Regulation of human vitamin D₃ 25-hydroxylases in dermal fibroblasts and prostate cancer LNCaP cells*

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Regulation of human vitamin D 25-hydroxylases

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Abbreviations:

CYP, cytochrome P450; CAR, constitutive androstane receptor; PXR, pregnane X receptor; VDR, vitamin D receptor; DMSO, dimethyl sulfoxide; HLPC, high performance liquid chromatography; calcitriol, 1α,25-dihydroxyvitamin D₃
ABSTRACT

In this study, we examined whether 1α,25-dihydroxyvitamin D₃ (calcitriol), phenobarbital and the antiretroviral drug efavirenz, drugs used by patient groups with high incidence of low bone mineral density, could affect the 25-hydroxylase activity or expression of human 25-hydroxylases in dermal fibroblasts and prostate cancer LNCaP cells. Fibroblasts express the 25-hydroxylating enzymes CYP2R1 and CYP27A1. LNCaP cells were found to express two potential vitamin D 25-hydroxylases - CYP2R1 and CYP2J2. The presence in different cells of nuclear receptors, VDR, PXR and CAR, was also determined. Phenobarbital suppressed the expression of CYP2R1 in fibroblasts and CYP2J2 in LNCaP cells. Efavirenz suppressed the expression of CYP2R1 in fibroblasts but not in LNCaP cells. CYP2J2 was slightly suppressed by efavirenz whereas CYP27A1 was not affected by any of the two drugs. Calcitriol suppressed the expression of CYP2R1 in both fibroblasts and LNCaP cells, but had no clear effect on the expression of either CYP2J2 or CYP27A1. The vitamin D₃ 25-hydroxylase activity in fibroblasts was suppressed by both calcitriol and efavirenz. In LNCaP cells, consumption of substrate (1α-hydroxyvitamin D₃) was used as indicator of metabolism since no 1α,25-dihydroxyvitamin D₃ product could be determined. The amount of 1α-hydroxyvitamin D₃ remaining in cells treated with calcitriol was significantly increased.

Taken together, 25-hydroxylation of vitamin D₃ was suppressed by calcitriol and drugs. The present study provides new information indicating that 25-hydroxylation of vitamin D₃ may be regulated. Also, the current results may offer a possible explanation for the impaired bone health after treatment with certain drugs.
The metabolic activation of vitamin D is initiated by 25-hydroxylation of the side chain followed by a 1α-hydroxylation. 1α,25-Dihydroxyvitamin D₃ (calcitriol), the biologically most active form of vitamin D₃, is known as a calcium-regulating hormone but is involved also in other processes such as modulation of the immune system and cell proliferation and differentiation. Currently, at least four enzymes capable of 25-hydroxylation of vitamin D₃ and/or vitamin D₂ have been described in humans, including the mitochondrial CYP27A1 and the microsomal CYP2R1, CYP2J2 and CYP3A4 (Prosser and Jones, 2004; Ohyama and Yamasali, 2004). CYP3A4 is reported to prefer the non-physiological form vitamin D₂ over vitamin D₃ (Gupta et al., 2004). Thus, several possible candidates as vitamin D₃ 25-hydroxylase have been suggested but from a regulatory perspective, the physiological roles of these proposed 25-hydroxylases remain poorly defined. In this context, it is interesting that vitamin D₃ 25-hydroxylation occurs also in certain extrahepatic tissues, e.g. the prostate. Regulation of human vitamin D₃ 25-hydroxylation may be particularly important in extrahepatic tissues and might be a means of controlling cellular levels of 25-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃. This might influence cell function because these vitamin D₃ metabolites are reported to be active hormones in human prostate cells and to have antiproliferative properties (Tokar and Webber, 2005; Lou et al., 2004).

In previous studies, both vitamin D metabolites and drugs affecting bone health have been shown to affect the promoter of the porcine vitamin D 25-hydroxylase, CYP2D25, in liver-derived HepG2 cells (Ellfolk et al., 2006; Hosseinpour et al., 2007). In the present study, the expression and regulation of human 25-hydroxylating enzymes are examined in dermal fibroblasts and prostate cancer LNCaP cells. The effects on gene expression and endogenous 25-hydroxylase enzyme activity by 1α,25-dihydroxyvitamin D₃ and the drugs phenobarbital and efavirenz are examined. Phenobarbital is an antiepileptic drug, often used in combination with other drugs. Efavirenz is an antiretroviral drug, always used in
combination therapy. High incidence of low bone mineral density is a concern for patients using antiepileptic drugs and for HIV-infected patients.
Materials and Methods

Materials. The dermal fibroblast cell line BJ and the prostate cancer cell line LNCaP were purchased from American Type Culture Collection (Rockwell, MD). 1α-Hydroxyvitamin D₃ was a gift from Leo Pharma, Denmark. Cell cultivation media, fetal bovine serum, non essential amino acids, antibiotics and sodium pyruvate were purchased from Gibco. Efavirenz was a gift from Dr. Filip Josephson, Karolinska institutet. 1α,25-Dihydroxyvitamin D₃ was obtained from Solvay (Duphar, The Netherlands). 24R,25-Dihydroxy [23,24(n)-³H]cholecalciferol was purchased from Amersham Life science (Little Chalfont, England). RNeasy Mini Kit and Rnase-free Dnase set were purchased from Qiagen and the Reverse Transcription Kit from Promega. The SYBR Green Master Mix, TaqMan Master mix and TaqMan probes were purchased from Applied Biosystems. The PCR primers for semi-quantitative PCR were obtained from Thermo Fischer Scientific GmbH (Ulm, Germany).

Cell culture. Dermal fibroblast cells (BJ) were cultured in MEM supplemented with 10 % fetal bovine serum, 1 % non essential amino acids, 1 % antibiotic-antimycotic and 1% sodium pyruvate. LNCaP cells were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. Both types of cells were grown in 5% CO₂ at 37°C. The cells were seeded the day before treatment and were kept in their growth medium overnight.

Treatment of cells. Cells were treated with either calcitriol (0.1 nM), phenobarbital (1.5 mM) or efavirenz (2.5 μg/ml) for 24 h prior to extraction of total RNA or addition of 1α-hydroxyvitamin D₃. The treatments were carried out in serum free media. 1α,25-Dihydroxyvitamin D₃ and efavirenz were dissolved in 99% ethanol. The vehicle, ethanol (99%) was used as control treatment.
Assay of vitamin D3 25-hydroxylase activity. Endogenous vitamin D3 25-hydroxylase activity in fibroblasts and LNCaP cells in the presence of either 1,25-dihydroxyvitamin D3 or efavirenz was measured by incubation with 1α-hydroxyvitamin D3 (0.85 μM) dissolved in DMSO. After incubation for 24 or 48 h (LNCaP and fibroblasts, respectively), the cells were harvested and medium collected for analysis for 1,25-dihydroxyvitamin D3 and 1α-hydroxyvitamin D3. Medium collected from the plates was extracted with ethyl acetate (5 ml ethyl acetate/ 1.5 ml medium). To control extraction efficiency (recovery) 24R,25-dihydroxy [23,24(n)-3H]cholecalciferol was added to the plates before the medium was collected.

HPLC. Vitamin D metabolites were analysed on straight phase HPLC-UV at the wavelength 265 nm. The mobile phase used was hexane:isopropanol, 90:10, the flow rate was 0.5 ml/min and the column used was LiChrosphere Si 60, 5 µm (Merck, Rahway, NJ). The detector was a 2151 Variable Wavelength Monitor (from LKB). To control for extraction efficiency 24R,25-dihydroxy[23,24(n)-3H]cholecalciferol was added to the plates immediately before the medium was collected. The retention time of the vitamin D metabolites were determined using authentic reference compounds. In this system 24R,25-dihydroxycholecalciferol had a retention time of 4 min and 1,25-dihydroxyvitamin D3 a retention time of 9 min.

Extraction of RNA. After 24 h of treatment, total RNA from LNCaP cells and fibroblasts was extracted using RNeasy Mini Kit (Qiagen) together with the RNase free DNase set (Qiagen) according to the manufacturer’s protocol. Reverse transcription of the obtained RNA was performed using Reverse transcription Kit (Promega) according to the manufacturer’s protocol. The produced cDNA was then used for studying expression levels of the human vitamin D 25-hydroxylases (CYP2J2, CYP2R1, CYP3A4 and CYP27A1) and nuclear
receptors of interest in gene regulation by 1α,25-dihydroxyvitamin D₃, phenobarbital and the antiretroviral drug efavirenz.

**Analysis of CYP2J2, CYP2R1, CYP27A1 and CYP3A4 mRNA expression.** Analysis of endogenous CYP2J2, CYP2R1, CYP27A1 and CYP3A4 expression in cultured fibroblasts (BJ), LNCaP, HepG2 and RWPE cells was carried out by semi-quantitative RT-PCR. The primers used were described for the CYP2J2 forward primer (Nishimura et al., 2002), and for the CYP2J2 reverse primer (Marden et al., 2003). The CYP2R1 primers were those found in PrimerBank (Wang and Seed, 2003). The forward primer is the forward primer of Primer Pair 1, with the Primer BankID 33591222a1 and the reverse primer is the reverse primer from PrimerPair 3 with the Primer BankID 33591222a3. The primers for CYP3A4 were those described previously (Hakkola et al., 1996). The primers for CYP27A1 were as follows: forward, 5´-AGT ACG GAA CGA CAT GGA GC-3´, and reverse, 5´-GCA GAG TCT CCT TAA GCA CAG C-3´ (Garuti et al., 1996). GAPDH was used as internal control. The PCR cycles were as follows: 95°C for 10 min, and then 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 60°C) and extension (1 min at 72°C) followed by a final extension for 10 min at 72°C.

**Quantitation of CYP2J2, CYP2R1 and CYP27A1 mRNA by real-time RT-PCR.** Quantitation of CYP2J2, CYP2R1 and CYP27A1 mRNA in fibroblasts and LNCaP cells was performed by realtime RT-PCR using a Bio-Rad iCycler, according to the manufacturer’s recommendations.

CYP2J2 and CYP27A1 were analysed by TaqMan assay (probes used:Applied biosystems Hs00356035_m1 and Hs00168003_m1, respectively) and Eukaryotic 18S rRNA (Applied biosystems Hs99999901_s1) was used as endogenous control. Human liver mRNA
was used as positive control. In cells treated with vehicle only, the Ct values for CYP27A1 were 30.2±0.6 (fibroblasts) and for CYP2J2 30.8±0.8 (LNCaP cells).

CYP2R1 was analyzed by the SYBR Green assay. The primers and PCR cycles for CYP2R1 were those used for semi-quantitative RT-PCR. The endogenous control was GAPDH. LNCaP RNA was used as positive control. In cells treated with vehicle only, the Ct values for CYP2R1 mRNA were 27.4±0.4 (fibroblasts) and 25.4±0.6 (LNCaP cells).

Targets and controls were amplified in triplicates as singleplex assays. All expression data were normalized to the endogenous control. The relative mRNA expression levels were calculated according to the standard curve method as described by Applied Biosystems. Standard curves were generated by serial five-fold dilutions of the cDNA of the positive control.

**Analysis of vitamin D receptor, pregnane X receptor and constitutive androstane receptor expression.** Analysis of endogenous VDR, PXR and CAR expression in fibroblasts (BJ) and LNCaP cells was carried out by semi-quantitative RT-PCR. The primers used were those described for VDR (Pascussi et al., 2005), for PXR (Noreault et al., 2005) and for CAR (Chang et al., 2003). Human liver mRNA was used as positive control.

**Statistical analysis.** Statistical analysis was performed using one-way ANOVA. P values <0.05 were considered statistically significant.
Results

Expression of human 25-hydroxylases in different cultured human cell lines. Different cell lines were tested for the expression of the human vitamin D 25-hydroxylases CYP2J2, CYP2R1, CYP3A4 and CYP27A1. The cell lines examined were the human dermal fibroblast cell line BJ, human hepatoma cell line HepG2, the prostate cancer cell line LNCaP and the prostate cell line RWPE-1. As positive control human liver mRNA was used, showing expression of all four 25-hydroxylating enzymes. CYP2J2 was expressed in HepG2 and LNCaP cells, CYP2R1 was expressed in fibroblasts and LNCaP cells, CYP3A4 was expressed in HepG2 cells and CYP27A1 was found in fibroblasts and HepG2 cells. Prostate RWPE cells showed no or very low expression of the 25-hydroxylases (Table 1). The human fibroblast cell line (BJ) and prostate cancer LNCaP cells were considered particularly useful for studies on regulation of human vitamin D₃ 25-hydroxylases.

Expression of vitamin D receptor, pregnane X receptor and constitutive androstane receptor in human dermal fibroblasts and prostate cancer LNCaP cells. Experiments were also performed in order to examine the expression in fibroblasts and LNCaP cells of VDR, PXR and CAR, receptors of relevance for regulation of the vitamin D 25-hydroxylating CYP enzymes. VDR is involved in regulation of gene expression by 1α,25-dihydroxyvitamin D₃ and PXR and CAR are involved in gene regulation by xenobiotics.

Both cell lines expressed VDR, indicating that vitamin D metabolites may affect the regulation of vitamin D sensitive genes in these cells. CAR was not expressed in any of the two cell lines. PXR was expressed only in the fibroblasts, indicating that genes expressed in fibroblasts might be more sensitive to regulation by xenobiotics than genes in LNCaP cells (results not shown).
Effects of phenobarbital on the mRNA levels of human vitamin D 25-hydroxylases. In a previous study, using the pig as model, we reported direct effects by phenobarbital on the expression of CYP27A1 and CYP2D25, two important 25-hydroxylases. In the current study, the effects of phenobarbital treatment on human vitamin D₃ 25-hydroxylases were studied in prostate cancer LNCaP cells and dermal fibroblasts.

LNCaP cells were treated with phenobarbital (1.5 mM) in order to study its effects on the mRNA expression of the two vitamin D 25-hydroxylases CYP2J2 and CYP2R1. Phenobarbital significantly suppressed the expression of CYP2J2 by about 50 %, whereas CYP2R1 expression was unaffected in this cell line (Fig. 1A). Fibroblasts were treated with phenobarbital in the same way to study its effects on the mRNA expression of the two vitamin D 25-hydroxylases CYP2R1 and CYP27A1. Phenobarbital significantly suppressed the expression of CYP2R1 by about 35 %, whereas CYP27A1 expression was unaffected (Fig. 1B).

Effects of efavirenz on the mRNA levels of human vitamin D 25-hydroxylases. LNCaP cells were treated with the antiretroviral drug efavirenz to study its effects on the mRNA expression of CYP2J2 and CYP2R1. Efavirenz significantly suppressed the levels of CYP2J2 by 25% (Fig. 2A). Fibroblasts were treated with efavirenz in the same way to study its effect on the expression of CYP2R1 and CYP27A1. Efavirenz significantly suppressed CYP2R1 mRNA expression in fibroblasts by about 60%, whereas CYP27A1 did not respond to this treatment (Fig. 2B). It may be concluded that efavirenz suppresses CYP2R1 mRNA expression in fibroblasts but not in LNCaP cells. This difference in regulation by efavirenz on CYP2R1 is correlated with expression of PXR in fibroblasts but not in LNCaP cells.
Effects of 1α,25-dihydroxyvitamin D₃ on the mRNA levels of human vitamin D 25-hydroxylases. LNCaP cells were treated with the active vitamin D metabolite 1α,25-dihydroxyvitamin D₃ to study its effects on the mRNA expression of CYP2J2 and CYP2R1. 1α,25-Dihydroxyvitamin D₃ significantly suppressed CYP2R1 by about 50%, whereas it had no clear effect on the levels of CYP2J2 (Fig. 3A). Fibroblasts were treated with 1α,25-dihydroxyvitamin D₃ in the same way to study its effect on the expression of CYP2R1 and CYP27A1. 1α,25-Dihydroxyvitamin D₃ significantly suppressed the expression of CYP2R1 by about 50%, whereas CYP27A1 expression was only slightly suppressed by the vitamin D metabolite (Fig. 3B). The results indicate that 1α,25-dihydroxyvitamin D₃ downregulates the expression of CYP2R1 in fibroblasts and LNCaP cells, both cell types expressing VDR.

Effects of efavirenz on the vitamin D 25-hydroxylase activity in fibroblasts. On incubating cultured fibroblasts with 1α-hydroxyvitamin D₃ as substrate, 25-hydroxylation could be determined by the appearance of the product 1α,25-dihydroxyvitamin D₃. The treatment concentration of efavirenz (2.5 µg/ml) is within the therapeutic window (1000-4000 µg/L) (Marzolini et al., 2001). The production of 1α,25-dihydroxyvitamin D₃ was suppressed about 30% by efavirenz (Fig. 4A). The suppression by efavirenz was statistically significant.

Efavirenz was not used as treatment for experiments on enzyme activity in prostate cancer LNCaP cells because its effects on the mRNA levels of the two 25-hydroxylases present in LNCaP cells as described above were found to be marginal.

Effects of 1α,25-dihydroxyvitamin D₃ on the vitamin D 25-hydroxylase activity in fibroblasts. The 25-hydroxylase activity in fibroblasts was determined by assay of the
product $1\alpha,25$-dihydroxyvitamin D$_3$. The treatment concentration of $1\alpha,25$-dihydroxyvitamin D$_3$ is within the physiological range (0.1 nM). This concentration of $1\alpha,25$-dihydroxyvitamin D$_3$ corresponds to 0.12 ng in the culture plates used. This amount is not detectable in HPLC analysis and thus does not interfere with assay of 25-hydroxylase activity. The 25-hydroxylase activity was slightly suppressed in fibroblasts by $1\alpha,25$-dihydroxyvitamin D$_3$ (Fig. 4B).

**Metabolism of $1\alpha$-hydroxyvitamin D$_3$ in LNCaP cells**

In incubations of LNCaP cells with $1\alpha$-hydroxyvitamin D$_3$ no $1\alpha,25$-dihydroxyvitamin D$_3$ could be detected. Instead consumption of substrate was used as a way to assay metabolism of $1\alpha$-hydroxyvitamin D$_3$, which is known to undergo 25-hydroxylation as the first step. Treatment with $1\alpha,25$-dihydroxyvitamin D$_3$ decreased the consumption of the substrate compared to the control (treatment with vehicle) (Fig. 5). In the presence of ethanol (vehicle) only 6% of the substrate could be detected after 24 h. However, in the presence of $1\alpha,25$-dihydroxyvitamin D$_3$ about 17% of the substrate remained after 24 h, indicating decreased metabolism. The results are statistically significant.

The LNCaP cells express two vitamin D 25-hydroxylases (CYP2R1 and CYP2J2) as well as the catabolism enzyme, CYP24A1. This enzyme metabolizes the 25-hydroxylated product, $1\alpha,25$-dihydroxyvitamin D$_3$, to more polar compounds. The results of these experiments may be influenced by further metabolism by CYP24A1. However, we believe that the major part of the substrate consumption observed in our experiments should reflect the initial 25-hydroxylation. These results indicate that the metabolism of $1\alpha$-hydroxyvitamin D$_3$ in LNCaP cells can be suppressed in the presence of physiological concentrations of $1\alpha,25$-dihydroxyvitamin D$_3$. 
Discussion

In previous studies, vitamin D metabolites and phenobarbital, a drug affecting bone health, have been found to suppress the promoter activity of the porcine vitamin D 25-hydroxylase, CYP2D25 (Ellfolk et al., 2006; Hosseinpour et al., 2007). In this study, we report that phenobarbital and the antiretroviral drug efavirenz, as well as the active hormone 1α,25-dihydroxyvitamin D3 (calcitriol) suppress the expression levels also of human 25-hydroxylating enzymes. In humans, several possible candidates for vitamin D₃ or vitamin D₂ 25-hydroxylase have been suggested, including CYP27A1 (Theodoropoulos et al., 2002), CYP2R1 (Cheng et al., 2003), CYP2J2 (Aiba et al., 2006), and CYP3A4 (Gupta et al., 2003). In recent years, the bioactivation of vitamin D₃ in extrahepatic cells has been subject to increased interest due to the regulating functions ascribed to 1α,25-dihydroxyvitamin D₃ (calcitriol) in cell proliferation and differentiation. In particular, the formation of the active vitamin D hormone has been studied in prostate cells and other cells in relation to cancer (Lou et al., 2004). Whereas regulation of the 1α-hydroxylation step, catalyzed by CYP27B1, has been studied extensively, the regulation of the different 25-hydroxylating enzymes in the bioactivation of vitamin D₃ in various tissues is still unclear (Prosser and Jones, 2004). The current results show that the 25-hydroxylating enzymes are expressed differently in various human cell types. Also the nuclear receptors VDR and PXR, needed for regulation of the gene expression by 1α,25-dihydroxyvitamin D₃ and drugs, were found to be expressed differently in various cells.

Prolonged therapy with phenobarbital may cause vitamin D deficiency. Several explanations for this have been suggested. Recently, PXR-mediated induction of CYP24A1 or CYP3A4 has been suggested to increase the metabolism of 1α,25-dihydroxyvitamin D₃ and explain the drug-induced osteomalacia by phenobarbital (Pascussi et al., 2005; Xu et al., 2006; Zhou et al., 2006). In a previous report using the pig as model, we presented results
indicating a possible novel mechanism for drug-induced osteomalacia by phenobarbital, involving phenobarbital-mediated suppression of hepatic 25-hydroxylation of vitamin D₃ (Hosseinpour et al., 2007). The current results showing a phenobarbital-mediated suppression also of human CYP2R1, an important human 25-hydroxylase, support the hypothesis that phenobarbital suppresses the bioactivation of vitamin D₃. From the results it may be concluded that phenobarbital regulates the expression of CYP2R1 differently in fibroblasts and LNCaP cells. Our finding that PXR, a nuclear receptor involved in gene regulation by several xenobiotics, is expressed in fibroblasts but not in LNCaP cells may offer an explanation for this cell-specific difference in effect of phenobarbital on CYP2R1 expression. The data in the present study support our previous hypothesis that vitamin D₃ 25-hydroxylation may be suppressed by phenobarbital (Hosseinpour et al., 2007). A down-regulation of 25-hydroxylation by phenobarbital may explain, at least in part, the increased risk of osteomalacia, bone loss and fractures in long-term phenobarbital therapy.

Osteomalacia and severe vitamin D deficiency diagnosed after introduction of antiretroviral therapy including efavirenz have been described in a case report (Gyllensten et al., 2006). It was suggested that CYP450 enzyme induction by efavirenz might have affected vitamin D metabolism in this patient, resulting in an aggravation of a pre-existing vitamin D insufficiency caused by nutritional habits and less vitamin D synthesis in the skin. The results of the current study suggest an alternative explanation, implying that efavirenz is able to suppress the first step in the bioactivation of vitamin D₃ mediated by the human vitamin D₃ 25-hydroxylase CYP2R1. It may also be concluded that efavirenz suppresses CYP2R1 mRNA expression in fibroblasts but not in LNCaP cells. This difference in regulation by efavirenz on CYP2R1 is correlated with expression of PXR in fibroblasts but not in LNCaP cells.

The relative roles of various human 25-hydroxylating enzymes in the
bioactivation of vitamin D₃ under different conditions are not completely clear. CYP2J2 and CYP3A4 are reported to prefer vitamin D₂ over the physiological form vitamin D₃ and appear less important in the bioactivation of vitamin D₃ (Aiba et al., 2006, Gupta et al., 2004). Both CYP27A1 and CYP2R1 preferentially hydroxylate vitamin D₃ and therefore might be more interesting for human vitamin D₃ bioactivation. CYP27A1 has a high Kₘ for vitamin D₃ and has been suggested to be an important enzyme when vitamin D₃ concentrations are high. Patients with mutations in the CYP27A1 gene do not always have decreased 25-hydroxyvitamin D₃ levels whereas cholesterol metabolism, where CYP27A1 is involved, is seriously affected (Moghadasian et al., 2002). CYP2R1 has a Kₘ within the physiological range of vitamin D₃ concentration and mutations in the CYP2R1 gene have been reported to be associated with disturbances of vitamin D status. From a regulatory point of view, however, this enzyme has been poorly defined. The present study showing that CYP2R1, in contrast to the other 25-hydroxylating enzymes, appears to be regulated further supports an important physiological role for this enzyme as a 25-hydroxylase in the bioactivation of vitamin D₃.

The perhaps most important finding in the current study is that human CYP2R1 in extrahepatic cells can be regulated by vitamin D metabolite(s) and some drugs. The results indicate that both the expression of CYP2R1 mRNA and the metabolism of 1α-hydroxyvitamin D₃ in dermal fibroblasts and prostate cancer LNCaP cells may be subject to VDR-mediated suppression in the presence of physiological concentrations of 1α,25-dihydroxyvitamin D₃. Such an autocrine regulation of 25-hydroxylase by vitamin D metabolites or exogenous compounds in prostate as well as other cells might be a previously unknown means of controlling cellular levels of 25-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃. Since these vitamin D₃ metabolites are reported to be active hormones
affecting both normal and cancerous cells in several ways, this regulatory mechanism might have a fundamental impact on cell function.
References


Footnotes

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Figure legends

**Fig. 1.** Levels of CYP2R1 and CYP2J2 mRNA expression in LNCaP cells (A) and levels of CYP2R1 and CYP27A1 mRNA expression in fibroblast BJ cells (B) after 24 h of phenobarbital (PB) treatment. The cells were treated with 1.5 mM PB which was dissolved directly into the growth medium. Untreated cells received growth medium. After 24 h of treatment, RNA was extracted and the mRNA levels were determined using real-time RT-PCR. The mRNA levels are shown as fold change compared to untreated cells. In cells treated with vehicle only, the Ct values for CYP2R1 mRNA were 27.4±0.4 (fibroblasts) and 25.4±0.6 (LNCaP cells), for CYP27A1 30.2±0.6 (fibroblasts) and for CYP2J2 30.8±0.8 (LNCaP cells). Data are given as mean ±S.D. (n=3-6). Statistically significant difference (**P<0.001).

**Fig. 2.** Levels of CYP2R1 and CYP2J2 mRNA in LNCaP cells (A) and levels of CYP2R1 and CYP27A1 mRNA in fibroblast BJ cells (B) after 24 h of treatment with efavirenz (efz). The cells were treated with 2.5 µg/ml efz which was dissolved in 99% ethanol. Untreated cells received 99% ethanol. After 24 h of treatment, RNA was extracted and the mRNA levels were determined using real-time RT-PCR. The mRNA levels are shown as fold change compared to untreated cells. In cells treated with vehicle only, the Ct values for CYP2R1 mRNA were 27.4±0.4 (fibroblasts) and 25.4±0.6 (LNCaP cells), for CYP27A1 30.2±0.6 (fibroblasts) and for CYP2J2 30.8±0.8 (LNCaP cells). Data are given as mean ±S.D. (n=3-6). Statistically significant difference (*P<0.05 and **P<0.001).

**Fig. 3.** Levels of CYP2R1 and CYP2J2 mRNA in LNCaP cells (A) and levels of CYP2R1 and CYP27A1 mRNA in fibroblast BJ cells (B) after 24 h of treatment with 1,25D3.
(calcitriol). The cells were treated with 0.1 nM 1,25D3 which was dissolved in 99% ethanol. Vehicle-treated cells received 99% ethanol. After 24 h of treatment, RNA was extracted and the mRNA levels were determined using real-time RT-PCR. The mRNA levels are shown as fold change compared to vehicle-treated cells. In cells treated with vehicle only, the Ct values for CYP2R1 mRNA were 27.4±0.4 (fibroblasts) and 25.4±0.6 (LNCaP cells), for CYP27A1 30.2±0.6 (fibroblasts) and for CYP2J2 30.8±0.8 (LNCaP cells). Data are given as mean ± S.D. (n=3-6). Statistically significant difference (*P<0.05 and **P<0.01).

Fig. 4. Effect of efavirenz treatment (A) and of 1,25D3-treatment (B) on the production of 1,25D3 (calcitriol) in fibroblast BJ cells. The cells were treated with ethanol (EtOH), efavirenz (efz) or 1,25D3 (calcitriol) for 24 h prior to the addition of substrate (1α-hydroxyvitamin D3). The cells were incubated with the substrate for 48 h. Data are given as mean ± S.D. (n=3-6). The amount of product formed in the experiments shown in Fig. 4A was 61±10 ng and 36±6 ng, respectively. The difference in production rate between ethanol-treated (EtOH) and efavirenz-treated (efz) cells is statistically significant (*P<0.02).

Fig. 5. Amount of substrate (1α-hydroxyvitamin D3) remaining after 6, 12 and 24 h of incubation in LNCaP cells treated with 1α,25-dihydroxyvitamin D3 (1,25D3) or ethanol for 24 h prior to the addition of substrate. Data are given as mean ± S.D. (n=3-6). Statistically significant difference (**P<0.01).
Table 1.

Expression of human vitamin D 25-hydroxylases in different cell lines determined by semi-quantitative PCR. + indicates expression, - is used when no expression could be seen.

<table>
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<th>CYP2J2</th>
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Fig. 5

amount of substrate (1α-D₃) remaining after 6, 12 or 24 h incubation