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The transcriptional regulation of the human *CYP2C* genes

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Abstract

In humans, four members of the CYP2C subfamily (CYP2C8, CYP2C9, CYP2C18, and CYP2C19) metabolize more than 20% of all therapeutic drugs as well as a number of endogenous compounds. The CYP2C enzymes are found predominantly in the liver, where they comprise ~20% of the total cytochrome P450. A variety of xenobiotics such as phenobarbital, rifampicin, and hyperforin have been shown to induce the transcriptional expression of *CYP2C* genes in primary human hepatocytes and to increase the metabolism of CYP2C substrates *in vivo* in man. This induction can result in drug-drug interactions, drug tolerance, and therapeutic failure. Several drug-activated nuclear receptors including CAR, PXR, VDR, and GR recognize drug responsive elements within the 5' flanking promoter region of *CYP2C* genes to mediate the transcriptional upregulation of these genes in response to xenobiotics and steroids. Other nuclear receptors and transcriptional factors including HNF4 α , HNF3 γ , C/EBP α and more recently RORs, have been reported to regulate the constitutive expression of *CYP2C* genes in liver. The maximum transcriptional induction of *CYP2C* genes appears to be achieved through a coordinative cross-talk between drug responsive nuclear receptors, hepatic factors, and coactivators. The transcriptional regulatory mechanisms of the expression of *CYP2C* genes in extrahepatic tissues has received less study, but these may be altered by perturbations from pathological conditions such as ischemia as well as some of the receptors mentioned above.

Keywords

Human CYP2C; transcription regulation; drug induction; hepatic nuclear receptor; hypoxia

Introduction

The cytochrome P450s (CYP) are a superfamily of enzymes that catalyze the metabolism of xenobiotic drugs and environmental chemicals as well as many endogenous compounds. The human CYP2C subfamily consists of four members clustering at the chromosomal location 10q24 as Cen- *CYP2C18-CYP2C19-CYP2C9*- and *CYP2C8*-Tel, and they comprise approximately 20% of the P450 enzymes in the human liver. Except for CYP2C18, which is expressed at the mRNA level but does not appear to be expressed at the protein level in any tissue, the CYP2C proteins are expressed predominantly in the liver (2C9>2C8>2C19). However, they are expressed to variable extents in a number of other extrahepatic tissues such as kidney, gut, brain, heart, aorta, and lung [1,2]. The CYP2C enzymes are well-known clinically important enzymes that metabolize more than twenty percent of all pharmaceutical drugs. CYP2C substrates include some of the most frequently prescribed drugs, such as the anticoagulant drug coumadin, the anticonvulsant drug phenytoin, the anti-diabetic drugs tolbutamide, glipizide, and rosiglitazone, and numerous nonsteroidal anti-inflammatory drugs

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such as celecoxib, flurbiprofen, ibuprofen, and diclofenac [3,4]. CYP2C19 metabolizes the prototype drug *S*-mephenytoin, antiulcer drugs such as omeprazole and other proton pump inhibitors, diazepam [3,4], and the platelet inhibitor clopidogrel [5], while CYP2C8 metabolizes rosiglitazone and the anticancer drug paclitaxel [3,4]. CYP2C8/9 enzymes are also responsible for the hydroxylation of retinoic acid [6], and the CYP2C enzymes are important in the generation of biologically active molecules such as epoxyeicosatrienoic acids (EETs) and hydroxyeicosatrienoic acids (HEETs) from arachidonic acid in both liver and extrahepatic tissues[7].

All of the *CYP2C* genes exhibit genetic polymorphisms, some of which produce large phenotypic inter-individual variability in the metabolism of certain CYP2C substrates [3,4, 8-12]. In particular, null polymorphisms of *CYP2C19* dramatically affect the metabolism of a number of substrates of this enzyme. When single-nucleotide polymorphisms (SNPs) occur in the coding region, they can result in amino acid changes (some of which alter activity, affinity and turnover number of substrates) or produce premature stop codons, resulting in null (completely nonfunctional) alleles. SNPs can destroy or create new splice sites, producing frame shifts which also produce null alleles. Single or multiple base pair deletions can also cause frame shifts. SNPs also occur in the regulatory regions, and one such SNP produces an ultra-rapid metabolizer allele of *CYP2C19* [13]. SNPs of *CYP2C9* are well-known to affect dosage and serious bleeding episodes of coumadin [4,14,15]. A recent report has linked an intron SNP of *CYP2C8* to bisphosphonate-related osteonecrosis of the jaw [16]. Moreover, patients treated with clopidogrel who are carriers of the *CYP2C19* defective alleles have an increase in death from cardiovascular causes and an increase in stent failures [5].

Another factor contributing to inter-individual variability in expression of the CYP2C proteins is their inducibility after exposure of humans to xenobiotics. Studies *in vitro* in primary human hepatocytes clearly indicate that the expression of CYP2C enzymes is induced by prior exposure to various drugs, including glucocorticoids, rifampicin, paclitaxel and phenobarbital [17,18]. Moreover, studies *in vivo* are consistent with changes in the half-life of CYP2C substrates (e.g. tolbutamide, glipizide, *S*-mephenytoin) in man after prior exposure to drugs such as rifampicin [19-22]. This could potentially result in diminished effectiveness of the drug and possibly therapeutic failure.

Because of the pharmaceutical and physiological significance of the CYP2C enzymes, it is important to understand the transcriptional modulation of the constitutive and inducible expression of *CYP2C* genes to better understand the basis for inter-individual variability and predict adverse drug-drug interactions. This review will focus on the significant progress over the past few years in unraveling the molecular regulatory mechanisms for both the basal and drug-induced upregulation of human *CYP2C* genes in liver. The transcriptional regulation of *CYP2C* genes in extrahepatic tissues as well as in pathological situations is also discussed here.

Induction of CYP2C enzymes by drugs and xenobiotics

A number of clinical reports suggest that the metabolism of CYP2C9, CYP2C8, and CYP2C19 substrates is increased when humans are exposed to a variety of clinical drugs (see Table 1 for references). This induction after prior treatment with drugs results in a faster drug clearance rate, a shorter half-life, and a lower plasma level of drugs that are primarily metabolized by CYP2C enzymes, including coumadin, glyburide, and glipizide (CYP2C9), rosiglitazone and pioglitazone (CYP2C8), and *S*-mephenytoin and omeprazole (CYP2C19). Administration of some herbal medicines also induces the activity of CYP2C. For example, long-term treatment with St. John's wort, a widely used herbal antidepressant, decreased the plasma concentrations of coumadin and glipizide (CYP2C9) as well as *S*-mephenytoin and omeprazole (CYP2C19). Due to clinical concerns resulting from the induction of CYP2C enzymes by drugs, a careful

dose increase could be necessary for drugs which are CYP2C substrates to avoid therapeutic failure when co-administered with drugs that are inducers of *CYP2C* genes.

Information concerning the inducibility of *CYP2C* genes has been frequently obtained from *in vitro* induction studies in isolated human primary hepatocytes, which are cited in Table 1. With this cell model, it has been reported that CYP2Cs are induced significantly at the levels of mRNA, protein, and activity by therapeutic reagents, hormones such as glucocorticoid, vitamin D, and the endogenous metabolite lithocholic acid, which was shown to induce CYP2C8. Compared to other *CYP* genes such as *CYP3A4* and *CYP2B6*, which are strongly induced after exposure to drugs, the *CYP2C* genes are modestly induced (2-8 fold) [17,18, 23]. The inducibility of *CYP2C* genes in liver can be generally ranked as *CYP2C8* ≥ *CYP2C9* > *CYP2C19*. Certain molecules act as inducers for all three *CYP2C* genes, including phenobarbital, rifampicin, hyperforin (an active ingredient of St. John's wort), and dexamethasone. The induction of *CYP2C19* protein and mRNA shows high inter-individual variability in human livers. Polymorphisms in this gene and its low constitutive expression in uninduced liver contribute to this variability in induction.

Nuclear receptor-mediated transcriptional activation of *CYP2C* genes by drugs in the liver

The transcriptional activation of most P450 genes is mediated by drug responsive nuclear receptors, which are transcriptional factors sensing foreign substances. The nuclear receptors CAR and PXR contain a DNA binding domain (DBD) and a ligand binding domain (LBD). After activation by exposure to xenobiotics, the nuclear receptors bind to the responsive elements as monomers or homo- or hetero-dimers, recruit coactivators to affect chromatin structure, and increase the transcription of target genes [24].

Several nuclear receptors have been identified that mediate the xenobiotic-induced transcriptional activation of the human *CYP2C* genes (Table 2). The nuclear receptor CAR is responsible for the transcriptional activation of *CYP2C9* (in human primary hepatocytes and HepG2 cells), *CYP2C8* (in human primary hepatocytes) and *CYP2C19* (in HepG2 cells and primary hepatocytes). CAR agonists include drugs such as phenobarbital and artemisinin as well as the chemical CITCO {6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl oxime)}. CITCO is a high affinity ligand for human CAR (hCAR) which activates hCAR in primary hepatocytes. But it only modestly increases promoter activity in the typical cell-based reporter assays, probably because CAR accumulates in the nucleus in immortalized cells while it is found primarily in the cytoplasm in primary hepatocytes and liver [25]. CAR is constitutively active without ligand, and many xenobiotics (phenobarbital and phenytoin) act primarily by causing its nuclear translocation rather than acting as ligands [26-28]. Another receptor, the human pregnane X receptor (PXR), has been shown to mediate induction of the *CYP2C* genes by drugs such as rifampicin, artemisinin, and hyperforin, all of which act as ligands for PXR. Dexamethasone, a glucocorticoid mimic drug activates the *CYP2C* promoters in HepG2 cells via the glucocorticoid receptor (GR). The Vitamin D receptor (VDR) has been reported to produce a modest 2-fold induction of *CYP2C9* in human primary hepatocytes by 1 α ,25-dihydroxyvitamin D₃ [29]. It may also mediate the induction of *CYP2C8* by lithocholic acid (LCA) in HepG2 cells [30].

CAR, PXR and VDR form heterodimers with the retinoid X receptor (RXR) while GR forms homodimers which are recognized by specific response elements within the *CYP2C* promoters. A typical nuclear receptor response element is composed of two half sites related to the hexamer AGGTCA separated by 3-6 bases. Fig (1) presents responsive elements within the *CYP2C9*, *2C8* and *2C19* upstream promoter regions that have been identified as binding sites for CAR, PXR, GR and VDR *in vitro* by gel shift assays [29,31-35]. The response elements of the

CYP2C genes exhibit similar but distinct features. Both the *CYP2C9* and *2C19* promoters contain a single similar proximal direct repeat spaced with 4 bp nucleotides (DR4) CAR/PXR-RE (located at -1839/-1824 from the translation start site of *2C9* and -1892/-1877 from the translation start of *2C19* respectively), differing by one nucleotide at the 3' prime end [35]. Both sites showed strong binding to CAR and PXR *in vitro*, and exchange of these two elements between the two *CYP2C* promoter constructs did not alter the activation of these two promoters by CAR in a transient transfection assay (Chen, unpublished observations).

CYP2C9 harbors a second DR5 type CAR/PXR-RE at -2897/-2881 which binds CAR and PXR in gel shift assays [32]. At a similar location in the *CYP2C8* promoter there is a DR4 (at -2796/2780) that binds CAR/PXR in gel shift assays but mutation of this element does not affect activation of the *CYP2C8* promoter in human hepatocytes by CAR or PXR agonists [33]. In the far upstream region of the *2C8* promoter, another DR4 element was identified at -8805/-8790 that strongly binds to CAR and PXR. Mutation of this element prevents activation of the *CYP2C8* in promoter by CAR or PXR agonists in human hepatocytes [33].

Additionally, the three *CYP2C* promoters harbor a putative DR3 type glucocorticoid response element (GRE) in their proximal regions (at -1697/-1682 for *2C9*, -1927/-1913 for *2C8*, and -1751/-1737 for *2C19*) [31,33,35], and the *2C9*-GRE was shown to bind hGR in gel shift assays [31]. The core sequences of the GREs are identical for *CYP2C9* and *2C19*, with a few nucleotides differing in the 5' flanking region. One base pair in the 5' half site of the GRE of the *CYP2C8* promoter differs from the GREs of *2C9* and *2C19*, which results in a change from TGA ACT (*2C9* and *2C19*) to TTA ACT. The proximal CAR/PXR-RE of *2C9* has also been shown to bind VDR *in vitro*.

To evaluate the responsiveness of the *CYP2C* promoters to induction by xenobiotics and the functionality of putative responsive elements, transient transfection has usually been performed in hepatic carcinoma cell lines such as HepG2 or human primary hepatocytes. *CYP2C9* and *2C19* promoters are significantly activated by cotransfection of CAR, PXR (in the presence of rifampicin), and GR (in the presence of dexamethasone) in HepG2 cells [31-35]. Unlike *CYP2C9* and *2C19*; however, induction of the *2C8* promoter by CAR and PXR ligands was observed in human primary hepatocytes but was not observed in HepG2 cells [33], suggesting the possibility that certain factors that are necessary for *CYP2C8* induction in primary hepatocytes are low or absent in HepG2 cells.

Both CAR/PXR-REs appear to contribute to activation of the *CYP2C9* promoter by PXR and CAR, but the site at -1839 is more important. For example, mutation of the CAR/PXR-RE at -2897 alone decreased rifampicin/PXR activation by ~30%, while mutation of the PXR binding site at -1839 bp alone nearly abolished rifampicin/PXR mediated promoter activation [34]. This data suggests that the site at -1839 bp is essential for induction, while the site at -2897 cooperates with the site at -1839 bp. The CAR/PXR-RE at -1839 is further shown to be required for transactivation in the context of a 12kb *CYP2C9* promoter by PXR and rifampicin in HepG2 cells [34].

Although activation of the *CYP2C19* promoter by CAR and PXR/rifampicin in HepG2 cells was more modest than the activation of the *CYP2C9* promoter, mutation of the CAR/PXR-RE at -1892/-1877 completely abolished this activation. Mutation of the CAR/PXR-RE of *CYP2C8* at -8805/-8790 completely abolished induction of *CYP2C8* promoter activity by CITCO and rifampicin in primary human hepatocytes, but mutation of the putative site at -2796/-2780 had no effect on promoter activation, suggesting that only the distal site is involved in activation of the *CYP2C8* gene by CAR and PXR ligands.

Each *CYP2C* promoter has also been shown to be activated by GR and its ligand dexamethasone via one GRE which is located within the first 2kb of the three promoters. The induction by dexamethasone was much higher for *CYP2C9* (>15-fold) than for *2C8* and *2C19* (3-fold and 4-8 fold respectively) in transfection assays in HepG2 cells [31,33,35]. Mutation of the GR elements of *CYP2C9*, *CYP2C19*, and *CYP2C8* abolished dexamethasone induction [31,33, 35]. The differing extent of dexamethasone induction among the three *CYP2C* genes is independent of the element itself, since *2C9* and *2C19* share an identical GRE. Possibly promoter context or nucleotides flanking the GRE could play a role. Only the *CYP2C9* gene has been examined for upregulation by the VDR ligand 1,25-(OH)₂D₃ in human primary hepatocytes [29]. The proximal CAR/PXR-RE at -1839/-1824 binds VDR *in vitro*. When this element was linked to the TK promoter and transfected into HepG2 cells, a modest but reproducible induction (~1.8 fold) by 1,25-(OH)₂D₃ was observed in VDR-transfected HepG2 but not in VDR-nontransfected cells. However, the TK promoter is a strong promoter, and the role of this VDR-RE in the induction of *CYP2C9* by 1 α , 25-dihydroxyvitamin D₃ has not been confirmed in the context of the original *CYP2C9* promoter.

It is of note that the CAR/PXR-REs in the promoters of all three human *CYP2C* genes are activated by both CAR or PXR, and gel shift assays confirm that both receptors bind strongly to the identified responsive elements in the human *CYP2C* gene promoters. These data suggest a symmetrical cross-talk between CAR and PXR in upregulation of human *CYP2C* genes, though CAR appears much more important for induction of the murine genes *Cyp2c29* and *Cyp2c37* based on studies in CAR and PXR knockout mice [26,36]. A similar cross-talk could occur between VDR and CAR/PXR for the expression of *CYP2C9*, since all three receptors are reported to bind to the proximal CAR/PXR-RE. A corresponding mutual inhibition of induction of the *CYP2C9* gene by PXR ligands and vitamin D may occur, as has been observed for *CYP3A4*, where two PXR binding sites (the distal DR3 motif and proximal everted repeat separated by six base pairs (ER6) bind VDR competitively [29].

Transcriptional regulation of the constitutive expression of CYP2C enzymes in liver and pathological conditions

The human *CYP2C* enzymes are expressed primarily in the liver, and a number of liver-enriched transcription factors (LETs) have been shown to regulate the constitutive expression of P450 genes, including the hepatic nuclear factors HNF1 α , HNF4 α , HNF3 γ , HNF6, C/EBP, and DBP as summarized in Table 3. The retinoic acid-related orphan receptors (RORs) have recently been identified as receptors which regulate *CYP2C8*. [37].

HNF4 α , an orphan nuclear receptor primarily expressed in the liver, kidney, intestine and pancreas, is well known to play a significant role in the regulation of many P450 genes and HNF4 α binding sites (described as HepG2-specific P450 2C factor-1 (HPF-1) motifs) were first discovered in rabbit *CYP2C* genes by Kemper and coworkers [38]. Using adenoviral HNF4 α antisense RNAs, Jover et al. [39] were able to reduce endogenous HNF4 α and observed a significant (40-45%) reduction of *CYP2C9* mRNA content in human primary hepatocytes. A slight but significant decrease in the mRNAs of *CYP2C8* and *2C19* was observed with adenoviral siRNA for HNF4 α in primary human hepatocytes [40]. These data indicate that HNF4 α influences the constitutive expression of all three *CYP2C* genes. The expression levels of *CYP2C8*, *2C9*, and *2C19* were recently found to be strongly associated with HNF4 α content in a study with 20 human liver samples (partial regression analysis), further supporting the role of HNF4 α as a predominant regulator for the basal *CYP2C* gene expression in human liver [41].

HNF4 α binds as a homodimer to a DR1 type element and also to the HPF-1 motif (5' RRRNCAAAGKNCANY) [42]. These sites are present in the basal promoters of all human

CYP2C genes except *CYP2C18* (Fig. 1). Both *CYP2C9* and *2C19* have two identical HPF-1 motifs located at a similar site in their promoters (at -150/-138 and -185/-173 for *2C9*; at -152/-140 and -187/-175 for *2C19*) [43,44]. Gel shift assays show that, both *in vitro* translated HNF4 α protein and nuclear extract from HepG2 cells bind to these sites, with the distal element displaying weaker binding than the proximal one. However, the *CYP2C9* basal promoter was significantly activated by HNF4 α when cotransfected in human hepatocarcinoma FLC7 and HepG2 cells, while the 2kb of *2C19* basal promoter was not [43]. A chromatin immunoprecipitation (ChIP) study showed that HNF4 α associates with the basal *CYP2C9* promoter region *in vivo* in human liver but was not detected in association with the *CYP2C19* promoter. Based on these results, it was proposed that *CYP2C19* is expressed at lower levels than *CYP2C9* in liver due to the lack of sufficient HNF4 α binding to the two HNF4 elements within the basal *CYP2C19* promoter. However, it is not clear why the basal *CYP2C19* promoter is not activated by HNF4 α , since it contains *two* HNF4 sites identical to those found in *2C9*. One HPF-1 motif has been also identified in the *CYP2C8* promoter at -152/-140 that interacts with HNF4 α *in vitro*. Again, cotransfected HNF4 α does not enhance the activity of the *CYP2C8* promoter in HepG2 cells, but does transactivate the *2C8* promoter construct in HeLa cells [33]. Recent unpublished studies in our laboratory have identified a second HPF-1 motif in the *CYP2C8* promoter.

In HepG2 cells, where HNF4 α is expressed endogenously, the HPF-1 motif at -185/-173 appears to be critical for HNF4 α activation of *CYP2C9*, while mutation of a site at -150/-138 bp leads to a significant reduction in HNF4 α activation. However, in the FLC7 cells in which HNF4 α is not expressed, Kawashima et al [43] observed that both HNF4 α responsive elements contributed equally to activation of the *CYP2C9* gene by HNF4 α . They also found that the region from -255/-195 bp of the *CYP2C9* promoter was necessary for HNF4 α to up-regulate the transcription of the *CYP2C9* gene and suggested that some other factors might bind to this region and assist HNF4 α in this upregulation. Consistent with their results, we recently reported preliminary results identifying a third HNF4 α binding element at -211/-199 of the *CYP2C9* promoter (Chen, unpublished data). This element aligns in the *CYP2C* promoter in a reverse orientation with respect to the other two proximal HNF4 α sites (Fig. 1) and specifically binds nuclear proteins from HepG2 cells as well as *in vitro* transcribed and translated HNF4 α protein. Three nucleotides in the core motif of a similar element within the *CYP2C19* promoter differ from that of *CYP2C9*, and this difference results in a weaker interaction between this element and HNF4 α in gel shift assays. When these three nucleotides were introduced into the *CYP2C9* promoter, *CYP2C9* activation by HNF4 α in HepG2 cells was 50% lower (unpublished data), but these results still do not completely explain the relative unresponsiveness of *CYP2C19* to HNF4 α compared to that of *CYP2C9*.

HNF3 γ and CCAAT/enhancer-binding protein α (C/EBP α) are two other liver-enriched transcriptional factors implicated in regulating the constitutive expression of the *CYP2C* genes in the liver [45,46]. During the isolation and culture of hepatocytes, these two factors have been found to be greatly downregulated, along with a concomitant downregulation of the expression of *CYP2C9* [46]. C/EBPs are basic leucine zipper (bZIP) transcription factors with a DNA-binding basic region and a leucine zipper dimerization domain. Homo- or heterodimerized C/EBPs recognize the CCAAT box motif in the promoter region and have been found to regulate the transcription of genes involved in the differentiation of hepatocytes. One factor, C/EBP α , begins to decay in a very early stage of primary hepatocyte culture and continues to decay very rapidly [46]. Moreover, in HepG2 cells the levels of C/EBP α mRNA are only ~15% of those in human hepatocytes, while the expression of all of three *CYP2C* genes is much lower in these cells than in liver. The re-expression of this factor in HepG2 cells increased the expression of *CYP2C9* (mRNA and protein) while the levels of other liver-enriched factors such as HNF4 α were not changed. These data further suggest that C/EBP α may play an important role in maintenance of the expression of *CYP2C* genes [46]. All of the three *CYP2C* promoters

harbor a CCAAT box in the 5' flanking region [2,47], and the deletion of this element significantly decreases the transcriptional activities of the *CYP2C9* promoter [48]. It still remains to be established to what extent C/EBP α regulates the constitutive expression of the *CYP2C* genes.

HNF3 γ , a member of the forkhead family of transcription factors, is expressed strongly in adult derivatives of the endoderm posterior to the liver [45]. These transcription factors bind to DNA as monomers and have a distinct conserved winged helix DNA-binding domain that is homologous to the *Drosophila* homeotic protein called Fork head. This factor also decays rapidly during the culture of human primary hepatocytes, although not as rapidly as C/EBP α , and the level of HNF3 γ mRNA in HepG2 cells is only ~25% of that found in liver [46]. Several putative HNF3 γ binding sites have been identified within the 5' flanking regions of the four human *CYP2C* genes [45]. The adenoviral expression of ectopic HNF3 γ in HepG2 cells resulted in an enhancement in endogenous mRNA levels of *CYP2C9* (4.5-fold) and *2C19* (50-fold), as well as *2C8* (20-fold) after cells were treated with a deacetylase inhibitor [45]. Promoter studies in HepG2 cells revealed that HNF3 γ activated the promoter activity of *CYP2C8* (25-fold), *2C9* (4-fold) and *2C19* (4-fold) [45]. Additional studies are needed to confirm the extent of the regulatory role of HNF3 γ in hepatic expression of individual *CYP2C* genes, such as whether knock-down of endogenous HNF3 γ reduces the expression of *CYP2C* genes, and which putative elements are required for HNF3 γ binding and its activation of the *CYP2C* promoters.

In addition, several other hepatic transcriptional factors have been shown to be implicated in the regulation of hepatic expression of some rodent *CYP2C* genes, including HNF1, HNF6, C/EBP β and albumin D-site binding protein (DBP) [38]. The extent to which these factors control the expression of human *CYP2C* genes remains uncertain.

Recently, we identified retinoid related orphan nuclear receptors (RORs) as new transcriptional regulators for *CYP2C8* but not *CYP2C9* or *CYP2C19* [37]. RORs are constitutively active orphan nuclear receptors. Some natural compound ligands such as cholesterol and all trans-retinoic acid have been found to bind to RORs and modulate their activity [49]. It has been shown that the expression of certain murine P450 genes including Cyp2c70 is altered in ROR knock-out mice [50]. We found that cotransfection of ROR α 4 and γ 1 significantly increased the promoter activity of a ~3kb construct of *CYP2C8* but not that of *CYP2C9* and *CYP2C19* in HepG2 cells. Two ROR-REs (at -2289 and -2045 bp within the *CYP2C8* promoter region) were identified which bound both ROR α 4 and γ 1 generated *in vitro*, but binding of the proximal site was stronger and mutagenesis studies confirmed that the proximal site was the essential one mediating the ROR activation of the *CYP2C8* promoter in HepG2 cells. Overexpression of either ROR α 4 or γ 1 elevated the endogenous *CYP2C8* mRNA in HepG2 cells and human primary hepatocytes, while knock-down of either endogenous ROR α 4 or γ 1 decreased the *CYP2C8* expression in HepG2 cells. RORs are also expressed in other extrahepatic tissues including the brain, where *CYP2C8* mRNA is preferentially expressed over other *CYP2C* mRNAs. The role of RORs in regulating *CYP2C8* in these extrahepatic tissues is not yet known.

The cooperativity of transcription factors and complexity in transcriptional regulation of human *CYP2C* genes

In addition to their direct interaction with the responsive element and regulation of the transcription of target genes, nuclear receptors often cooperate with each other or with other factors, such as coactivators and corepressors, to achieve precise modulation of target genes. Moreover, the expression of nuclear receptors can be regulated by endogenous or other receptors exogenous compounds, e.g., glucocorticoids induce the expression of CAR, PXR,

and RXR via a direct transactivation mediated by GR and the GR responsive elements within the promoter regions of these nuclear receptors, thus enhancing the expression of target genes including *CYP2C9* and *CYP2C8* [51,52]. HNF4 α is also known to increase CAR and fetal PXR as well [53-55]. On the other hand, the mRNA expression of CAR, PXR and RXR has been shown to be markedly decreased by the proinflammatory cytokines interleukin (IL)-1 β and IL-6. Consistent with these results, the constitutive and inducible mRNA expression of the typical CAR and PXR target genes *CYP2C9* and *2C8* are specifically inhibited by these cytokines in human primary hepatocytes [56,57]. Further studies demonstrated that the inflammatory stimuli by lipopolysaccharides (LPS) and IL-1 β caused the nuclear accumulation of NF- κ Bp65, which acts as an inhibitor of GR and trans-represses the activation of the CAR promoter by glucocorticoid and GR. A ChIP assay also revealed that dexamethasone induced histone H4 acetylation of the proximal CAR gene promoter, while both LPS and IL-1 β dramatically inhibited this increased acetylation in human primary hepatocytes [56]. However, recent work shows that the *CYP2C* genes are downregulated by different inflammatory cytokines in a gene-specific manner in human primary hepatocytes [58].

Recently, transcription factors and coactivators have been found to cooperate in the transcriptional regulation of human *CYP2C* genes (Fig. 2). A synergy between HNF4 α and CAR/PXR was first reported for the *CYP3A4* gene, where coexpression of HNF4 α and PXR dramatically increased the activity of the *CYP3A4* promoter in the presence of PXR ligands [54]. HNF4 α has also been shown to synergize with CAR and PXR to enhance the induction of *CYP2C9* mediated by these two nuclear receptors in HepG2 cells [44]. This synergy differs from that reported for the *CYP3A4* promoter, where the HNF4 α binding site essential for the synergy is immediately upstream of two CAR/PXR-REs within the distal XREM. The two HNF4 α sites in the *CYP2C9* promoter are located at -185 bp and -150 bp, far downstream of the CAR/PXR-RE (at -1839 bp). Mutation of the HNF4 α sites essentially abolished the drug induction of *CYP2C9* mediated by CAR and PXR, clearly indicating the HNF4 α sites are required for the drug responsiveness of the *CYP2C9* promoter. In contrast, rifampicin induction of *CYP3A4* remained when the HNF4 α site was mutated or deleted [54]. Because of the distance between the drug responsive element and HNF4 α binding sites in the *CYP2C9* promoter, an indirect cross-talk between the receptors was proposed as a likely underlying mechanism for the synergistic activation of the *CYP2C9* gene by HNF4 α and CAR/PXR. This cross-talk would bridge HNF4 α and CAR/PXR via cofactors or other transcriptional factors rather than involving direct interaction between the two nuclear receptors. This hypothesis has gained experimental support from a recent discovery that the nuclear receptor coactivator NCoA6 interacts with CAR and HNF4 α and appears to bridge the CAR-RE to the HNF4 α sites to cause a synergistic activation of the *CYP2C9* promoter in HepG2 cells (Fig. 2) [59]. ChIP analysis showed that NCoA6 interacted with both the HNF4 α sites and the CAR sites. Knockdown of NCoA6 disrupted this bridge and decreased the synergistic elevation in expression of *CYP2C9* mRNA by CAR and HNF4 α .

A number of coactivators are involved in the indirect modulation of the *CYP2C* genes. Coactivators are a class of protein factors which do not bind to DNA directly but interact with DNA binding transcription factors and are thus recruited to chromatin. Coactivators recruit histone acetyltransferases and methyltransferases to the promoter region where nuclear receptors bind and facilitate chromatin remodeling, allowing access of general transcriptional machinery to the promoter of the target gene. Two other coactivators have been implicated in the regulation of the *CYP2C* genes by interacting with the receptor HNF4 α : the nuclear receptor coactivator (SRC-1) and the peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1 α) [60]. Each coactivator activated the *CYP2C9* promoter when transfected into human hepatic carcinoma cells. PGC-1 α is a known coactivator for HNF4 α [61]. Activation of *CYP2C9* by PGC-1 α may depend largely on the presence of HNF4 α in HepG2 cells, since activation of the *CYP2C* promoter in HeLa cells was dependent on the presence of exogenous

HNF4 α . The deletion of a short *CYP2C9* basal promoter region which harbors the HNF4 α sites completely destroyed the activation of the *CYP2C9* promoter by HNF4 α as well as PGC-1 α [60]. It has been proposed that the large reduction of these two cofactors in human carcinoma cells results in a lower expression of *CYP2C9* compared to the level in liver or human primary hepatocytes. Consistent with this suggestion, viral-transduced PGC-1 α and SRC-1 significantly increased the amount of *CYP2C9* mRNA in these cells [60].

Both SRC-1 and PGC-1 α have been demonstrated to act as coactivators for other nuclear receptors such as GR, CAR, and PXR, as well as VDR which are known to regulate the induction of human *CYP2C* genes. They are thus possibly involved in the inducible transcription of *CYP2C* genes by coactivation of these nuclear receptors. Of note is that the PGC-1 α gene is responsive to energy metabolic homeostasis, induced in the liver by fasting and decreased by insulin. This suggests the possibility that target genes such as *CYP2C9* could also be regulated by factors that affect energy homeostasis. In fact, *CYP2C9* mRNA was decreased in HepG2 cells and human primary hepatocytes treated with insulin [60]. In sum, the transcriptional regulation of *CYP2C9* might be subject not only to environmental stimulation by xenobiotic drugs but also affected by various physical conditions such as fasting.

Transcriptional regulation of *CYP2C* genes in extrahepatic tissues and pathological conditions

Human *CYP2C* enzymes are widely distributed in a variety of extrahepatic tissues, but the level of human *CYP2C* transcripts and proteins in these tissues is lower than that in liver [1, 2]. Moreover, the pattern of expression of the individual *CYP2C* enzymes and transcripts differ in these organs, suggesting that the regulatory control of the *CYP2C* genes differs in various extra-hepatic tissues. However, the regulatory control of the *CYP2Cs* in extrahepatic tissues has received less study than that of liver.

In the human intestine, *CYP2Cs* are the second most abundant subfamily of P450 enzymes (25% of total immunoquantified P450s) [62]. Treatment with the PXR ligand rifampicin in healthy humans significantly increases the mRNA and protein level of *CYP2C9*, *2C8* and *2C19* as well as their enzymatic activity in the small intestine [63-65]. The order of inducibility is similar to that in hepatic *CYP2Cs*: *2C8*>*2C9*>*2C19* [64], but the induction response is reported to be weaker (about 1.5-fold at the mRNA level) in the small intestine than in the liver, as quantified using intestinal biopsies [65]. Notably, CAR, PXR, and HNF4 α are also expressed in the small intestine.

In the kidney, *CYP2Cs* are well known renal arachidonic acid epoxygenases, and their metabolites, EETs, play an antihypertensive role [66]. In human kidneys, the mRNAs and proteins of *CYP2C9* and *CYP2C8* have been detected [2,67], and *CYP2C8* has been suggested to be responsible for the generation of active renal vasodilatory epoxygenases (EETs) [68]. The expression and activity of murine renal Cyp2c44 and rat *CYP2C23* are decreased in several hypertension animal models (salt-sensitive [66], diet-induced [69], and obesity-related albuminuria [70]). Several lines of experimental evidence have suggested that increased levels of cytokines such as TGF- β and TNF α might be involved in downregulation of the renal *CYP2C* genes [71,72]. Recently, EETs have been identified as potent ligands for human PPAR α and - γ *in vitro* and shown to transactivate both receptors in human hepatic carcinoma cells [73]. The expression of murine renal Cyp2c44 was increased by ligands for PPAR α [74,75]. However, there is no human equivalent of Cyp2c44 [76,77], and at present there are no reports as to whether renal *CYP2C8* or *2C9* can be modulated by PPAR agonists.

In the brain, *CYP2C8* mRNA is expressed at a higher level than other *CYP2C* mRNAs, and *CYP2C8* mRNA is expressed at higher levels in brain than any other extrahepatic tissues we

tested (Delozier and Goldstein, unpublished). Low levels of CYP2C9 and 2C19 mRNAs were reported in the whole brain [2,78,79], where these enzymes could be implicated in the local metabolism of psychoactive drugs and xenobiotics as well as possibly in the regulation of the cerebral blood flow through production of EETs. mRNAs of *CYP2C* subfamily members such as CYP2C8 and 2C9 have also been identified in human astrocytoma cells [80]. Cocaine treatment reduced mRNAs or proteins of CYP2C8 and 2C9 in human U373 MG astrocytoma cells, along with a simultaneous downregulation of CAR and GR, two nuclear receptors which could be involved in this decrease [80]. RORs are newly identified as transcriptional regulators of *CYP2C8* in HepG2 cells [81]. ROR α and - β are well expressed in different regions of the brain, where they play a role in the control of circadian rhythm. It would be of interest to examine whether ROR and CYP2C8 are colocalized in the brain, and whether *CYP2C8* is upregulated by RORs in the brain.

Of note is the expression of *CYP2C8* and *2C9* in human endothelial cells (ECs) [82,83], where they metabolize endogenous arachidonic acid into vasoreactive EETs. CYP2C9 appears to be predominant in the heart, aorta, and cardiac vessels, while CYP2C8 is found in the heart [1]. EETs play critical roles in vascular homeostasis as endothelial-derived hyperpolarizing factors (EDHF) [84]. More importantly, they act as signal molecules that elicit multiple cellular activities, including promotion of endothelial cell proliferation, migration and angiogenesis [85]. Because of the cardioprotective role of EETs in cardiovascular disease [86], it is important to understand the regulation of the expression and activity of *CYP2C* genes in ECs. Accumulating evidence has demonstrated that the expression of the *CYP2C* genes in ECs is affected by multiple stimuli, such as hemodynamic and physio-chemical forces [87] as well as the glucocorticoid cortisol [88]. A dramatic enhancement in the expression of the *CYP2C* genes was reported to be elicited by the Ca²⁺ antagonist nifedipine in human umbilical endothelial cells (mRNA) and porcine coronary arteries (mRNA & protein) [89]. Some HMG-CoA reductase inhibitors, such as cerivastatin, fluvastatin, and lovastatin, have been found to induce the expression of CYP2C mRNA and protein in native and cultured endothelial cells, but not that of the *CYP3A* or *CYP2J* genes [90,91]. The mechanisms underlying the induction of the *CYP2C* genes by nifedipine remain to be elucidated. There have been suggestions that the induction by certain statins may be mediated by CAR. For example, CAR has been shown to be activated by statins including cerivastatin, fluvastatin, and atorvastatin in hepatocellular carcinoma FLC7 cells stably transfected with hCAR [92].

Chronic hypoxia has been reported to induce human *CYP2C* genes and angiogenesis in human endothelial cells [93]. It is also known that hepatic expression of *CYP2C* genes is markedly increased (including mRNA, protein, and activity) in sudden infant death syndrome (SIDS) patients with an unchanged overall hepatic P450 content although the mechanism is unknown [94,95]. Recently, the expression of *CYP2C8* or *2C9* mRNA and production of EETs were found to be augmented in human endothelial cells upon exposure to hypoxia [93]. The activity of the *CYP2C9* promoter was also reported to be modestly enhanced by hypoxia treatment in human endothelial cells [93]. The mechanism of this proposed upregulation of the transcription of the *CYP2C* genes has not been defined. The vascular endothelial growth factor (VEGF) plays a key regulatory role in physiological and pathological angiogenesis. Hypoxia induces its expression by stabilizing the hypoxia inducible factor-1 (HIF-1), which binds to the hypoxia response element (HRE) within the VEGF promoter and strongly enhances its transcription. VEGF was recently reported to activate the promoter of *CYP2C9* and enhance the expression of *CYP2C8* mRNA and protein in endothelial cells [96]. This enhanced expression is dependent upon the phosphorylation of the AMP-activated protein kinase (AMPK), an energy sensor which is activated under stress situations such as hypoxia. Over-expression of wild type AMPK increased CYP2C expression without VEGF, while the dominant negative AMPK mutant prevented induction of CYP2C expression by VEGF. In the liver, AMPK is activated by PB and some PB-type drugs [97] and this induced AMPK activity has been reported to be essential

for the PB induction of P450 in human hepatoma cells and primary hepatocytes [98-100]. Intracellular production of the mitochondrial reactive oxygen species (ROS) triggered by PB seems to be necessary for AMPK activation and induction of P450s by PB since interference with ROS production diminished the phosphorylation of AMPK and decreased PB induction of P450 genes in male leghorn chick hepatoma LMH cells [99].

Interestingly, the CYP2C8/9 metabolite 11, 12-EET was recently shown to increase the level of HIF-1 α in human umbilical artery endothelial cells (HUAEC) and human hepatoma cells (Hep3B), possibly via the stabilization of HIF-1 α [101]. Induction of VEGF mRNA by hypoxia was enhanced by overexpression of CYP2C8 but efficiently inhibited in HUAEC by sulfaphenazole (10 μ M), a high affinity inhibitor of CYP2C9. Although sulphaphenazole also inhibits CYP2C8, the IC₅₀ for CYP2C8 is two orders of magnitude lower (IC₅₀ 130 μ M) than for CYP2C9 (IC₅₀ 0.6 μ M) [102]. However, the activity of the luciferase promoter containing the hypoxia response element (HRE) from the VEGF promoter as an enhancer was induced by exogenous 11, 12- EET but suppressed by 10 μ M sulfaphenazole under hypoxia in HUAEC [101].

Although a self-positive feedback mechanism could be proposed for the induction of *CYP2C* by hypoxia, it is unclear how EETs increase HIF-1 α protein and how phosphorylated AMPK activates the transcription of the *CYP2C* genes. mRNA of HIF-1 α was not increased by EETs [101]; therefore the observed enhancement in induction of HIF-1 α proteins by EETs under hypoxia is not due to augmented transcription. EETs have been shown to activate the PI3K/Akt pathway to promote tube formation [103], while this pathway has been shown to be required for protection of HIF-1 α from degradation [104]. Possibly EETs could stabilize HIF-1 α via activation of the PI3K/Akt pathway to induce the expression of VEGF. More research is needed to clarify the possible effects of hypoxia on *CYP2C* genes and the mechanism(s) involved.

Conclusions

Human CYP2C enzymes metabolize 20% of clinical drugs and also metabolize arachidonic acid to produce EETs, important endogenous signal molecules that regulate many physiological processes such as vasodilation and angiogenesis. The expression of *CYP2C* genes is transcriptionally upregulated by exposure to xenobiotics. Drug-responsive nuclear receptors as well as hepatic transcriptional factors bind to cis elements within *CYP2C* gene promoters to regulate the transcription of *CYP2C* genes. HNF4 α is probably the most important receptor for upregulating the constitutive expression of the CYP2Cs in liver. Variability in expression of the CYP2C enzymes has been shown to correlate with levels of HNF4 α in human liver. Moreover, cross-talk between HNF4 α sites and PXR/CAR sites appears to be necessary for optimal induction in response to drugs. Other regulatory factors, such as coactivators, corepressors, and signal pathways indirectly modulate the expression of human *CYP2C* genes. Very little progress has yet been made on the transcriptional regulation of the extrahepatic CYP2Cs.

Animals carrying both transgenic human nuclear receptors and human *CYP2Cs* would be a promising experimental model for better understanding the transcriptional regulation of human *CYP2C* genes *in vivo* [105], due to the lack of direct orthologs for human *CYP2C* genes in animals. There are also ligand/agonist differences between rodent and human nuclear receptors such as PXR and CAR; therefore, it would be beneficial to use mice with humanized nuclear receptors. For example, Scheer and coworkers [106] have established murine lines with human PXR and human CAR. These mice could be used to establish human *CYP2C* models. Human primary hepatocytes grown under different matrices also remain an *in vitro* model for

answering some of these questions. Future studies will undoubtedly address how pathological/physiological conditions and stresses perturb *CYP2C* expression.

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Abbreviation

AMPK AMP activated protein kinase

CAR	the constitutive androstane receptor
C/EBP α	CCAAT/enhancer-binding protein α
CITCO	[6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime]
DR	direct repeat
EETs	epoxyeicosatrienoic acids
ER	everted repeat
GR	the glucocorticoid receptor
HEETs	hydroxyeicosatrienoic acids
HIF-1	hypoxia inducible factor-1
HNF	hepatic nuclear factor
HPF-1	HepG2-specific P450 2C factor-1
HRE	hypoxia responsive element
LCA	lithocholic acid
PGC-1 α	the peroxisome proliferator-activated receptor gamma coactivator-1 α
PI3K/Akt	phosphoinositide 3-kinase/protein kinase B
PPAR	the peroxisome proliferator-activated receptor
PXR	the pregnane X receptor
RE	responsive element
ROR	Retinoic acid related-related orphan receptors
ROS	reactive oxygen species
RXR	the retinoid X receptor
siRNA	silencing RNA
SRC-1	the nuclear receptor coactivator-1
VDR	the vitamin D receptor
VEGF	vascular endothelial growth factor
XREM	xenobiotic response element

Figure 1.

Summary of the known response elements for nuclear receptors regulating the *CYP2C8*, *2C9*, and *2C19* genes. This figure summarizes our current view of the regulatory elements within the three human *CYP2C* gene promoters. The hexamer sequences are shown in capital letters for each element along with their exact locations within each promoter. The arrows indicate the direct repeat of each element. The elements which bind nuclear receptors in vitro but are identified as nonfunctional by mutagenesis in luciferase promoter studies are shown with grey lines. Newly identified HNF4α sites in *CYP2C9* and *CYP2C8* are indicated with dark parallel lines (Chen unpublished).

Figure 2.

Interactions between nuclear receptors and coactivators precisely modulate the transcription of *CYP2C9*. The coactivator NcoA6 bridges the HNF4 α binding site(s) within the basal promoter and the proximal CAR/PXR-RE site by interacting with HNF4 α and CAR, producing a synergistic transactivation of the *CYP2C9* promoter. The coactivators PGC-1 α and SRC-1 also interact with HNF4 α , and probably also with GR, to regulate the activity of the *CYP2C9* promoter.

Table 1
Induction of human CYP2C in liver

CYP2C Genes	Transcription Inducers	Clinical Studies	<i>In vitro</i> Studies
CYP2C9	Phenobarbital		[17,18,23]
	Rifampicin	[22,107,108]	[17,18,23,109]
	Hyperforin & St John's wort	[110,111]	[112]
	Avasimibe	[113]	[113]
	Ritonavir, Nelfinavir & Lopinavir	[114,115]	[116]
	Dexamethasone		[17,18]
	Cyclophosphamide or Ifosfamide		[117]
	Nifedipine, Nicardipine, BK8644 & Isradipine		[118]
	Carbamazepine	[119]	
	Aprepitant	[120,121]	
CYP2C8	Phenobarbital	[122]	[17,18,23,33]
	Phenytoin	[123]	[33]
	Rifampicin	[124-126]	[17,18,23,109]
	Hyperforin		[33,127,128]
	Dexamethasone		[17,18,23,33]
	Ritonavir & Nelfinavir		[116,127]
	Cyclophosphamide or Ifosfamide		[117]
	Lithocholic acid		[33]
	Paclitaxel	[129]	[33,130]
	Gemfibrozil, Fenofibric acid & Clofibric acid		[131]
CYP2C19	Ritonavir & Nelfinavir		[116]
	Rifampicin	[132,133]	[17,23,109]
	Hyperforin & St. John's wort	[134,135]	
	Dexamethasone		[17]
	Artemisinin	[136]	[137]

Table 2
The transcriptional regulation of human *CYP2C* genes by nuclear receptors and their inducers in liver

CYP2C Genes	Nuclear Receptors	Inducers
CYP2C9	PXR	Rifampicin [32,34], Hyperforin [34], Phenobarbital [34]
	CAR	Phenobarbital [31], CITCO [33]
	GR α	Glucocorticoid [31]
	VDR	Vitamin D [29]
CYP2C8	PXR	Rifampicin [33], Retonavir [127]
	CAR	CITCO [33]
	GR α	Glucocorticoid [33]
	VDR	Lithocholic acid [30]
CYP2C19	PXR	Rifampicin [35], Artemisinin [137]
	CAR	Phenobarbital [35], Artemisinin [137]
	GR α	Dexamethasone [35]

Table 3
The hepatic transcriptional factors of human *CYP2C* genes

CYP2C Genes	HNF4α	HNF3γ	C/EBPα	RORα & γ
CYP2C9	↑ [39-41,44,48,60]	↑ [45]	↑ [46]	
CYP2C8	↑ [33,40,41]	↑ [45]		↑[37]
CYP2C19	↑ [40,41]	↑ [45]		