Expression of CYP3A4, CYP2B6 and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes

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List of abbreviations

CYP, cytochrome P450; FDP : fructose 1,6-biphosphatase, ANF : atrial natriuretic factor, RIF, rifampicin.

The official nomenclature system for the nuclear receptor superfamily has been used in this work (1): GR, glucocorticoid receptor (NR3C1); PXR, pregnane X receptor (NR1I2); RXR retinoid X receptor (NR2B1); CAR, constitutive androstane receptor (NR1I3); VDR, vitamin D receptor (NR1I1).
ABSTRACT

The fully active di-hydroxylated metabolite of vitamin D₃ (1α,25(OH)₂D₃) induces the expression of CYP3A4 and to a lesser extent of CYP2B6 and CYP2C9 genes in normal differentiated primary human hepatocytes. Electrophoretic mobility shift assays and co-transfection in HepG2 cells using wild-type and mutated oligonucleotides revealed that the vitamin D receptor (VDR) binds and transactivates those xenobiotic-responsive elements (ER6, DR3 and DR4) previously identified in CYP3A4, CYP2B6 and CYP2C9 promoters and shown to be targeted by the Pregnane X Receptor (PXR) and/or the Constitutive Androstane Receptor (CAR). Full VDR-response of various CYP3A4 heterologous/homologous promoter-reporter constructs requires both the proximal ER6 and the distal DR3 motifs, as observed previously with rifampicin-activated PXR. Co-transfection of a CYP3A4 homologous promoter-reporter construct (including distal and proximal PXR-binding motifs) and of PXR or CAR expression vectors in HepG2 cells revealed the ability of these receptors to compete with VDR for transcriptional regulation of CYP3A4. In conclusion, this work suggests that VDR, PXR and CAR control the basal and inducible expression of several CYP genes through competitive interaction with the same battery of response elements.
INTRODUCTION

Cytochrome P450 enzymes (CYP) are mainly expressed in the liver and catalyze the metabolic conversion of xenobiotics, including environmental pollutants and drugs, to more polar and easily disposable derivatives (2,3). CYP genes from the CYP2 and CYP3 families are inducible by many xenobiotics including notably, barbiturates and rifampicin. Two nuclear receptors, the Pregnane X Receptor (PXR, NR1I2) and the Constitutive Androstane Receptor (CAR, NR1I3) have recently been shown to mediate CYP2 and CYP3 gene induction in animals and in man (4-6). Both PXR and CAR form heterodimers with the Retinoic Acid Receptor (RXR, NR2B1). PXR is activated by a wide spectrum of xenobiotics and steroids (4,7,8) and controls CYP3A4 and CYP3A7 induction by targeting two specific responsive elements present in the regulatory region of these genes (4,7-12). The first of these is the proximal PXR responsive element (pPXRE) located at –160. It consists of an everted repeat of the nuclear receptor half-site AGGTCA separated by 6 nucleotides (ER6); this element is necessary but not sufficient for full transactivation of the CYP3A4 promoter. Indeed, full PXR-mediated induction requires the presence of a second distal xenobiotic response element (dPXRE), located between –7800 and -7200 (9). This element is composite and consists of two direct repeats separated by 3 nucleotides (DR3), encompassing an ER6 motif. In contrast to PXR, CAR is sequestered in the cytoplasm and translocates into the nucleus upon activation, notably in response to phenobarbital (6,13). Several groups have identified a complex phenobarbital-response element module (PBREM) which consists of two nuclear receptor binding sites (termed NR1 and NR2) and one NF1 binding site (12,14). Both NR1 and NR2 are imperfect DR4 motifs and essential for phenobarbital induction of CYP2B genes. In human CYP2B6, PBREM is located between –1684 and –1733, and has been shown to bind to and be transactivated by CAR and by PXR (12,15).
Previous reports revealed that 1α, 25-dihydroxy vitamin D3 (1α,25(OH)2D3), the most active metabolite of vitamin D3, behaves as a transcriptional inducer of *CYP3A4* in the colic carcinoma Caco-2 cell line and in human intestinal LS180 cell line (16,17). Vitamin D3 function is mediated through the vitamin D receptor (VDR, NR1I1) which, after binding 1α,25(OH)2D3 with high affinity forms heterodimers with RXR (18-20). The heterodimer then binds to and transactivates the vitamin D response elements (VDRE) present in the regulatory region of target genes (21). The classical VDREs consist of a direct repeat of nuclear receptor half-sites separated by three nucleotides (DR3) (18). In the classic vitamin D-responsive organs including the intestine, bone, kidney and parathyroid gland, vitamin D3-activated VDR plays a central role in the regulation of calcium and phosphate homeostasis, bone mineralisation and resorption, inhibition of cell growth and parathyroid hormone synthesis (22). VDR is also expressed in many other non-classic vitamin D-responsive organs including the liver, muscle, skin, immune system, pancreas, brain and cancer cells (23) in which it controls a number of biological processes including immunomodulation, tissue regeneration, inhibition of cell growth and apoptosis, and cell differentiation (24-26).

In an exploratory part of this work we found that 1α,25(OH)2D3 is an inducer of *CYP3A4* in human hepatocytes, as previously observed by others in intestinal cell lines (16,17). We therefore thought that VDR might be able to target PXR- and/or CAR-responsive elements of *CYP3A4*. We further reasoned that, if the hypothesis is correct, 1α,25(OH)2D3 could be an inducer of other *CYP* genes controlled by these receptors. The data presented here show that 1α,25(OH)2D3 not only induces *CYP3A4*, but also *CYP2B6* and *CYP2C9* in primary human hepatocytes. In addition, we show that VDR is able to bind and transactivate different motifs recognized by xenobiotic-activated PXR and CAR, in the promoter of these *CYP* genes.
MATERIALS AND METHODS

Materials

Ham F-12 and William’s E culture media, 1α,25(OH)2D3, vitamins and hormones, collagenase (Type IV), dimethylsulfoxide (DMSO), and dexamethasone were purchased from Sigma (St Quentin Fallavier, France). Collagen-coated culture dishes were obtained from Corning (Iwaki, Japan). α-(32P) dCTP, α-(32P) UTP and ECL developing reagents were purchased from Amersham-Pharmacia (Buckinghamshire, England).

Plasmids

The ΔATG-hPXR expression plasmid, was generated by PCR amplification of cDNA encoding amino acids 1-434 of hPXR (kindly provided by Dr S Kliewer, Glaxo-Wellcome, Research Triangle Park NC) using oligonucleotides 5′-GGGTGTGGGGAA-TTTACCACCATGGAGGTGAGACCCAAAGAAAGC and 5′-GGGTGTGGGGGATCCTCAG-CTACCTGTGATGCCG and insertion into pSG5 digested with EcoR1/BamH1. The mCAR expression vector (pCR3-mCAR) was kindly provided by Dr M Negishi (NIEHS, Research Triangle Park, NC).

Homologous construct plasmids. The CYP3A4 5′-flanking fragment (-262/+11), containing the pER6 element, was generated by PCR from a previously isolated genomic clone (27) used as a template, and from oligonucleotides which create artificial cloning sites in 5′ (Kpn I) and 3′ (Sma I). This fragment was cloned into pGL3-basic (Promega, Madison, WI) upstream of a luciferase reporter gene to generate the homologous construct plasmid p(3A4-pER6)-LUC. Plasmids p(3A4-5′dDR3/dER6/3′dDR3/pER6)-LUC, and p(3A4-5′dDR3/dER6/pER6)-LUC were generated by inserting the -7800/-7200 or -7800/-7600 region of CYP3A4 (9) amplified by PCR from human genomic DNA, into plasmid p(3A4-pER6)-LUC digested with Kpn1.
**Heterologous construct plasmids.** Plasmids p(3A4-5’dDR3/dER6/3’dDR3)-tk-LUC and p(3A4-5’dDR3/dER6)-tk-LUC were constructed as indicated above for homologous constructs except that the amplified regions were cloned in pGL3-basic upstream of the luciferase reporter gene, driven by the thymidine kinase (tk) promoter. Plasmid p(2C9-(DR4)₄)-SV40-LUC was generated by cloning four copies of the oligonucleotide 2C9-DR4 (28) upstream of a luciferase reporter gene driven by the SV40 promoter in pGL3 vector. Plasmid p(2B6-(NR1)₃)-tk-LUC was generated by cloning three copies of the NR1 (2B6-3’DR4) motif of human CYP2B6 (12) upstream of a luciferase reporter gene driven by the tk promoter in pGL3 vector. Plasmids p(3A4-(dDR3)₃)-tk-LUC and p(3A4-(pER6)₃)-tk-LUC were generated by cloning three copies of the respective motif of human CYP3A4 (9) upstream of a luciferase reporter gene driven by the tk promoter in pGL3 vector.

**Cell culture and transfections**

HepG2 cells (Human hepatocarcinoma) purchased from the European Collection of Cell Cultures (Salysbury, England) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 100µg/ml penicillin and 100µg/ml streptomycin (Life Technology, Inc.). Transfection of plasmid DNA was performed in single batches with Fugene-6 (Roche Diagnostics Corporation, Indianapolis, USA) as recommended by the manufacturer. Transfections were performed using 100,000 cells, 250 ng of reporter plasmid and 50ng of pSG5-hVDR expression vector (provided by P. Balaguer, INSERM U439, Montpellier). For competition experiments, we used 500ng of reporter plasmid and 100ng of pSG5-hVDR expression vector. Cotransfection of hPXR or mCAR were performed using increasing concentrations (10, 50, 100, 300ng) of both expression vectors and pSG5 (Stratagene, La Jolla, CA) empty vector was added to normalize the total concentration of transfected plasmid DNA. As an internal control of transfection, 25ng of pSV-galactosidase
(Progema Madison WI) was used in all experiments. After 16 hours, the medium was changed, and fresh medium containing 0.1% dimethylsulfoxide or inducers were added. Cells were harvested in reporter lysis buffer (Promega, Madison, WI) after 24 hours incubation, and cell extracts were analysed for luciferase and β-galactosidase activities as described elsewhere (11).

**Liver samples and hepatocyte cultures**

Hepatocytes were prepared from liver lobectomy segments resected from adult patients for medically required purposes unrelated to our research program. The use of these human hepatic specimens for scientific purposes has been approved by the French National Ethics Committee. Three different cultures from three different liver donors were made in this work: FT181 (51 year-old male who became an organ donor after a car accident, liver not transplanted because of the presence of a kidney tumor), FT187 (67 year-old male who underwent a liver lobectomy for a metastasis of a colon tumor) and FT189 (48 year-old male who underwent a liver lobectomy for a metastasis of a sigmoid colon tumor). Hepatocytes were prepared and cultured according to the previously published procedure (29,30). The cells were plated into 100-mm plastic dishes precoated with collagen at 10x10⁶ cells per plate in a total volume of 6 ml of a hormonally and chemically defined medium consisting of a mixture of Williams’E and Ham F12 (1:1 in volume). Forty-eight hours after plating, cells were cultured in the presence of the indicated concentrations of 1α,25(OH)₂D₃ for an additional 24 hours. Total RNA and protein were isolated using Trizol reagent (Gibco BRL, Cergy-Pontoise France) according to the manufacturer’s instructions.
RT-PCR experiments

Reverse transcription was performed from 1 µg of mRNA using the MMLV-RT Life Technology Kit (Gibco BRL, Cergy-Pontoise France) according to the manufacturer’s instructions. One tenth of the RT reaction was then subjected to PCR.

Quantitative PCR: Quantification of CYP3A4, CYP2B6, CYP2C9 and GAPDH mRNA was performed using the Roche Light Cycler apparatus. For CYP3A4, CYP2B6, and GAPDH the following program was used: denaturation step 95°C, 8 min; 40 cycles of PCR (denaturation 95°C, 15 sec; annealing 70°C, 6 sec; extension 72°C, 12 sec). In all cases, the quality of the PCR-product was assessed by monitoring a fusion step. For CYP2C9: the same program was used, except for the annealing which was performed at 60°C. Sense and reverse primers were as follows, respectively: CYP3A4: 5’-CACAAACCGGAGGCTTTTG-3’ and 5’-

ATCCATGCTGTAGGCCCCAA-3’,
GAPDH: 5’-GGTCGGAGTCAACGGATTTGGTGC-3’ and 5’-
CAAAAGTGTGCTATGGATGACC-3’,
CYP2B6: 5’-GGCCATACGAGGCAGCCCTT-3’ and 5’-AGGGCCCCTTGGATTTCCG-3’,
CYP2C9: 5’-TCCTATCCATTACTTCTCC-3’ and 5’-
AACTGCACTGTTTCTCAAGC-3’.

FBP: 5’-CCCCGCGCTCTACCCGGTTCA-3’ and 5’-TGTGTGAGACAAAAGGTCCA-3’ (31).

In vitro translation and electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed using VDR and RXR prepared by in vitro translation using a transcription-translation coupled system (Promega). Proteins were incubated for 20 minutes at room temperature with 50,000 cpm of T4 polynucleotide kinase-labelled oligonucleotides in 10 mM Tris (pH 8), 6% glycerol, 1mM DTT, 1µg/µl poly-(dI-dC)
(Pharmacia, Orsay, France). The mixture was then submitted to electrophoresis using a 4% polyacrylamide gel in 0.5x TBE (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA). The following oligonucleotides were used either as radiolabelled probes or as competitors (sense strand is shown, with hexanucleotides in bold):

CYP3A4-pER6: 5’-TAGAATA TGAACT CAAGGAG GTCA GTGAGT-3’;
CYP3A-5’dDR3: 5’-GAATGA ACCT GTGACCC CTCTTCT-3’;
CYP3A4-5’dDR3mutant: 5’-GAATGCCCATG CTAATCT TTCTTCT-3’,
CYP3A4-dER6: 5’-CCCTTGAAAATCATGC GGTTCA AGCA-3’
CYP2B6-DR4: 3’-ACTGTACT TTCTGACC CTGAGA AGGA-5’,
CYP2C9-DR4: 5’-AACCAA GCTCTTGTAC CCCTCTCAATCTAGTCAAC TGGG-3’,
Rat Atrial Natriuretic Factor VDRE: 5’-GTCAG AGGTCA TGA AGGACA TTACA-3’ (32).

Anti-RXRα (N197, sc 774 X, Santa Cruz Biotechnology) was used for the “supershift” assays. Autoradiography was carried out by exposing the dried gel to Kodak X-AR film.
RESULTS

Induction of CYP genes by 1α,25(OH)₂D₃ in human hepatocytes

Forty eight hours after plating, hepatocytes were treated either with increasing concentrations (1 to 100nM) of 1α,25(OH)₂D₃ or, in parallel, with 10µM rifampicin, for 24 hours. Then, CYP mRNAs were analysed by both classical and real-time quantitative RT-PCR (Light Cycler, Roche). In a preliminary series of experiments, we verified that hepatocytes respond as expected to 1α,25(OH)₂D₃ in our culture model. For this purpose, the expression of fructose 1,6-biphosphatase gene (FBP), a gene known to be induced through VDR activation (31), was evaluated in response to increasing concentrations of 1α,25(OH)₂D₃. The results are shown in Figure 1. Expression of FBP mRNA as assessed by RT-PCR analysis was induced as expected. Analysis of the same RNA samples revealed that 1α,25(OH)₂D₃ was a potent and concentration-dependent inducer of CYP3A4 mRNA and a modest inducer of CYP2B6 and CYP2C9 mRNAs, the maximum accumulation being reached at 10 nM (Figure 1). Next, real-time quantitative RT-PCR was used to evaluate the induction ratios (mRNA levels in treated cells compared to control cells) obtained from the analysis of three different cultures from three different liver donors. Induction ratios were as follows: 15±2 for CYP3A4; 3.5±1 for CYP2B6 and 2.6±1 for CYP2C9 mRNAs. In comparison, rifampicin induction ratios were respectively: 50±15 for CYP3A4, 10±3 for CYP2B6 and 3.3±1.5 for CYP2C9 mRNAs. This last gene was recently shown to be positively regulated by rifampicin and phenobarbital through CAR/PXR activation (28,33). GAPDH mRNA levels used as quality controls of RNA preparations were not affected significantly by 1α,25(OH)₂D₃. The finding that 1α,25(OH)₂D₃ induces CYP mRNAs within the nanomolar concentration range suggested a classical vitamin D3 receptor-mediated mechanism of induction. Although the consensus VDRE is a DR3 motif, this nuclear receptor has been shown to bind other motifs including DR4, DR6 and inverted palindromes (32,34).
therefore suspected that CYP2B6, CYP2C9 and CYP3A4 induction by 1α,25(OH)2D3 could be mediated by VDR through the previously identified PXR and CAR responsive elements.

VDR-RXR heterodimer binds the PXR responsive elements of the CYP3A4 promoter

The PXR responsive elements of CYP3A4 consist of a proximal ER6 (-160), hereafter referred to as 3A4-pER6, and a distal enhancer (-7800/-7200) containing three nuclear receptor motifs, referred to hereafter as 3A4-5’dDR3, 3A4-dER6, and 3A4-3’dDR3 (Figure 2). These elements correspond to dNR1, dNR2 and dNR3, identified by Goodwin et al. (9), respectively. dNR1 and dNR3 have been reported to be the key elements conferring enhancer activity. Gel mobility shift assays were performed in order to determine whether VDR interacts with these elements.

First, we checked the binding of in vitro translated VDR-RXR heterodimer to a consensus VDRE oligonucleotide (rANF-DR3-type) by gel shift assay, as shown in Figure 3A. As expected, a retarded band was observed when both VDR and RXR were incubated with the target oligonucleotide (lane 4) but not when these receptors were incubated alone (lanes 2 and 3). Anti-RXR antibodies produced a supershift (lane 5) while an excess of unlabelled rANF DR3 oligonucleotide suppressed the retarded band (not shown). In addition, the specific VDR-RXR-DNA complex was suppressed, in a dose-dependent manner, when incubated in the presence of a 5- to 50-fold molar excesses of unlabelled 3A4-5’dDR3 (lanes 8-9) or 3A4-pER6 (lanes 10-11). This suggests that these elements can be targeted by the VDR/RXR heterodimer. In contrast, excess of the 3A4-dER6 oligonucleotide did not produce any suppression of the VDR/RXR-VDRE complex (lanes 6-7).

In the next series of experiments, we used the same assay to investigate the binding of VDR to both 3A4-5’dDR3 and 3A4pER6 oligonucleotides. As expected, no complex was observed when the probes were incubated with VDR or RXR alone (Figure 3B lanes 2-3, and
In agreement with data presented in Figure 3A, a retarded band was observed when 3A4-5’dDR3 (Figure 3B lane 4) or 3A4pER6 (Figure 3C lane 2) were incubated in the presence of the VDR/RXR complex, and anti-RXR antibodies produced a supershift of the band (Figure 3B lane 5 and 3C lane 3). The specificity of the interaction was confirmed by competition experiments using 10- and 100-fold molar excesses of unlabelled oligonucleotides, including the consensus rANF-DR3 or the 3A4pER6 (Figure 3B lanes 6-7 and 8-9, respectively, and Figure 3C, lanes 4-5).

**VDR-RXR heterodimer binds the PXR/CAR responsive elements of the CYP2B6 and CYP2C9 promoters**

A 51 bp sequence termed the phenobarbital-responsive element (PBRE) has been shown to be necessary and sufficient for phenobarbital induction of mouse Cyp2b10 gene (35-37). Sequence analysis of various CYP2B PBREs reveals the presence of two conserved imperfect DR4 motifs (NR1 and NR2) which appear to be essential for a full response to phenobarbital. In the human CYP2B6 gene, these elements are oriented in opposite directions with respect to those of the mouse and the rat, and are located in the region –1733/–1684 (12). They are hereafter referred to as 2B6-3’DR4 and 2B6-5’DR4, respectively. Recently, we identified a functional CAR-responsive element (CAR-RE) in the –1856/-1783 region of human CYP2C9 (28). Sequence analysis revealed the presence of an imperfect DR4 motif, hereafter referred to as 2C9-DR4. This element was shown to bind to and be transactivated by CAR as well as by PXR, albeit to a lower extent.

As shown in Figure 4, VDR-RXR heterodimer binds efficiently both the 2C9-DR4 and 2B6-3’DR4 motifs as assessed by gel mobility shift assay. This binding was only observed in the presence of the heterodimerization partner RXR and the specificity of the interaction was confirmed by competition experiments using 10- and 100-fold molar excesses.
of unlabelled consensus rANF-DR3 oligonucleotide (Figure 4 lanes 4-5 and 11-12). Note, however, that the binding of the VDR-RXR heterodimer to 2C9-DR4 seems to be of much lower affinity compared with the binding to the other CYP3A4 and 2B6 PXR/CAR elements. In sum, these observations show that the VDR-RXR heterodimer binds to the major PXR/CAR-responsive elements of CYP3A4, CYP2B6 and CYP2C9.

**VDR transactivates the PXR responsive elements of CYP3A4**

Transactivation of the PXR responsive elements of CYP3A4 (shown to bind to the VDR-RXR heterodimer) by the 1α,25(OH)2D3-activated VDR was analysed by transient transfection assays in HepG2 cells. Cells were co-transfected with the various CYP3A4-specific heterologous and homologous promoter-reporter plasmids and with the VDR expression plasmid or the empty expression plasmid as a control. Cells were then treated with increasing concentrations of 1α,25(OH)2D3 for 24 hours and reporter gene activities were measured. The results are shown in Figure 5. 1α,25(OH)2D3 strongly increased the transcriptional activity of the heterologous promoter constructs containing 3A4-5’dDR3 and 3A4-pER6 (by factors of 12 and 40, respectively) in a concentration-dependent manner (the maximum being reached at 1 nM), Figure 5A-B. This effect was observed only in cells co-transfected with the VDR expression vector. In the absence of VDR, the transcriptional activity of these elements was only modestly increased by 1α,25(OH)2D3 (by factors of 2 and 5, respectively) suggesting weak VDR expression in HepG2 cells. Note that 1α,25(OH)2D3 had no significant effect on the transcriptional activity of the mutated 3A4-5’dDR3 element (Figure 5A), of 3A4-dER6 and of pGL3 reporter (not shown). Similar experiments were then carried out with different CYP3A4 homologous promoter-reporter constructs with the aim of comparing the pattern of transcriptional activity of these constructs in response to VDR with that observed in response to PXR. For this purpose, the -7800/-7200 region (harboring
elements 5’dDR3, dER6 and 3’dDR3) was fused upstream of the –262/+11 region of CYP3A4 (harboring element pER6), in front of the LUC reporter gene (Figure 2). This construct (CYP3A4-5’dDR3/dER6/3’dDR3/pER6-LUC, construct A) has been shown to be fully responsive to PXR (9) and this was confirmed in this work (see Figure 7A). Several deletions of this construct (constructs B and C, Figure 2) were then generated and their transcriptional activity was measured in response to 1α,25(OH)₂D₃-activated VDR. The results are presented in Figure 5C. VDR strongly transactivated (by factors of 15 to 20) construct A in a 1α,25(OH)₂D₃ concentration-dependent manner. In contrast, all other constructs exhibited only a modest transcriptional activity. The absence of the 3’dDR3 resulted in a > 60% inhibition of transcriptional activity (construct C), while the proximal promoter containing the pER6 alone was only slightly affected by VDR (by factors of 2 to 3, construct B). Interestingly, when the proximal promoter of CYP3A4 (-262/+11) was replaced by a minimal thymidine kinase promoter (corresponding to the loss of the proximal ER6), the transcriptional activity of 5’dDR3/dER6/3’dDR3 (construct D) and of 5’dDR3/dER6 (construct E) was less than 50% of that measured with construct A, suggesting a cooperative interaction between the dPXRE region and the pER6 element as previously reported for PXR-mediated transactivation of these elements (9). Finally, in control experiments, neither PXR nor CAR were activated by 1α,25(OH)₂D₃ (not shown).

In sum, these results show that both the proximal region containing pER6 and the distal enhancer dPXRE containing the dDR3 motifs are necessary to confer full VDR-response and that, in the context of the CYP3A4 homologous promoter, transactivation by 1α,25(OH)₂D₃-activated VDR parallels transactivation by xenobiotic-activated PXR.

**VDR transactivates the CAR/PXR responsive elements of CYP2B6 and CYP2C9**
Similar experiments were carried out with the CAR/PXR responsive elements identified in *CYP2B6* and *CYP2C9*. The results are shown in Figure 6. A modest but significant and reproducible activation of both 2B6-3’DR4 and 2C9-DR4 constructs was observed in the presence of 1α,25(OH)2D3-activated VDR. Indeed, VDR-mediated transactivation of the major *CYP3A4* responsive elements was much greater than the activation observed here. This is consistent with the finding that in primary hepatocytes the induction ratio of CYP3A4 mRNA in response to 1α,25(OH)2D3 is much greater than that of both *CYP2B6* and *CYP2C9* mRNAs (Figure 1B).

**Competition of VDR-mediated CYP3A4 transactivation by PXR and CAR**

Since VDR binds and transactivates PXR and CAR responsive elements, the next step of our investigation was to determine whether PXR and CAR compete with VDR. For this purpose plasmid p(3A4-dPXRE/pER6)-LUC (construct A in figure 2), was transfected in HepG2 cells in the presence of a fixed amount of VDR expression vector (100 ng) and in the absence or presence of increasing amounts of PXR or CAR expression vectors (10 to 300 or 10 to 100 ng, respectively). Cells were then cultured for 24 hours in the absence or presence of : i) 1nM 1α,25(OH)2D3, 10µM rifampicin (PXR activator) or a mix of both, or ii) 1nM 1α,25(OH)2D3, 5µM androstenol (mCAR de-activator (10) or a mix of both, and reporter gene activities were measured.

Data on the PXR/VDR competition are shown in Figure 7A. The fold induction ratios are presented here because no significant change in reporter gene activity was observed in cells cultured in the absence of inducers (untreated cells). A week transactivation (< 2) was observed in the absence of receptor; this might reflect the low endogenous level of receptors in HepG2 cells. VDR was not activated by rifampicin (lane 0) and PXR was not activated by 1α,25(OH)2D3 (first lane from right). Transactivation of the dPXRE/pER6 construct by the
combinations VDR/PXR, in the presence of 1nm 1\alpha,25(OH)_{2}D_{3} alone decreased from 14-fold (no PXR) to approximately 1-fold, as observed with PXR alone, as the amount of transfected PXR increased. In contrast, in the same experiment, transactivation of the construct, in the presence of rifampicin alone, increased from 1- (no PXR) to 7-fold, as observed with PXR alone, as the dose of PXR increased. When cells were treated with both rifampicin and 1\alpha,25(OH)_{2}D_{3}, transactivation of the construct varied from a “pure” vitamin D/VDR response to a “pure” rifampicin/PXR response, that is, from 11- (maximum observed with VDR alone) to 7-fold (the maximum observed for PXR alone). These results suggest a competition between PXR and VDR for the CYP3A4 promoter elements.

Data on the CAR/VDR competition are shown in Figure 7B. The results are presented here as luciferase activity (normalized to \beta Gal activity) to emphasize the increase in basal transactivation of dPXRE/pER6 construct (approximately 4-fold) as the amount of CAR increases in the absence of any ligand (UT). This reflects the well established fact that CAR is constitutively active when transfected in cell lines such as HepG2. These results also show the androstenol-mediated inhibition of CAR (UT vs Androstenol), as previously described (10). In addition, the results show that the transcriptional activity of VDR and CAR are not affected by androstenol and by 1\alpha,25(OH)_{2}D_{3}, respectively. Transactivation of the dPXRE/pER6 construct by the combinations VDR/CAR, in the presence of 1nm 1\alpha,25(OH)_{2}D_{3} alone decreased from approximately 3.5 (no CAR) to approximately 1 luciferase activity (arbitrary unit), as observed with CAR alone, as the amount of transfected CAR increased. When cells were treated with both androstenol and 1\alpha,25(OH)_{2}D_{3}, transactivation of the construct varied from a “pure” vitamin D/VDR response to a “pure” androstenol/CAR response, that is, the maximum activity observed with VDR alone (approximately 3.5) to the minimum activity (approximately 0.25) observed with CAR alone in the presence of androstenol. The reason for this observation is that in the presence of androstane, CAR is inactivated because the co-
activator recruitment is blocked, but it is still able to bind to its responsive element. These results suggest a competition between CAR and VDR for the CYP3A4 promoter elements. Finally, these results are in agreement with the gel shift experiments showing that VDR can bind to PXR (Figure 3) and CAR (Figure 4) responsive elements and therefore confirm that, in the context of the CYP3A4 homologous promoter, the sites targeted by VDR overlap with those recognized by PXR and CAR.
DISCUSSION

In this study, we have shown that 1\(\alpha\),25(OH)\(_2\)D\(_3\) induces the expression of CYP3A4 gene in normal differentiated primary human hepatocytes, and to a lesser extent CYP2B6 and CYP2C9. Data obtained from electrophoretic mobility shift assays, co-transfection experiments with various oligonucleotides and heterologous/homologous promoter-reporter constructs, and competition experiments between nuclear receptors suggest that the 1\(\alpha\),25(OH)\(_2\)D\(_3\)-activated VDR is responsible for this induction by transactivating those responsive elements previously identified in the promoter of these genes and shown to be targeted by PXR and/or CAR in response to xenobiotics.

Vitamin D (vitamins D2 and D3) is a provitamin that requires a two step biotransformation for full activation including first hydroxylation at position 25 occurring mainly in the liver through mitochondrial CYP27A, and second hydroxylation at position 1\(\alpha\) occurring mainly in the kidney through mitochondrial CYP27B (22). This leads to the production of 1\(\alpha\),25(OH)\(_2\)D\(_3\), the most biologically active form of vitamin D. This metabolite is then catabolized mainly in the kidney through hydroxylation at position 24 by CYP24, as well as by another minor pathway involving the formation of a lactone derivative (22). Thus, although our culture medium contains significant amounts of vitamin D2 (approximately 250nM), 1\(\alpha\),25(OH)\(_2\)D\(_3\) cannot be produced nor catabolized in our cultured hepatocytes, since the kidney biotransformation pathways obviously are missing. In this work, cells were treated therefore with a range of concentrations of 1\(\alpha\),25(OH)\(_2\)D\(_3\) (0.1-100nM) that reflects the blood level in the normal adult (19-190nM). Although it was considered in the past that VDR could be absent or expressed at very low level in the liver, it was recently demonstrated that this receptor is present in fetal, neonatal and adult rat liver, by RT-PCR and immunohistochemistry (38). Control experiments, using the inducible expression of FBP,
previously shown to be controlled by VDR (31), have clearly shown that VDR is expressed and activated in our cultures after treatment with 1\(\alpha\),25(OH)\(_2\)D\(_3\).

Although each nuclear receptor binds preferentially to a specific DNA sequence (39,40, 1), there have recently been indications that a given receptor (whatever the family it belongs to) may bind to and transactivate different responsive elements. Thus for example, the steroid hormone receptors (NR3C subfamily) bind classically, and almost exclusively, as homodimers to palindromic sequences separated by 3 nucleotides. However, the glucocorticoid or the estrogen receptors have been shown to bind to DRs with different spacings between half sites (including DR2, DR5, DR6, DR9) as well as to an ER9, although binding to these motifs is weaker than to the palindrome (41). Zhou et al (42) reported that the androgen receptor may bind to a DR1 motif in addition to the classical palindrome. On the other hand, VDR, PXR and CAR belong to the NR1I subfamily which form heterodimers with RXR. Their responsive motifs consist of a hexanucleotide consensus sequence (AGGTCA), which can be configured into different motifs including direct repeats (DR), everted repeats (ER) and inverted repeats (IR). Several authors have reported that CAR and PXR can transactivate \textit{CYP2} or \textit{CYP3} genes \textit{via} the same response elements in a xenobiotic-dependent manner. Thus, for example PXR is able to transactivate \textit{CYP2B} genes \textit{via} recognition of the phenobarbital responsive DR4 element (43) and reciprocally, CAR is able to transactivate human \textit{CYP3A4} through the PXR-response elements, pER6 and dDR3 (15). The existence of a possible cross-talk between these two nuclear receptor signaling pathways has accordingly been suggested. This apparent versatility in the ability of a given nuclear receptor to target similar but distinct DNA sequences is believed to result from the flexibility of either the ligand and/or DNA binding domains, the intervening linker region, or the DNA template itself.
The results presented here suggesting that VDR binds to and transactivates DR4 and ER6 motifs, in addition to the more classical DR3 elements, are therefore not surprising and clearly offer another example of this nuclear receptor versatility. Indeed, other VDRE motifs have been previously identified including a DR4 (for which VDR exhibited an higher affinity than for DR3), a DR6 and an inverted palindrome IP9 (32,44). In addition, sequence comparison with other members of the nuclear receptor family shows that VDR and PXR isoforms share the greatest similarity (64%) in their DNA binding domain (4). The versatility of these nuclear receptors in their DNA binding capacity stands in contrast to their distinct specificity in ligand binding. Indeed, VDR was not activated by rifampicin nor by phenobarbital and neither PXR nor CAR was activated by 1α,25(OH)2D3; this is consistent with the finding that the similarity in the ligand binding domain of VDR and PXR is only 37%. On the other hand, the extent of induction of CYP3A4, CYP2B6 and CYP2C9 mRNA expression in response to 1α,25(OH)2D3 correlated with the relative binding to and transactivation of the respective PXR- or CAR-responsive elements by VDR (compare Figure 1 and Figures 5A and 6). This most likely reflects the fact that deletion or insertion of a single (or several) base pair(s) in the nuclear receptor half-site spacer region are expected to alter both the distance and the rotation angle between the half-sites, thus altering both the binding affinity of the receptor heterodimer and its ability to interact with the different transcriptional factors and/or the various coactivators or corepressors.

Recently, we have shown that the expression of PXR, RXR and CAR is under the control of the glucocorticoid receptor in primary human hepatocytes (45-47). Whether VDR expression is controlled by this receptor as well, is not known. Thus, a fully activated glucocorticoid receptor is a prerequisite for maximum CYP2/CYP3 induction by xenobiotics. We observed in the same model that interleukin-6 decreases the expression of PXR and CAR (48) thus leading to a decrease in CYP2 and CYP3 gene expression. The present and previous
results (16,17) showing that vitamin D affects CYP gene expression increase the list of those physiological compounds able to interfere with the metabolism of xenobiotics. Actually, our results suggest that, in the absence of xenobiotic, the basal expression of CYP2 and CYP3 genes may be, at least in part, controlled through VDR activation. In the presence of xenobiotics able to activate either PXR or CAR, these receptors then will compete efficiently with VDR (see Figure 7) on CYP gene promoter response elements. In this respect, it has to be noted that the extent of CYP3A4 and CYP2B6 mRNA induction in primary human hepatocytes, was much greater in response to rifampicin than in response to \(1\alpha,25(OH)_2D_3\).

Finally, although the results presented here suggest that the implication of VDR on CYP3A4 basal expression is substantial at physiological concentrations of vitamin D, its implication on CYP2C9 and CYP2B6 appears to be relatively modest, so that, the physiological significance of vitamin D effects on these genes is less clear. In this respect it is worth emphasizing that, CYP2C9 appears to be a primary glucocorticoid receptor-responsive gene (28) the expression of which, under normal physiological conditions, is maintained at a substantial level and this may account for the fact that xenobiotic- and vitamin D-mediated induction of this gene is modest.

Vitamin D can be obtained from different sources (22). A few components of the diet including fish oils, egg yolks, milk and liver contain naturally significant amounts of vitamin D3, while some plants contain vitamin D2. Many other foods are fortified with these vitamins. Another source is the skin in which ultraviolet light induces the photoconversion of 7-dehydrocholesterol to previtamin D3, followed by thermal isomerisation to vitamin D3. It is therefore possible that interindividual differences in dietary and/or light exposure habits may partly account for interindividual variations in CYP2/CYP3 basal expression and related processes such as drug and xenobiotic metabolism as well as prodrug and procarcinogen
activation. These considerations provide another reasonable basis for the occurrence of xenobiotic-dietary compound interactions.

Finally, the reason why these genes are controlled by VDR is unclear. CYP2B6, CYP2Cs and CYP3A4 have not been shown to be involved in the metabolism of vitamin D (49-51). However, it has been observed that prolonged therapy with rifampicin can cause vitamin D deficiency (52). In 8 healthy subjects, rifampicin treatment reduced circulating levels of 25 hydroxy vitamin D and 1α,25(OH)2D3 by 34 and 23% respectively. In addition, rifampicin and phenobarbital are two of the drugs most frequently associated with osteomalacia, a metabolic bone disease characterized by a defect of bone mineralization frequently due to an alteration of vitamin D metabolism (53). This suggests that CAR and/or PXR might be involved in the control of genes involved in vitamin D synthesis or catabolism.

In conclusion, this work suggests that VDR, PXR and CAR control the basal and inducible expression of several CYP genes through competitive interaction with the same battery of response elements (ER6, DR3, DR4). In consequence, we suggest that the expression of VDR-controlled genes might be affected by xenobiotics such as rifampicin through the PXR and/or CAR pathway. This possibility is under current evaluation in our laboratory.

Acknowledgements

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REFERENCES


Figure 1: Induction of CYP3A4, CYP2B6, CYP2C9 and fructose 1,6-biphosphatase mRNAs by 1α,25(OH)2D3 in human hepatocytes

Forty eight hours after plating, hepatocytes were untreated (UT) treated with increasing concentrations of 1α,25(OH)2D3 (from 1 nM to 100nM). Twenty four hours later, total RNA was extracted and analysed by RT-PCR.

A : Results obtained with culture FT187. Expression of CYP3A4, CYP2C9, CYP2B6, GAPDH and fructose 1,6-biphosphatase (FBP) mRNAs as assessed by semi-quantitative RT-PCR as indicated under Materials and Methods. PCR products exhibited the expected size and were analysed on agarose gel after exposition to 1% ethidium bromide.

B : CYP3A4, CYP2C9, CYP2B6, and GAPDH mRNAs were quantified by real time RT-PCR analysis using the Light Cycler apparatus (Roche) and the quality of the PCR-products was controled through fusion step analysis, at the end of each PCR run. Data presented are means (from three different cultures from three different liver donors, FT181, FT187 and FT189) of the ratio of mRNA levels in vitamin D-treated cells to corresponding levels in untreated cells, normalized with respect to GAPDH mRNA levels, which themselves exhibited no significant variation.

Figure 2: PXR-responsive elements present in CYP3A4 gene

Schematic representation of CYP3A4 constructs used in this work.

A, B, C : homologous constructs ; D, E : heterologous constructs with the thymidine kinase gene promoter upstream of luciferase reporter gene (LUC).

Figure 3: Analysis of CYP3A4 xenobiotic-responsive element binding to VDR by electrophoretic mobility shift assay
A- Analysis of ANF-VDRE binding to VDR. Radiolabeled ANF-VDRE oligonucleotide (50,000cpm $^{32}$P) was incubated in the absence (lane 1) or presence of RXR (lane 2), VDR (lane 3) or both proteins (lane 4) produced by an in vitro coupled transcription and translation system before loading onto the gel. In parallel experiments, incubation was performed in the presence of anti-RXR antibodies (Ab-RXR, 1µg, lane 5) or of a 5- to 50-fold molar excess of unlabelled 3A4-dER6 (lanes 6-7), 3A4-5’dDR3 (lanes 8-9) or 3A4-pER6 oligonucleotide (lanes 10-11), see Figure 2.

B- Analysis of 3A4-dDR3 binding to VDR. Radiolabeled dDR3 oligonucleotide (50,000cpm $^{32}$P) was incubated as indicated above for ANF-VDRE (lanes 1-5). In parallel experiments, incubation was performed in the presence of a 10- to 100-fold molar excess of unlabeled ANF-VDRE (lanes 6-7) or unlabeled pER6 (lanes 8-9).

C- Analysis of 3A4-pER6 binding to VDR. Radiolabeled pER6 oligonucleotide (50,000cpm $^{32}$P) was incubated as indicated above (lanes 1-3). In parallel experiments, incubation was performed in the presence of a 10- to 100-fold molar excess of unlabeled ANF-VDRE (lane 4-5). S: shift ; SS: supershift.

Figure 4: Analysis of CYP2B6 and CYP2C9 xenobiotic-responsive element binding to VDR by electrophoretic mobility shift assay

Radiolabeled 2B6-5’DR4 and 2C9-CAR-RE oligonucleotides (50,000cpm $^{32}$P) were incubated in the absence or presence of RXR (lanes 1 and 7), VDR (lanes 2 and 8) or both proteins (lanes 3 and 9) prepared by in vitro translation using a transcription-translation coupled system (Promega), before loading onto the gel. In parallel experiments, incubation was performed in the presence of anti-RXR antibodies (Ab-RXR, 1µg, lane 10) or of a 5- to 50-fold molar excess of unlabelled ANF-VDRE (lanes 4 and 5, and lanes 11 and 12). Lane 14: same assay as lane 4 of Figure 3A.
Figure 5: Transactivation of the xenobiotic-responsive elements of CYP3A4 in heterologous and homologous promoter constructs by VDR-RXR heterodimer

HepG2 cells were co-transfected with the various CYP3A4 heterologous and homologous promoter-reporter (LUC) constructs (see Figure 2) and with the VDR expression plasmid or the empty expression plasmid and the pSVβ–galactosidase expression vector, as controls. Cells were then treated with increasing concentrations of 1α,25(OH)2D3 for 24 hours and reporter gene activities were measured. The mean LUC induction (expressed as the ratio of activity in vitamin D-treated cells to activity in untreated cells, normalized to the β-galactosidase signal) determined in triplicate independent experiments is presented.

A: 3A4-5’dDR3 (wild type and mutated element, in plasmid p(3A4-(dDR3)3)-tk-LUC).
B: 3A4-pER6 in plasmid p(3A4-(pER6)3)-tk-LUC.
C: 3A4 homologous and heterologous promoter constructs. The -7800/-7200 region of CYP3A4 (harboring elements 5’dDR3, dER6 and 3’dDR3, see Figure 2) was fused upstream of the –262/+11 region (harboring element pER6), in front of the LUC reporter gene. Several deletions of this construct were then generated (Figure 2) and their transcriptional activity was measured in response to 1α,25(OH)2D3-activated VDR.

Figure 6: Transactivation the xenobiotic responsive elements of CYP2B6 and CYP2C9 by the VDR-RXR heterodimer

HepG2 cells were co-transfected with p(2B6-(NR1) 3)-tk-LUC (A) or p(2C9-(DR4) 4)-SV40-LUC (B) constructs and with the VDR expression plasmid or the empty expression plasmid and the pSVβ–galactosidase expression vector, as controls. Cells were then treated with increasing concentrations of 1α,25(OH)2D3 for 24 hours and reporter gene activities were measured. The mean LUC induction (expressed as the ratio of activity in vitamin D-treated...
cells to activity in untreated cells, normalized to the β-galactosidase signal) determined in triplicate independent experiments is presented.

**Figure 7: Competitive effect of PXR and CAR on the transactivation of CYP3A4 homologous promoter by VDR**

HepG2 cells were transfected with 500 ng of construct A (Figure 2), various concentrations of PXR or CAR, and pSVβ–galactosidase vectors as described in materials and methods. The amount of PXR or CAR varied from 0 to 300 ng depending on experiments, the total amount of expression vector being kept constant by addition of corresponding amounts of empty vectors, pSG5 (PXR) or pCR3 (CAR), while the amount of VDR expression vector was constant in all experiments. Twenty-four hours after transfection, cell were cultured without foetal calf serum for 16 hours before determination of luciferase and β–galactosidase activities. Luciferase activities were normalised to β–galactosidase. The data shown represent:

A. Effect of PXR in the absence or presence of 10 μM rifampicin, 1 nM 1α,25(OH)2D3 or 10 μM rifampicin + 1 nM 1α,25(OH)2D3. The results are represented in fold induction: ratio of luciferase activity in vitamin D or xenobiotic-treated cells to corresponding levels in untreated cells (UT), and are the mean values of triplicate transfections from two independent experiments.

B. Effect of CAR in the absence or presence of 5 μM androstenol, 1 nM 1α,25(OH)2D3 or 5 μM androstenol + 1 nM 1α,25(OH)2D3. The results are represented in absolute values of luciferase activity normalized to the β-galactosidase activity in order to evaluate CAR basal transactivation and are the mean values of triplicate transfections from two independent experiments.
A

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CYP3A4
CYP2B6
CYP2C9
GAPDH
FBPase

B

mRNA Fold induction

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10 nM 1α,25 (OH)₂ D₃

Drocourt et al. Figure 1
Drocourt et al. Figure 2
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Drocourt et al. Figure 4
A: dDR3

![Bar chart showing fold induction for wild-type and mutated dDR3](image)

B: pER6

![Bar chart showing fold induction for with and without VDR](image)

Drocourt et al. Figure 5A, 5B
Drocourt et al. Figure 5C
A 2B6-NR1

B 2C9-CAR-RE

Fold induction

UT 0.1 nM 1 nM 10 nM
1,25(OH)2 D3

UT 0.1 nM 1 nM 10 nM
1,25(OH)2 D3

Drocourt et al. Figure 6
Expression of CYP3A4, CYP2B6 and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes
Lionel Drocourt, Jean-Claude Ourlin, Jean-Marc Pascussi, Patrick Maurel and Marie-José Vilarem

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