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To cite this version:
Jean Marc Pascussi, Agnes Robert, Minh Nguyen, Odile Walrant-Debray, Michèle Garabedian, et al.. Possible involvement of pregnane X receptor-enhanced CYP24 expression in drug-induced osteomalacia.. Journal of Clinical Investigation, American Society for Clinical Investigation, 2005, 115, pp.177-86. <10.1172/JCI200521867>. <inserm-00086875>
Possible involvement of pregnane X receptor–enhanced CYP24 expression in drug-induced osteomalacia

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Vitamin D controls calcium homeostasis and the development and maintenance of bones through vitamin D receptor activation. Prolonged therapy with rifampicin or phenobarbital has been shown to cause vitamin D deficiency or osteomalacia, particularly in patients with marginal vitamin D stores. However, the molecular mechanism of this process is unknown. Here we show that these drugs lead to the upregulation of 25-hydroxyvitamin D3 (25(OH)D3)–24-hydroxylase (CYP24) gene expression through the activation of the nuclear receptor pregnane X receptor (PXR; NR1I2). CYP24 is a mitochondrial enzyme responsible for inactivating vitamin D metabolites. CYP24 mRNA is upregulated in vivo in mice by pregnenolone 16α-carbonitrile and dexamethasone, 2 murine PXR agonists, and in vitro in human hepatocytes by rifampicin and hyperforin, 2 human PXR agonists. Moreover, rifampicin increased 24-hydroxylase activity in these cells, while, in vivo in mice, pregnenolone 16α-carbonitrile increased the plasma concentration of 24,25-dihydroxyvitamin D3. Transfection of PXR in human embryonic kidney cells resulted in rifampicin-mediated induction of CYP24 mRNA. Analysis of the human CYP24 promoter showed that PXR transactivates the sequence between –326 and –142. We demonstrated that PXR binds to and transactivates the 2 proximal vitamin D–responsive elements of the human CYP24 promoter. These data suggest that xenobiotics and drugs can modulate CYP24 gene expression and alter vitamin D hormonal activity and calcium homeostasis through the activation of PXR.

Introduction

Vitamin D is essential for the maintenance of calcium homeostasis and for the development and maintenance of bones. Before acquiring its biological potential, vitamin D is transformed into 25-hydroxyvitamin D3 (25(OH)D3), the major form of vitamin D in the circulation, by two 25-hydroxylases (the mitochondrial CYP27A, and the microsomal CYP2R1) mainly expressed in the liver (1, 2). The 25(OH)D3 is further hydroxylated in the 1α position to form 1α,25-dihydroxyvitamin D3 [1α,25(OH)2D3], by the CYP27B1 mainly expressed in the kidney (3). The active form of vitamin D3, 1α,25(OH)2D3, elicits most of its biological effects by binding to a high-affinity receptor, the vitamin D receptor (VDR; NR1I1) (4). After binding 1α,25(OH)2D3, VDR forms heterodimers with the retinoid X receptor (RXR; NR2B1) and then binds to and transactivates the vitamin D–responsive elements (VDREs) present in the regulatory region of target genes (5). The classical VDREs consist of a direct repeat of nuclear receptor half-sites separated by 3 nucleotides (DR3) (6). In the classical 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 mineralization, inhibition of cell growth, and parathyroid hormone synthesis (3, 7). VDR is also expressed in many other nonclassical vitamin D–responsive organs, including the liver (8, 9).

The potent role of 1α,25(OH)2D3 in the regulation of serum calcium and phosphate levels requires a tight control of its blood concentration. This is reached through the balance between the rates of synthesis and degradation. CYP24 is a multifunctional 24-hydroxylase and the major catabolic enzyme of vitamin D (10). It directs the side-chain oxidation and cleavage of 1,25(OH)2D3 and 25(OH)D3 to catabolic carboxylic acid end products. Notably, this enzyme converts 1α,25(OH)2D3 into 1α,24,25(OH)3D3, a product with decreased hormonal activity (3), and further into calcitroic acid, known as the final metabolite of 1α,25(OH)2D3 excreted in bile. Hypocalcemia increases the serum levels of 1α,25(OH)2D3 through stimulation of 1α-hydroxylase (2) and suppresses the CYP24 activity through increased levels of parathyroid hormone (10). Inversely, hypercalcemia depresses 1α-hydroxylase and increases the 24-hydroxylase activity, and high levels of 1α,25(OH)2D3 induce the 24-hydroxylase activity through VDR (11). The important role of the CYP24 gene has been further demonstrated in CYP24 knockout mice, which exhibit high ambient 1α,25(OH)2D3 levels and abnormal bone mineralization (12).

Osteomalacia is a metabolic bone disease, characterized by a defect of bone mineralization that can be caused by vitamin D deficiency or genetic alterations of vitamin D metabolism, vitamin D action, or other factors involved in the phosphocalcic metabolism. This pathology has also been suspected to result from the adverse effects of drugs. Some of the drugs most frequently associated with osteomalacia are phenobarbital, phenytoin, rifampicin,
and carbamazepine (13–17). Indeed, long-term antiepileptic drug therapy, such as phenobarbital or carbamazepine, is a known risk factor for bone loss and fractures (13). In addition, prolonged therapy with rifampicin can cause vitamin D deficiency or osteomalacia (18), particularly in patients with marginal vitamin D stores (19). Notably, 2 weeks of rifampicin treatment reduces the levels of circulating 25-hydroxyvitamin D and 1,25(OH)₂D₃ (19). This might be involved in this phenomenon. PXR has been shown to mediate the induction of target genes by forming heterodimers with RXR (25, 26) and is activated by a wide variety of commonly used drugs, notably rifampicin (25) and phenobarbital (25) but also carbamazepine (27) and phenytoin (27).

In close similarity to VDR and the enzymes involved in vitamin D biosynthesis and metabolism, PXR is expressed in intestine, kidney, and liver (25, 28), and both receptors share around 60% similarity of amino acids in their DNA-binding domains. Moreover, even though their ligand-binding domains share only 37% identity, lithocholic acid and derivatives act as ligands for both PXR and VDR (29). PXR and VDR share around 60% similarity of their ligand-binding domains. Moreover, even though their ligand-binding domains share only 37% identity, lithocholic acid and derivatives act as ligands for both PXR and VDR (29). PXR and VDR share around 60% similarity of their ligand-binding domains.

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Figure 1

Effect of 1α,25(OH)₂D₃, rifampicin, hyperforin, carbamazepine, and phenobarbital on CYP24, CYP2R1, CYP27B, CYP27A, VDR, and CYP3A4 mRNAs in human hepatocytes. Human hepatocytes were cultured for 48 hours in the absence (UT) or presence of the indicated compounds: 50 nM 1α,25(OH)₂D₃ (VD3), 20 ìM rifampicin (RIF), 2 ìM hyperforin (HP), 20 ìM carbamazepine (CARBA), or 500 ìM phenobarbital (PB). Total RNA was isolated using TRIzol reagent. One microgram of total RNA was reverse-transcribed, and CYP3A4, CYP24, CYP2R1, CYP27B, VDR, and GAPDH mRNAs were quantified by real-time RT-PCR analysis using the LightCycler apparatus (Roche Diagnostics Corp.). Data presented are means ± SE (from 5 different cultures from 5 different liver donors) of the ratio of mRNA levels in treated cells to corresponding levels in untreated cells, normalized with respect to GAPDH mRNA levels, which themselves exhibited no significant variation. Statistically significant inductions compared with those in untreated cells are marked with asterisks: * P < 0.05 and ** P < 0.01.

Results

Induction of CYP24 gene expression and CYP24 activity by PXR activators in human hepatocytes. We first investigated the effects of PXR activators on the expression of key proteins involved in vitamin D₃ homeostasis, i.e., the mitochondrial 25-hydroxylase CYP27A, the microsomal 25-hydroxylase CYP2R1, the 1α,25-hydroxylase CYP27B, the 24-hydroxylase CYP24, and VDR. Forty-eight hours after plating, human hepatocytes were treated with 1α,25(OH)₂D₃ (50 nM) or PXR activators such as rifampicin (10 ìM), hyperforin (2 ìM), carbamazepine (20 ìM), and phenobarbital (500 ìM) for 48 hours. CYP24, CYP2R1, VDR, CYP27B, and CYP27A mRNAs were then analyzed by real-time quantitative RT-PCR using the LightCycler apparatus (Roche Diagnostics Corp.), in parallel with CYP3A4 mRNA as a control for PXR activation. We evaluated the induction ratios (mRNA levels in treated cells compared with control cells after normalization with respect to GAPDH signal) obtained from the analysis of 5 different cultures from 5 different liver donors. GAPDH and β-actin mRNA levels (data not shown) were used as quality controls of RNA preparations and RT reactions. As expected, vehicle-treated cells expressed very low levels of CYP24 (cycle threshold [Ct] = 37 ± 2 cycles), VDR (Ct = 32 ± 4), and CYP27B (Ct = 32 ± 2) mRNAs compared with CYP27A (Ct = 24 ± 1), CYP3A4 (Ct = 25 ± 5), CYP2R1 (Ct = 26 ± 2), and GAPDH (Ct = 17 ± 2).
As shown in Figure 1, expression of CYP3A4 mRNA was significantly induced by all of the compounds tested, although more modestly by 1α,25(OH)₂D₃ (5-fold induction). In addition, expression of CYP24 mRNA was strongly induced by 1α,25(OH)₂D₃ (47-fold induction). Interestingly, expression of CYP24 mRNA was significantly (P < 0.05) increased by rifampicin (20-fold induction), hyperforin (11-fold induction), and phenobarbital (12-fold induction). Carbamazepine was a weak, nonsignificant (P > 0.07) inducer of CYP24. The relative induction potency of rifampicin, hyperforin, carbamazepine, and phenobarbital was identical for CYP3A4 and CYP24 genes: i.e., rifampicin > phenobarbital > hyperforin > carbamazepine. In contrast, 1α,25(OH)₂D₃ was a weak inducer of CYP3A4 compared with rifampicin or the other PXR activators but appeared as the most potent inducer of the CYP24 gene. As previously observed in other tissues (34), 1α,25(OH)₂D₃ produced a significant decrease (50% inhibition, P < 0.01) of the CYP27B1 mRNA level (Figure 1). In contrast, rifampicin, hyperforin, or phenobarbital did not affect the expression of this mRNA. The level of VDR mRNA in human hepatocytes was not affected by the drugs used, except by 1α,25(OH)₂D₃ (2.2-fold increase). The levels of mRNA encoding 2 enzymes involved in the 25-hydroxylation of vitamin D (i.e., the mitochondrial CYP27A and the recently “de-orphanized” microsomal CYP2R1) were not affected by any of the compounds tested, even by 1α,25(OH)₂D₃.

Next, we compared the extent of mRNA induction of the main PXR target gene, CYP3A4, to the extent of mRNA induction of CYP24 at various time points (from 6 to 48 hours with 20μM rifampicin; Figure 2A), and with increasing concentrations of rifampicin (from 1 to 20 μM after 48 hours of treatment; Figure 2B). As shown, both the time-course and the dose-response profiles of induction were similar for CYP3A4 and CYP24 mRNAs. To determine whether xenobiotics induce CYP24 mRNA directly or indirectly, human hepatocytes were treated with rifampicin in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX) for 24 hours, and RNA was analyzed by semiquantitative RT-PCR using primers for CYP24 and GAPDH as control. As shown in Figure 2C, the rifampicin-mediated induction of CYP24 mRNA was not affected by CHX, although the latter compound alone increased the expression of CYP24 mRNA. It is likely that this results from an mRNA stabilization effect of this compound. In contrast, the GAPDH mRNA level was not affected by these treatments.

To investigate whether CYP24 mRNA accumulation coincided with an increase in the rate of 24-hydroxylation of vitamin D₃, the metabolism of [³H-25(OH)D₃] was evaluated. For this purpose, human hepatocytes were treated with 1α,25(OH)₂D₃ (50 nM) or 20 μM rifampicin for 72 hours, and then incubated for 4 hours with [³H-25(OH)D₃]. A significant increase of 24,25(OH)₂D₃ production was observed both after 1α,25(OH)₂D₃ treatment (3.79-fold induction, P < 0.001) and after rifampicin treatment (1.58-fold induction, P < 0.02) (Table 1). The potency of these compounds to increase the rate of 24-hydroxylation of 25(OH)D₃ parallels their ability to induce CYP24 mRNA accumulation [i.e., 1α,25(OH)₂D₃ > rifampicin].

In vivo induction of mouse CYP24 mRNA and 24,25(OH)₂D₃ plasma concentration by PXR agonists. To determine whether activators of PXR induce CYP24 in vivo, mice (n = 5) were treated for 6 days with dexamethasone or pregnenolone 16α-carbonitrile, 2 well-known murine PXR agonists (28), or corn oil plus 5% DMSO (vehicle). Total RNA was prepared from liver and kidney and analyzed for cyp3a11, cyp24, and cyclophilin (internal control) gene expression by real-time PCR. As expected, the cyp3a11 mRNA level was significantly (P < 0.01) and strongly increased by dexamethasone (16.3-fold) and pregnenolone 16α-carbonitrile (9.7-fold) in the liver of treated mice in comparison to the level in vehicle-treated mice (Figure 3A). More interestingly, dexamethasone and pregnenolone 16α-carbonitrile provoked a significant increase of cyp24 mRNA expression in kidney, 4.6-fold (P = 0.0005) and 2.2-fold (P = 0.0019) induction, respectively (Figure 3B). In addition, we measured changes in the plasma concentration of vitamin D metabolites [24,25(OH)₂D₃ and 25(OH)D₃] after 1 week of pregnenolone 16α-carbonitrile treatment. As shown in Figure 3C, pregnenolone 16α-carbonitrile provoked a significant increase of 24,25(OH)₂D₃ plasma level (2.01-fold induction, P < 0.03), while no significant effect was observed on 25(OH)D₃ circulating level (Figure 3D).

PXR transactivates human CYP24 gene promoter in Hek293 cells. The results presented above suggest that PXR is involved in the regula-
tion of the CYP24 gene. To further document this point, we first established a stable human embryonic kidney cell line (Hek293) expressing the human PXR protein. For this purpose, Hek293 cells were transfected with pIRES-hPXR/neo expression plasmid and cultured 3 weeks in a selection medium containing 600 μg/ml G418. Several colonies were amplified and tested for their ability to express PXR protein, and 1 of them was used for subsequent studies. As shown in Figure 4A, this cell line (Hek293-hPXR) expresses human PXR protein, as assessed by Western blotting performed with an anti–human PXR serum, while the parental Hek293 cells do not. Moreover, in contrast to the parental cells, these cells respond to rifampicin, as observed in transient transfection assays using a CYP2B6 promoter that confers response of the promoter to both human PXR and RXR ligands (37).

**Table 1**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>UT (n = 11)</th>
<th>RIF (n = 12)</th>
<th>VD3 (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24,25(OH)2D3</td>
<td>34.4 ± 4.3</td>
<td>54.0 ± 3.0</td>
<td>130.5 ± 11.9</td>
</tr>
<tr>
<td>(fmol/well/240 min)</td>
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Human hepatocytes (from 4 different cultures from 4 different liver donors) were cultured in the absence (UT) or presence of 50 nM 1α,25(OH)2D3 (VD3) or 1 μM rifampicin (RIF) for 72 hours, and then incubated for 4 hours with 1.6 nM 24,25(OH)2D3. The 24-hydroxylase enzyme activity was calculated as described in Methods. Values represent the mean ± SE. For each experiment, 2–3 determinations were performed. ""P < 0.02, ""P < 0.001 versus untreated cultures incubated in the same conditions.

The cloning of the human CYP24 promoter has been reported (35). To determine the nature of the cis-acting elements involved in PXR-mediated CYP24 gene expression, analysis of promoter transcriptional activity was undertaken. Various deletions of the human CYP24 promoter were constructed into a promoterless luciferase reporter vector (pGL3basic; Figure 5A). These constructs were tested for their ability to respond to PXR by transient cotransfection assays in Hek293 cells. In response to rifampicin, the luciferase activity of the 1.2-kb promoter fragment (~1,200 to ~22) exhibited a 4-fold increase under cotransfection with human PXR cDNA expression vector (Figure 5B). The magnitude of the response was identical to that observed with the ~326 to ~22 construct. However, the ~144 to ~22 construct did not respond to rifampicin. Similar results were obtained with pregnenolone 16α-carbonitrile and a mouse PXR expression vector. Therefore, the ~326 to ~144 region of the CYP24 promoter confers response to both human and murine PXR. Interestingly, this region is responsive to 1α,25(OH)2D3 even in the absence of cotransfection of the receptor expression vector (Figure 5B), because Hek293 cells express endogenous VDR (36). Indeed, 2 VDREs have been defined within the ~300 to ~150 region of the human and mouse CYP24 promoter that confer response of the promoter to both 1α,25(OH)2D3 (35) and RXR ligands (37).

**Figure 3**

In vivo modulation of CYP24 by PXR agonists. (A and B) Effect of pregnenolone 16α-carbonitrile and dexamethasone on cyp24 mRNA abundance. Mice (n = 5) were injected i.p. for 6 consecutive days with dexamethasone (DEX; 10 mg/kg/d), pregnenolone 16α-carbonitrile (PCN; 100 mg/kg), or corn oil (UT). Total RNA from liver or kidney was prepared, and 1 μg was reverse-transcribed. The relative levels of cyp3a11 and cyp24 mRNAs were determined in duplicate for each mouse by real-time PCR using cyp3a11-specific (A) and cyp24-specific (B) primers. Cyclophilin mRNA levels were used as a reference standard. Data are means ± SE of the ratio of mRNA levels in treated mice to corresponding levels in untreated mice, normalized with respect to cyclophilin mRNA levels. (C and D) Effect of pregnenolone 16α-carbonitrile on vitamin D3 metabolites in mouse plasma. Mice (n = 5) were injected i.p. for 6 consecutive days with pregnenolone 16α-carbonitrile (100 mg/kg) or corn oil and plasma samples. Pooled mouse plasma (1–2 mice, 300 μl, n = 3) or 50-μl plasma samples (n = 5) were analyzed for 24,25(OH)2D3 (C) or 25(OH)D3 metabolites (D), respectively, as described in Methods. Statistically significant expressions compared with untreated mice are marked with asterisks: *P < 0.05, **P < 0.01, and ***P < 0.005. Fold change relative to control mice is indicated.
Figure 5
PXR transactivates the proximal human CYP24 promoter. (A) VDREs present in the human CYP24 (hCYP24) gene. Schematic representation of the human CYP24 constructs used in this work. (B) Ligand-activated PXR transactivates the −326 to −143 promoter region of human CYP24. Hek293 cells were transiently transfected with human PXR (pSG5-ΔATG-hPXR), mouse PXR (pSG5-mPXR), or control expression vector (pSG5) in the presence of reporter vectors driven by the indicated human CYP24 promoter sequences, together with pRSV-β-gal transfection control plasmid. Cells were treated with vehicle (0.1% DMSO; UT), 50 nM 1α,25(OH)2D3 (VD3), 10 μM rifampicin (RIF), or 10 μM pregnenolone 16α-carbonitrile (PCN) for 24 hours. Cells were then harvested and analyzed for both luciferase and β-gal activities. Values represent β-gal–corrected luciferase activities normalized to the corresponding level in untreated Hek293 cells and are the average of duplicates ± SE. These were replicated in independent experiments.

Figure 6
Rifampicin induces the CYP24 gene in a Hek293 cell line stably transfected with PXR. (A) Expression of human PXR in parental and pIRES-hPXR/neo–transfected Hek293 cells. Whole-cell extracts were prepared from parental Hek293 cells or pIRES-hPXR/neo–transfected cells (Hek293-hPXR). Proteins were separated on 10% SDS-PAGE, and PXR and β-actin expression was monitored by Western blotting (Santa Cruz Biotechnology Inc.). (B) Rifampicin activates an NR1-LUC reporter gene in Hek293-hPXR cells. Parental Hek293 or Hek293-hPXR cell lines were transiently transfected with CYP2B6 NR1-LUC reporter gene together with pRSV-β-gal transfection control plasmid. After transfection, cells were treated with 0.1% DMSO (UT; white bars) or 10 μM rifampicin (RIF; black bars). Values represent β-gal–corrected luciferase activities normalized to the corresponding level in untreated Hek293 cells and are the average of duplicates ± SE. These were replicated in independent experiments. (C) Rifampicin and hyperforin induce the CYP24 gene in Hek293-hPXR cells. Parental Hek293 or Hek293-hPXR cell lines were untreated (UT) or treated with 50 nM 1α,25(OH)2D3 (VD3), 10 μM rifampicin (RIF), or 2 μM hyperforin (HP) in triplicate. Forty-eight hours later, total RNA was extracted and analyzed by quantitative RT-PCR for CYP24 and GAPDH mRNA content. Values represent the average ± SE.
constructs exhibited increased luciferase activity in response to 1α,25(OH)2D3, even in the absence of the cotransfected receptor (7- and 4-fold induction, respectively). This induction was increased in the presence of VDR. Interestingly, in both VDRE-I and VDRE-II reporter constructs, rifampicin produced an increase in reporter activity (approximately 4- to 6-fold induction) only in the presence of PXR cotransfection. In contrast, the luciferase activity of the site 3 reporter construct was affected neither by 1α,25(OH)2D3 treatment nor by rifampicin-activated PXR. We then introduced specific mutations into VDRE-I and VDRE-II motifs in the native CYP24 promoter (–326 to –22) by means of PCR-based site-directed mutagenesis. The effect of PXR on these mutated CYP24 gene promoters was investigated by transient transfection experiments. As shown in Figure 8B, the mutation in VDRE-I caused a strong decrease in both the VDR-dependent and the PXR-dependent luciferase induction. On the other hand, introduction of the mutation into VDRE-II retained half of the VDR response, while it completely abolished PXR-dependent luciferase induction. Double mutations in both VDRE-I and VDRE-II completely abolished both VDR and PXR response. These results demonstrate that PXR binds to and transactivates the 2 VDRE motifs present in the human CYP24 promoter.

**Discussion**

Recent studies have shown that the orphan nuclear receptor PXR plays a central role in the transcriptional regulation of enzymes and transporters involved in detoxification, such as CYP3A4 and MDR1 (38). PXR is activated by a number of pharmaceutical agents, including rifampicin, SR12813, taxol, clotrimazole, phenobarbital, hyperforin, the herbal antidepressant St. John’s wort (39), and HIV protease inhibitors such as ritonavir and saquinavir (40).

Figure 6

CYP2A4 VDREs compete with the CYP3A4 ER6 probe for binding to the PXR:RXRα heterodimer in electrophoretic mobility shift assay. (A) PXR-responsive region of CYP24 promoter sequence. VDRE sequences (VDRE-II, –294 to –274, and VDRE-I, –174 to –151) are overlined, and half-sites are indicated in boldface letters. The region between –316 and –291 (site 3), which contains sequences that are homologous to various direct repeats, is underlined. Circles under nucleotides denote those nucleotides that were changed in mutation constructs. The TATA box is indicated. (B) CYP24 VDRE-I and VDRE-II motifs compete with the CYP3A4 ER6 probe for binding to the PXR:RXRα heterodimer. Radiolabeled CYP3A4 ER6 oligonucleotide (50,000 cpm) was incubated in the presence of PXR and RXRα proteins prepared by in vitro translation using a transcription-translation coupled system (lane 1). In parallel experiments, incubation was performed in the presence of a 50-fold molar excess of the CYP24 site 3 region (lane 2), or a 10- to 50-fold molar excess of unlabelled CYP3A4 ER6 (lanes 3 and 4), CYP24 VDRE-I (lanes 5 and 6), and VDRE-II (lanes 7 and 8).

Figure 7

CYP2A4 VDREs compete with the CYP3A4 ER6 probe for binding to the PXR:RXRα heterodimer in electrophoretic mobility shift assay. Radiolabeled CYP2A4 VDRE-I (lanes 1–6) and VDRE-II (lanes 7–12) oligonucleotides (50,000 cpm) were incubated in the absence or presence of PXR, RXRα, or VDR protein, alone or in association as indicated, before being loaded onto the gel. Mock: control TNT lysate. NS, nonspecific band.
a multifunctional enzyme that directs the side-chain oxidation and cleavage of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ to catabolic carboxylic acid end products (43). It is well established that induction of the rat CYP24 gene by 1,25(OH)$_2$D$_3$ is mediated by 2 VDREs located about 100 bp apart within the 300-bp proximal promoter (35). In human hepatocytes, PXR-mediated CYP24 mRNA accumulation was not inhibited by CHX pretreatment, which suggests a direct effect of PXR on CYP24 gene expression. In addition, transfection studies in human embryonic kidney cells demonstrated that both human PXR and mouse PXR directly transactivate the CYP24 promoter. Moreover, we found that PXR binds to and transactivates the 2 proximal VDREs present in the CYP24 promoter. Point mutations within the S’ half-site of either the upstream VDRE (AVDRE-II) or the downstream VDRE (AVDRE-I) led to a total inhibition of PXR-mediated induction of luciferase activity, which suggests that both sites are required for an efficient PXR-mediated CYP24 gene expression. Such cooperation between several PXREs is frequently observed within PXR target genes. In summary, our results demonstrate that CYP24 is a VDR target gene as well as a PXR target gene. Moreover, we observed that, in vivo in mice, activation of PXR by pregnenolone 16a-carbonitrile significantly increased the circulating level of 24,25(OH)$_2$D$_3$ (2-fold induction). These results fully prove our hypothesis and are in agreement with those previously obtained by others. Indeed, pharmacological doses of dexamethasone, another mouse PXR agonist and cyp3a inducer (28), increase intestinal and other drugs now identified as PXR activators produce vitamin D deficiency and increase the risk of bone fracture. It is well-known that long-term treatment with phenytoin, phenobarbital, rifampicin, or carbamazepine impairs bioavailability of vitamin D and bone mineralization, leading to osteomalacia and osteoporosis (13, 15–18, 20, 45–47). Consistent with this, it has been observed that the circulating level of 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$ is decreased by these drugs (18, 20, 48, 49). In addition, osteopenia, osteoporosis, and osteonecrosis are the most significant bone disorders affecting patients suffering from HIV infection (50), and HIV-infected patients receiving antiretroviral therapy have a high prevalence of reduced bone mineral density (51). The mechanisms of these phenomena are complex and unclear. However, a part of these effects might be the consequence of CYP24 gene upregulation induced by the activation of PXR. Indeed, protease inhibitors have been suspected to play some role in this process (52, 53), and 2 of them, ritonavir and saquinavir (27, 40), are PXR agonists.

In the most classical view of molecular toxicology, undesirable effects of xenobiotics are primarily ascribed to the chemical reactivity of the parent compounds and/or of some of their metabolites that are produced during oxidative metabolism mediated notably by CYPs (e.g., covalent binding to proteins and DNA, effects on membrane lipids, etc.). A second aspect of the undesirable effects of xenobiotics results from the fact that these compounds may affect the level and activity of xenobiotic detoxification systems (interactions, induction, and inhibition). In this respect, although PXR evolved to protect the body from xenobiotic-mediated toxic effects, its activation by some drugs represents the basis for a common class of potentially life-threatening drug interactions in which one drug accelerates the metabolism of another (54). The cross-talk described here between PXR and VDR represents another aspect of the undesirable effects of xenobiotics, in which a xenobiotic-mediated increase in endobiotic-compound metabolism/catabolism can lead to endocrine system perturbation.

**Methods**

**Chemicals.** DMEM, DMSO, dexamethasone, rifampicin, 1α,25(OH)$_2$D$_3$, carbamazepine, hyuperfin, pregnenolone 16α-carbonitrile, and culture medium additives were from Sigma-Aldrich. The radioisotope γ-$^{[32P]}$d-ATP was from Amersham International. Gelatin was purchased from Touzart-Matignon, neutral charcoal from NORIT, and dextran 20 from OSI.
vitamin D activity, and 26,27-OH-3(Vector Biolabs) were purchased from Merck SA.

**Figure 9**

Proposed model for PXR-mediated drug-induced osteopenia or osteomalacia. While 1α,25(OH)₂D₃ maintains vitamin D homeostasis via downregulation of vitamin D₃ biosynthesis enzymes (CYP27B) and upregulation of the vitamin D-inactivating enzyme (CYP24), drugs that activate PXR may be responsible for the acceleration of vitamin D catabolism through the upregulation of CYP24, leading to vitamin D deficiency and eventually to osteopenia or osteomalacia.

**Pharmaceuticals.** Unlabeled 25(OH)D₃ was a gift from Laboratoires Rousset-Uclaf, 24,25(OH)₂D₃ was a gift from M.L. Uskokovic (F. Hoffmann-La Roche Ltd.), and 26,27-ΔH-dihydroxyvitamin D₃ (specific activity, 23 Ci/mmol) was purchased from Amersham Biosciences Europe GmbH. Chromatography solvents (n-hexane and isopropanol), methanol, and chloroform were purchased from Merck SA.

**Plasmids.** The following plasmids have been described previously: pSG5-Δ¹⁵⁻¹⁷-lux (PXR), pSG5-mPXRxR (8), pSG5-mRXXt (8), and pSG5-hVDVR (8). pRJES-hPXR/neo plasmid was generated by PCR amplification (High-Fidelity PCR system; F. Hoffmann-La Roche Ltd.) from cDNA encoding amino acids 1–434 of human PXR using oligonucleotides 5'-GGGTGTGGGGGATCCTCAGCTACCTGTGATGCCG and insertion into pIREsneo (BD Biosciences — Clontech) digested with EcoRI/BamHI.

Human CYP24 promoter deletions were obtained by PCR (Pfu polymerase; Promega Corp.) using CYP24 forward 18-mer oligonucleotide primers (–1,200 to –1,182, –326 to –308, or –144 to –126) with an overhang Sall site immediately upstream of the S′-most base of the deletion construct; a common 3′ primer, TGCAATTTATGGAGACAGACTGC (CYP24 –39 to –22); and the human CYP24 (–1,250 to –300 bp) BLCAT3 plasmid as a template (a gift from H.F. DeLuca, University of Wisconsin — Madison, Madison, Wisconsin, USA). PCR products were then digested by Sall and subcloned into Sall/Smal–linearized pG1 basic vector (Promega Corp.) to produce –1,200–LUC, –326–LUC, and –144–LUC constructs.

pG1LUC plasmid was obtained by ligation of the HindIII/BglII–polyA vector fragment (BD Biosciences — Clontech), corresponding to the minimal 147nt long tk promoter, into the HindIII/BglII–linearized pG1 basic vector. Oligonucleotides corresponding to the individual CYP2B6 NR1 (55), CYP2C site 3 (–316 to –291), and VDREs (VDRE-II, –294 to –274, and VDRE-I, –174 to –151) (37) were synthesized with BamHI and BglII ends, annealed, and ligated, and 3 copies were subcloned into the BamHI/BglII–digested pG1LUC. The resulting (NR1)₃-tkLUC, (site 3)₃-tkLUC, (VDRE-II)₃-tkLUC, and (VDRE-I)₃-tkLUC were verified by sequencing.

The specific mutations of the VDREs in the native CYP24 promoter (–326 to –22) were introduced by PCR-based site-directed mutagenesis (QuikChange PCR-mediated site-directed mutagenesis kit; Stratagene) using –326–LUC vector as a template to produce AVDRE-I (186ΔCCC mutated to GTT) and AVDRE-II (289ΔCACCC mutated to AAAA). The AVDRE-II vector was then used as a template to produce the AVDRE-I + AVDRE-II (AVDREs) construct.

**Primary culture of human hepatocytes.** Hepatocytes were prepared from 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 (Paris, France). Hepatocytes were prepared and cultured according to a previously published procedure (56). The cells were plated into collagen-coated dishes at 0.17 × 10⁶ cells per square centimeter in a hormonally and chemically defined medium consisting of a mixture of Williams’s E medium and Ham’s F12 medium (1:1 in volume). Forty-eight hours after plating, cells were cultured in the presence of the specific concentrations of compounds.

**Quantitative PCR.** Total RNA was extracted from cells or frozen mouse tissues using TRIZOL reagent (Invitrogen Corp.), according to the manufacturer’s instructions. Purified was confirmed by spectrophotometry. cDNAs were synthesized from 1–2 μg of total RNA using the SuperScript II first-strand synthesis system for PCR (Invitrogen Corp.) at 42°C for 60 minutes, in the presence of random hexamers (Amersham Pharmacia Biotech). Quantification of mRNAs was performed using the LightCycler apparatus (Roche Diagnostics Corp.). Two microliters of diluted RT reaction (1:10) was used for real-time PCR amplification. The following program was used: a denaturation step at 95°C for 8 minutes, and 50 cycles of PCR (denaturation, 95°C, 15 seconds; annealing, 67°C, 10 seconds; extension, 72°C, 20 seconds). In all cases, the quality of the PCR product was assessed by monitoring of a fusion step at the end of the run. Mouse sense and reverse primers, respectively, were as follows: cyp24a1, 5′-CTCCCTATGTTGAGATGTATGTAAGT and 5′-TTTAAAAACGTG-ATCAGTTGCTCATAACT; cyp3a11, 5′-GAAGGAAAGCCGGCTCGTATT and 5′-GGGTAGTGGCAGAAAAGTAA; cyclophilin, 5′-TGCAAGGAC-CAAGACAGACA and 5′-TGCCGGATGTCGAAATGAT. Human sense and reverse primers, respectively, were as follows: CYP3A4, 5′-CACAACCGCA-GAGCCTTTTTG and 5′-ATCCATTGCTTGAGCCCCCATC; GAPDH, 5′-GGTCCAGACCGAAGCTTGTCG and 5′-CAATGATTGCTGATGAC; CYP2C3 (NM_000782), 5′-GTCGCAAGCAGCGACCCCT and 5′-GGCCGAGGTGTTACAGGTG; CYP27B (NM_000785), 5′-CGCAAGGACCTGAGGATCTC and 5′-TCTGGCTCAGCCAGACCTTTCC; CYP27A2 (NM_000784), 5′-GCTTCCTCTCTCCACAGAAC and 5′-ACTGGCAGGCCCACTCTTCTA; VDR (NM_000376), 5′-AGACAGCGCATCTA-TGGC and 5′-GAGATGAGGACGCTGGAAGA.

Mouse and human CYP24, CYP2R1, CYP27A, and VDR mRNA amplified fragments were purified using the NucleoSpin extract kit (Macherey-Nagel Co.) and verified by sequencing.

25(OH)D₃ 24-hydroxylase assay. Human hepatocytes plated in collagen-coated dishes were treated for 72 hours with 50 nM 1,25(OH)₂D₃, 20 μM rifampicin, or 500 μM phenobarbital. After rinsing with medium, 1.6 nM 1,25(OH)₂D₃ (0.05 μCi) were added, and the cells were incubated for an additional 4 hours in 1 ml medium. The medium was removed and the 1,25(OH)₂D₃ derivatives present in the medium were extracted by addition of 2 ml methanol plus 2 ml chloroform. The chloroform extract was cochromatographed with 100 ng unlabeled 24,25(OH)₂D₃ using a straight-phase HPLC system (Beckman Coulter Inc.) equipped with a 4.6 × 250 mm Ultrasphere Si column (Altex; Beckman Coulter Inc.), and equilibrated with n-hexane and isopropanol (95:5) (flow rate 1.5 ml/min). Absorbance at 254 nm was continually monitored, and effluent fractions were collected every minute. After evaporation to dryness, fractions were dissolved in Ultima Gold scintillation fluid (Packard Instrument Co.), and the radioactivity was measured. The 24-hydroxylase enzyme activity (femtomoles per culture well per 240 minutes) was calculated based on the percentage of the
injected radioactivity recovered in the 24,25(OH)2D3 regions after HPLC. The data were subjected to an overall statistical evaluation using ANOVA. Test groups were compared with respective untreated control groups using the Mann-Whitney nonparametric U test.

Animals and treatments. Male mice (C57BL/6; Charles River Laboratories France) were housed in a pathogen-free animal facility under a standard cycle of 12 hours light, 12 hours dark. After 1 week of acclimatization, pregnenolone 16α-carbonitrile (100 mg/kg/d) or dexamethasone (10 mg/kg/d) was injected in corn oil plus 5% DMSO through 6 successive daily administrations (n = 5 for each group). The mice were killed 4 hours after the last pregnenolone 16α-carbonitrile administration, and plasma and tissues (liver and kidney) were snap-frozen in liquid nitrogen.

Measurements of vitamin D metabolites in mouse plasma. The 25(OH)D3 metabolite was extracted from 50-μl plasma samples (n = 5) with methanol-chloroform, and chromatographed on Amprep C18 Mini-Columns (RP1900; Amersham Biosciences Europe GmbH) to separate 25(OH)D3 from vitamin D (57). The 24,25(OH)2D3 metabolite was extracted from 300-μl plasma samples (pool of 1-2 mice, n = 3) with methanol-chloroform, and chromatographed on a straight-phase HPLC system (Beckman Coulter Inc.) using 92% n-hexane and 8% isopropanol as solvents. The 25(OH)D3 region from Amprep C18 chromatography and the 24,25(OH)2D3 region from HPLC were dried down and dissolved in ethanol. They were then assayed by the competitive protein-binding assay of Preece et al. (58), with 2 modifications: serum vitamin D-binding protein was obtained from 127, Δ12–25-hydroxyvitamin D, and vitamin D-deficient rats, and gelatin (3 g/l) was used instead of albumin. The quality of the results was controlled by simultaneous assessment of external standards (Vitamin D External Quality Assessment Scheme [DEQAS]).

Cell culture and transfections. Hek293 cells (human embryonic kidney cells) were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Corp.). Transfection of plasmid DNA was performed in single batches with FuGENE 6 (Roche Diagnostics Corp.) according to the manufacturer’s instructions. For reporter analysis, transfections were performed using 80,000 cells, 150 ng of luciferase-based reporter plasmids, and expression plasmids for mouse PXR (50 ng of pSG5-ΔATG-hPXR), or human VDR (25 ng of pRSV-β-gal; Promega Corp.) as internal control, 50 ng of pRSV-β-gal (Promega Corp.) was added. After 12–16 hours, the medium was changed, and fresh medium containing 5% delipidated charcoal-stripped serum (Sigma-Aldrich) and 0.1% DMSO (vehicle) or inducers was added. Twenty-four hours after the medium was changed, the cells were harvested in reporter lysis buffer (Promega Corp.), and cell extracts were analyzed for luciferase and β-gal activities as described previously (8). Luciferase values were normalized with β-gal values calculated by division of the maximal response by the β-gal values elicited with VDR.

Stable Hek293 clones expressing human PXR (Hek293-hPXR) were obtained by transfection of Hek293 cells with pIREs-hPXRneo and, after 3 weeks of selection, with 600 μg/ml G418. Individual clones were then amplified and analyzed for PXR mRNA and protein expression levels.

Western blot analysis. Whole-cell lysates (106 cells) were prepared from Hek293 or stable transfectant Hek293-hPXR cell lines, with 300 μl of 100 mM Tris (pH 8.5), 250 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM DTI, and anti-protease tablets from Roche Diagnostics Corp. Following centrifugation (12,000 g for 10 minutes at 4°C), the supernatant was collected and stored at −80°C. Protein content was determined by bichinchoninic acid (BCA) protein assay (Interchim). Fifty-microgram extracts were subjected to 10% SDS-PAGE and transferred to PROTRAN nitrocellulose membrane (Schleicher & Schuell BioScience Inc.). Immunoblotting was carried out with actin antibody (I-19) and PXR antibody (N-16), both purchased from Santa Cruz Biotechnology Inc. Chemiluminescence detection was performed using HRP-conjugated secondary antibodies and the Amersham Biosciences Europe GmbH ECL kit.

Electrophoretic mobility shift assays. Double-stranded oligonucleotides corresponding to the 2 VDREs and site 3 of human CYP24 were synthesized (Invitrogen Corp.): VDRE-I (−174 to −151), CCGAGGCGCTCGCTCACCTCGCTGA; VDRE-II (−294 to −274), CGAACACACCCCCGT-GAACTCCGG; site 3 (−316 to −291), GAGGGGCCGAGGGCGCTTGC-GAAG. The CYP3A4 ER6 probe and binding procedure have previously been described (8). Probes were end-labeled with [32P]ATP using T4 polynucleotide kinase (Promega Corp.) and then incubated for 20 minutes at room temperature with 1 μl of in vitro–synthesized PXR, VDR, or RXRα (transcription and translation–coupled [TNT-coupled] transcriptional translation; Promega Corp.). DNA-protein complexes were subjected to electrophoresis on a 4% polyacrylamide gel (30:1 acrylamide/bis-acrylamide) in 0.5x TBE (1x TBE = 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography with XAR film (Eastman Kodak Co. Scientific Imaging Systems).

Acknowledgments

We are grateful to Sanofi-Synthélabo France SA for financial support. We thank L. Pichard-Garcia for the preparation of human hepatocytes, and F. Lasserre and C. Béthoulières (Institut National de la Recherche Agronomique) for assistance in execution of animal studies.

Received for publication April 13, 2004, and accepted in revised form October 26, 2004.

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