous CYP2C8. We prepared adenoaviral constructs to overexpress wild-type mROR protein in HepG2 cells. CYP2C8 expression was measured for 4 days after infection. As confirmed in Western blots (Fig. 5, C and D), both the wild-type RORα4 and -γ1 proteins were overproduced in HepG2 cells. Compared with the LacZ control, adenoviral overexpressed RORα4 significantly increased the level of endogenous CYP2C8 mRNA (p < 0.001) in a time-dependent manner. By the 4th day after infection, a 9-fold increase in the CYP2C8 mRNA level was observed compared with cells infected with LacZ (p < 0.001) (Fig. 5A). Overexpression of RORγ1 also increased endogenous CYP2C8 mRNA, reaching a 4-fold higher level by day 4 compared with the control adenovirus expressing GFP (p < 0.001). No change in CYP2C8 transcripts was observed in cells infected with the GFP control (Fig. 5B).

Next, we examined whether exogenous RORs could elevate CYP2C8 expression in primary human hepatocytes. Adenoviruses expressing wild-type murine RORα4 and RORγ1 were used to infect primary human hepatocytes for 60 h. The relative amount of endogenous hRORA mRNA (relative to TBP) was 5.2 ± 0.1 in GFP-infected cells. After transfection, the level of mRORA expression was 200-fold higher (1209 ± 131) than that of endogenous hRORA. The amount of endogenous hRORγ in GFP-infected cells was 1.8 ± 0.2, whereas after transfection with mRORγ, RORγ levels were ~24 times higher (44 ± 5). It should be noted that amino acid identity between human and murine RORs is ~97% for RORα proteins and 89% for RORγ proteins, suggesting their biological effects should be very similar. Endogenous CYP2C8 mRNA was significantly increased, 5-fold by exogenous RORα4 and 7-fold by exogenous RORγ1 (p < 0.001) (as shown in Fig. 6, A and B). The effects of exogenous RORα and RORγ on CYP2C8 expression in HepG2 cells and primary hepatocytes suggest that RORs can activate the endogenous CYP2C8 gene in human liver.

**Endogenous CYP2C8 Was Reduced by the Decrease in Endogenous RORs in HepG2 Cells.** To gain more insight into the involvement of RORs in the transcriptional regulation of the CYP2C8 gene in vivo, we used siRNA technology to knock down endogenous ROR expression and examined its effect on endogenous CYP2C8 mRNA expression. siRNA oligos against human RORA and RORγ were transfected into HepG2 cells individually or in combination at low and high doses. As shown in Fig. 7, compared with transfection with the nontarget oligo, only the siRNAs that are specific for their target genes (RORA, RORγ, and GAPDH) could...
significantly reduce the mRNA levels of their targets; the siRNA for RORα/H9251 reduced endogenous RORα/H9251 mRNA specifically to 30% at the high dose 100 nM either used individually or in combination with siRNA for RORγ/H9253 (p < 0.001) (Fig. 7A). Endogenous RORγ mRNA was significantly reduced to 30% exclusively by its siRNA at the high dose (p < 0.001) (Fig. 7B). RORα and RORγ were decreased significantly more at the high dose of 100 nM siRNA than the low dose of 50 nM (p < 0.001). In accordance, CYP2C8 mRNA was reduced to 60% with the transfection of siRNA for RORα alone at both doses (p < 0.01 with 50 nM and p < 0.001 with 100 nM) and to 65% with the transfection of siRNA for RORγ at the high dose of 100 nM (p < 0.01). However, it should be noted that the level of RORα mRNA is approximately 3-fold higher than that of RORγ in HepG2 control cells, which is consistent with the slightly greater effect of knockdown of RORα on CYP2C8.

Fig. 3. Binding of RORα1 and RORα4 to the −2289 and −2045 ROREs within the CYP2C8 promoter by gel shift assays and supershifts with RORα antibody. 32P-labeled probes for the two CYP2C8 ROREs and the control RORE (m-pcp-2) were incubated at room temperature for 20 min with (+) or without (−) in vitro-transcribed and -translated ROR proteins (A, RORα4 and RORα1; B, RORα1) as described in Fig. 2. Wild-type but not mutated cold competitors effectively competed for both elements confirming the specificity of the binding. Antibody against RORα caused a supershift (SS) of the RORα complex with both m-pcp-2 (A, lane 6) and the −2045 RE (A, lane 16), whereas IgG did not (lanes 5 and 15). The supershifts were also formed with the complex with RORα1 by RORα antibody (B, lanes 9 and 18). The RORE at −2045 bp in the CYP2C8 promoter bound RORα1 much more strongly than the element at −2289 bp.
mRNA concentrations. When the two siRNAs were given in combination, CYP2C8 mRNA concentrations were reduced to 57% at 50 nM (p < 0.01) or 50% at 100 nM (p < 0.001). siRNA for GAPDH did not significantly affect the expression of CYP2C8 transcripts when transfected at 50 nM (Fig. 7D).

**Discussion**

In the present study, we show that the 3 kb of the human proximal CYP2C8 promoter is specifically activated by RORα1, RORα4, and RORγ1 in HepG2 cells, the forms of ROR known to be expressed in human liver. In contrast, neither the CYP2C9 nor CYP2C19 promoters are activated by RORs, which was confirmed with both 3- and 12-kb promoter constructs. Two putative ROREs were identified and shown to interact specifically with RORα1, -α4, and -γ1 proteins in gel shift assays; however, the proximal site at -2045 bp showed a much stronger affinity for all three isoforms than the more distal site. Mutation of the ROREs showed that only the proximal site plays a role in activation of the CYP2C8 promoter by RORs. In addition, endogenous CYP2C8 mRNA expression was up-regulated by exogenous RORα and -γ isoforms in HepG2 cells in the present study. Double knockdown of RORα and -γ isoforms produced a 50% decrease in CYP2C8 mRNA. These knockdown studies might underestimate the role of RORs in liver somewhat because of the fact the knockdown of RORs receptors was not complete. The present study indicates that the ROR nuclear receptor family plays a role in regulating the expression of the human P450 gene CYP2C8 in liver and perhaps other tissues. In addition, constitutive levels of CYP2C8 are probably regulated to some extent by HNF4α in liver (Ferguson et al., 2005).

The CYP2C8 enzyme oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics. It is important in the metabolism of arachidonic acid to physiologically active compounds, such as epoxyeicosatrienoic acids (EETs) (Zeldin, 2001). CYP2C8 is the principal enzyme responsible for the metabolism of clinically important drugs, such as the antitumor drug paclitaxel, the antimalarial drug amodiaquine, the antidiabetic drugs troglitazone and rosiglitazone, the antiarrhythmic drug amiodarone, and the calcium channel blocker verapamil (Totah and Rettie, 2005). The discovery that CYP2C8 is transactivated by RORα and -γ in hepatocytes suggests the possibility
that the clearance of these drugs might be modulated to some extent by ligands of RORs that modulate ROR activity. Recent X-ray crystal structure studies have demonstrated a series of natural compounds that can reversibly bind to RORs and act as agonists increasing its transactivational activity (Kallen et al., 2002, 2004). These include cholesterol and its structural derivatives 7-dehydrocholesterol and cholesterol sulfates. For example, depletion of cholesterol by the drug lovastatin in the osteosarcoma cells U-2OS in vitro has been proposed to modulate ROR transcriptional activity (Kallen et al., 2002). In addition, certain natural compounds, including several retinoids such as all-trans-retinoic acid and the synthetic retinoid ALRT1550 (Ligand Pharmaceuticals, San Diego, CA), can bind RORs and act as partial antagonists, inhibiting their transactivation activity (Stehlin-Gaon et al., 2003).

Recent studies have implicated RORs in the control of circadian rhythm, both in the central nervous system (RORα and -β) (Jetten and Joo, 2006) and in peripheral tissues such as liver (RORα and -γ) (Jetten and Joo, 2006). In mammals, many physiological and behavioral processes exhibit daily oscillations, including many hepatic enzyme activities, including those involved in energy metabolism, and a clear link between the control of circadian rhythm and metabolism has been established (Albrecht, 2006; Hastings et al., 2007). The clock oscillator consists of interlocked positive and negative transcriptional/posttranscriptional feedback loops between the clock genes Clock/Bmal1 and Per/Crys. By binding to the RORE within target gene promoters, RORs, along with the negative competitors Rev-Erbα and -β, control the expression of Bmal1 (Jetten and Joo, 2006). A number of rodent P450 enzymes display a daily fluctuation in their mRNA expression or catalytic activity, such as Cyp1a1 and 1b1, Cyp2a, Cyp2c, Cyp2e1, Cyp4a, Cyp7a1 and 7b1, and Cyp8b1 (Kang et al., 2007; Ohdo, 2007). RORα has been suggested to be involved in the oscillatory regulation of some of these P450 enzymes (Wada et al., 2008). In humans, the effect of circa-

![Fig. 5](image1.png)

**Fig. 5.** Overexpression of murine RORα4 and γ1 dramatically increases endogenous CYP2C8 mRNA in HepG2 cells. Adenoviruses containing FLAG-mRORα4 and the control LacZ (A) or FLAG-mRORγ1 and the control GFP/β-Gal (B) were utilized to infect HepG2 cells in triplicate for 1 to 4 days with a dose of 1000 particles/cell. Cells were harvested and used to isolate total RNA. cdnas were synthesized with MMLV, and then real-time quantitative PCR analyses were performed to determine the expression of CYP2C8 and TBP. Endogenous CYP2C8 mRNA gradually increased after infection of HepG2 cells with adenoviruses containing ROR over those infected with LacZ significantly (**p < 0.01; ***p < 0.001). Data represent means ± S.E. (n = 9). All data are normalized to TBP levels. The same ROR viruses and the GFP control were used to infect HepG2 cells in six-well plate in duplicates. After 2 days, the whole-cell lysates were prepared, and the Western blot was performed to detect the expression of exogenous FLAG-tagged ROR protein (C and D).

![Fig. 6](image2.png)

**Fig. 6.** Overexpression of murine ROR dramatically elevated endogenous CYP2C8 mRNA in human primary hepatocytes. Human primary hepatocytes were infected with adenoviruses for FLAG-mRORα4 (A) and FLAG-mRORγ1 (B) and the control GFP/β-Gal at a dose of 1000 particles/cell. Total RNA was isolated after 60 h, and real-time quantitative PCR analyses were performed to determine the expression of CYP2C8 relative to TBP. Both ROR viruses significantly increased endogenous CYP2C8 mRNA over the GFP adenoviral control (p < 0.001). Data represent means ± S.E. (n = 9). All data are normalized to TBP levels.
Diurnal rhythm on drug metabolism has not been studied extensively. However, studies from Ohdo and colleagues have suggested that the effectiveness of drugs depends on the time of day at which they are administered (Kang et al., 2007; Ohdo, 2007). Moreover, they showed that activity of CYP3A4 exhibits a 2.8-fold diurnal variation in humans based on 6-hydroxycortisol/cortisol ratios, which are a noninvasive index of CYP3A4 activity (Ohdo, 2007). It would not be surprising to find an oscillatory regulation in other human P450 enzymes such as CYP2C8 because the expression of a number of nuclear receptors, including RORs and HNF4α, CAR, peroxisome proliferator-activated receptor, estrogen-related receptor, and small heterodimer partner, display circadian rhythm (Yang et al., 2006).

CYP2C8 is the one CYP2C member that has the broadest tissue expression. It has been found to be expressed primarily in liver but also exists in many other tissues such as brain, heart, endothelial cells, intestine, and kidney (Klose et al., 1999). Both RORs and RORγ are widely expressed in most of these tissues (Jetten and Joo, 2006). This overlapping distribution is consistent with the possibility that RORs may also regulate CYP2C8 in some of these extrahepatic tissues. In brain, RORα is expressed in many regions, including the thalamus and cerebellum, where it plays an important role in circadian rhythm. RORγ is also found in brain. CYP2C8 mRNA has been detected at relatively high levels in many regions in human brain, including cerebellum, although unlike ROR, the distribution of CYP2C8 has not been studied in different cell types in the brain using immunohistochemical approaches (McFayden et al., 1998; Klose et al., 1999). RORα is known to be expressed in small intestinal epithelium and in the human colon cell line Caco-2 (Jetten and Joo, 2006).

The present study demonstrates that the CYP2C8 promoter can be activated by RORα1 in Caco-2 cells, indicating a possible regulatory role of RORs in transcription of CYP2C8 in colon and intestine. mRNA of RORα also has been detected in endothelial cells (Besnard et al., 2002), where CYP2C8 oxidizes arachidonic acid to produce 11,12- and 14,15-EETs that have vasodilatory roles and anti-inflammatory roles (Wray and Bishop-Bailey, 2008). If CYP2C8 expression is up-regulated by RORα1 in endothelial cells, this might increase EET formation, thus enhancing dilation and producing anti-inflammatory responses.

In summary, in the present study, we provide evidence demonstrating that RORα1, -α4, and -γ1 positively regulate CYP2C8 gene expression in human hepatocytes through an RORE in the CYP2C8 proximal promoter region. Because CYP2C8 catalyzes the metabolism of a number of clinically important drugs, and RORs function as ligand-dependent transcription factors, ROR (ant)agonists may be able to control the expression of CYP2C8 and, therefore, the metabolism of these drugs. In addition, because RORs play a role in the regulation of circadian rhythm, they might play a role in the possible circadian regulation of CYP2C8.

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References


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