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# Regulation of Vitamin D 25-hydroxylases

*Effects of Vitamin D Metabolites and  
Pharmaceutical Compounds on the Bioactivation of  
Vitamin D*

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#### **Abstract**

Ellfolk, M. 2008. Regulation of Vitamin D 25-hydroxylases. Effects of Vitamin D Metabolites and Pharmaceutical Compounds on the Bioactivation of Vitamin D. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 87. 47 pp. Uppsala. ISBN 978-91-554-7362-4.

A 700bp portion of the promoter of CYP2D25, the porcine microsomal vitamin D 25-hydroxylase was isolated and sequenced. The computer analysis of the sequence revealed the existence of a putative VDRE at 220 bp upstream of the transcription start site. A CYP2D25 promoter-luciferase reporter plasmid was constructed in order to study the transcriptional regulation of the gene. Treatment with the vitamin D metabolites calcidiol and calcitriol suppressed the promoter, provided that the nuclear receptors VDR and RXR were overexpressed. Phenobarbital was also capable of suppressing the promoter if the nuclear receptors PXR or CAR were overexpressed.

The 25-hydroxylases are not expressed solely in liver but in a wide array of other organs as well. It is therefore possible at least in theory to study the vitamin D 25-hydroxylation in human subjects using cells from extrahepatic organs, from which biopsy retrieval is easier than from the liver. Dermal fibroblasts are frequently used to study different pathological conditions in human subjects and they are easy to come by. Dermal fibroblasts were shown to express two vitamin D 25-hydroxylases: CYP27A1 and CYP2R1. The expression pattern of CYP2R1 displayed considerable interindividual variation. The fibroblasts were also capable of measurable vitamin D 25-hydroxylation, which makes dermal fibroblasts a possible tool in studying vitamin D 25-hydroxylation in human subjects.

Little is known about the regulation of expression and activity of the human vitamin D 25-hydroxylases. Therefore dermal fibroblasts – expressing CYP2R1 and CYP27A1 – and human prostate cancer LNCaP cells, that express CYP2R1 and CYP2J2, were treated with calcitriol and phenobarbital and efavirenz, two drugs that give rise to vitamin D deficiency. Treatment decreased the mRNA levels of CYP2R1 and CYP2J2 provided that the treated cells also expressed the necessary nuclear receptors. CYP27A1 did not respond to any of the treatments. The treatments also managed to decrease the 25-hydroxylating activity of the cells.

The results show that vitamin D 25-hydroxylases can be regulated by both endogenous and xenobiotic compounds.

*Keywords:* CYP2D25, vitamin D 25-hydroxylase, transcriptional regulation, vitamin D, CYP2R1, CYP2J2, CYP27A1, phenobarbital, efavirenz, fibroblast, LNCaP

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To my family



# List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I Ellfolk, M., Norlin, M. and Wikvall, K.  
Isolation and properties of the CYP2D25 promoter:  
Transcriptional regulation by vitamin D<sub>3</sub> metabolites.  
Biochem. Biophys. Res. Commun. 345, 568-572 (2006)
- II Hosseinpour, F., Ellfolk, M., Norlin, M. and Wikvall, K.  
Phenobarbital suppresses vitamin D<sub>3</sub> 25-hydroxylase expression:  
A potential new mechanism for drug-induced osteomalacia.  
Biochem. Biophys. Res. Commun. 357, 603-607 (2007)
- III Ellfolk, M., Norlin, M., Gustafsson, J. and Wikvall, K.  
Dermal fibroblasts: a possible tool for studies of vitamin D<sub>3</sub>  
25-hydroxylation in humans.  
Manuscript
- IV Ellfolk, M., Norlin, M. and Wikvall, K.  
Regulation of vitamin D<sub>3</sub> 25-hydroxylases in dermal fibroblasts  
and prostate cancer LNCaP cells.  
Submitted

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# Abbreviations

APC	Antigen presenting cell
bp	Base pair
CAR	Constitutive androstane receptor
CYP	Cytochrome P450
DBP	Vitamin D binding protein
DC	Dendritic cell
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPLC	High-pressure liquid chromatography
IL	Interleukin
MARRS	Membrane associated rapid response steroid
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NADH	Nicotinamide adenine dinucleotide, reduced form
ONPG	2-Nitrophenyl- $\beta$ -D-galactopyranoside
PXR	Pregnane X receptor
RANK	Receptor activator of nuclear factor- $\kappa$ B
RANKL	RANK ligand
RLU	Relative light units
RT-PCR	Reverse transcriptase-polymerase chain reaction
RXR	Retinoid X receptor
Th	T-helper cell
UV	Ultraviolet
VDR	Vitamin D receptor
VDRE	Vitamin D response element

# Introduction

## Vitamin D

### Vitamin D - its production, effects and mechanisms of action

#### Vitamin D production

Vitamin D exists in two natural forms: vitamin D3 or cholecalciferol and vitamin D2 or ergocalciferol. Vitamin D2 is found in plants and yeast whereas vitamin D3 is of animal origin (Figure 1).

Vitamin D3 is synthesized in the skin (the epidermis) from 7-dehydrocholesterol on exposure to UVB-light. The photons cleave the B-ring on the steroid skeleton thus producing pre-vitamin D which undergoes thermal isomerization to vitamin D3. Vitamin D3 is removed from the epidermis into circulation by binding to the vitamin D binding protein (DBP) (Holick, 1987). Ergocalciferol is formed when the plant sterol ergosterol is exposed to UVB-light.

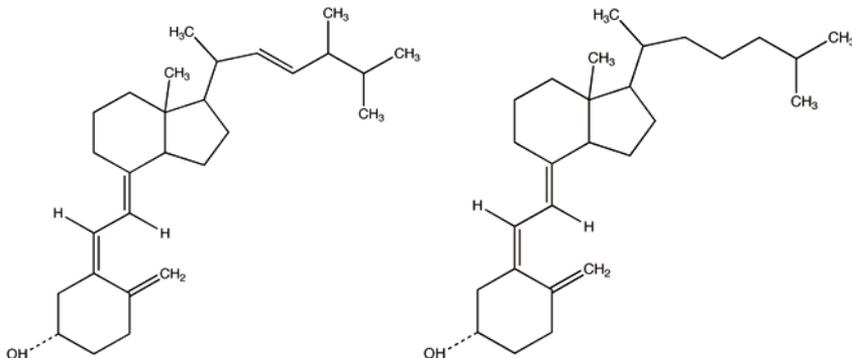


Figure 1. Structures of ergocalciferol (left) and cholecalciferol (right)

Vitamin D is also acquired from the diet. Good sources are fish, eggs and different oils. Some food stuffs are also fortified with vitamin D, like milk and margarine.

#### Vitamin D metabolic activation and inactivation

In order to gain biological activity vitamin D needs to be activated. The activation is carried out in two steps and by different enzymes (Figure 2).

The first activation step takes place in the liver, where a hydroxyl group is attached to carbon nr 25, rendering 25-hydroxyvitamin D or calcidiol. It has some biological activity and its normal serum concentration in humans is 20-250 nmol/L. A number of cytochrome P450-enzymes are capable of performing the 25-hydroxylation and in most organisms studied at least two 25-hydroxylases have been found – a mitochondrial and a microsomal one.

The second activation step takes place in the kidney, where CYP27B1 hydroxylates calcidiol into 1 $\alpha$ ,25-dihydroxyvitamin D or calcitriol. It is the most potent of the vitamin D metabolites and at its normal concentration of 20-250 pmol/L is considered to be a hormone (Brown et al., 1999; Dusso et al., 2005; Jones et al., 1998).

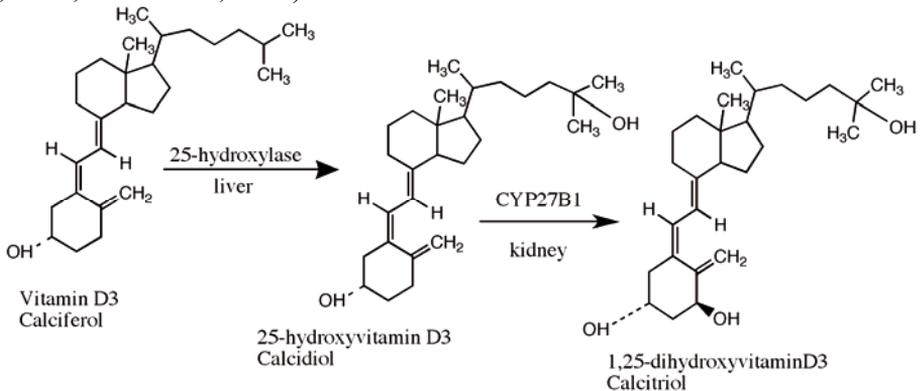


Figure 2. The bioactivation of vitamin D3. Vitamin D2 is activated in the same way.

Both calcidiol and calcitriol undergo further metabolism by CYP24A1. This enzyme 24-hydroxylates vitamin D metabolites and the 24-hydroxylation is a key point at which degradation of vitamin D begins. 24-hydroxylated vitamin D metabolites are less biologically active and are then further metabolized into less active substances. Finally, after several hydroxylations and ultimately side chain cleavage, calcitroic acid is formed. Calcitroic acid is then excreted into the bile (Makin et al., 1989; Reddy and Tserng, 1989; Zimmerman et al., 2001).

### Vitamin D effects

Rickets is a disease which was rampant in the industrialized cities of Europe. About a century ago sunlight and cod liver oil were found to cure rickets, and soon after the discovery vitamin D was found to be the preventing agent and of importance for skeletal health and over all calcium metabolism in the body.

About two decades ago vitamin D was found to harbour effects beyond calcium regulation – from stimulation of cell differentiation and inhibition of cell proliferation to modulation of the immune system.

### *Vitamin D, bone and calcium metabolism*

Vitamin D deficiency leads to skeletal disorders in humans: rickets in children and osteomalacia in adults. Both diseases are caused by poor skeletal mineralization resulting from poor calcium uptake due to vitamin D deficiency. Although vitamin D plays an important role in maintenance of healthy bone, its main function is to maintain calcium levels in serum within tight limits.

Vitamin D does not seem to have any direct effect on bone mineralization. Studies on mice suffering from rickets have shown that given very large amounts of calcium their bones start to develop normally and their rickets is cured, even if no vitamin D is present (Panda et al., 2004). These findings are supported by observations in humans as well: children with mutations in the VDR gene, which renders the receptors unfunctional, suffer from hereditary vitamin D resistant rickets. Their rickets cannot be cured with vitamin D as the receptor required for vitamin D function does not work. However, their rickets can be cured with either large oral amounts of calcium or intravenous administration of calcium (Malloy et al., 1999). On the other hand there are reports emerging, which indicate that vitamin D does have direct effects on bone formation. The vitamin D deficient mice whose rickets has been cured with large amounts of calcium still display morphological abnormalities in the growth plates of the bones (Panda et al., 2004).

It seems as though the beneficial effects of vitamin D on bone mineralization are transmitted indirectly through its effects on calcium and phosphate absorption. Vitamin D upregulates the expression of proteins (epithelial calcium channel TRPV6) involved in the active uptake of calcium from the intestinal lumen into the epithelial cells. Vitamin D also stimulates the expression of the protein responsible for pumping the calcium ions out of the epithelial cell into circulation (baso-lateral membrane pump PMCA1b). Vitamin D seemingly also upregulates the expression of calbindin, a protein which binds intracellular calcium and is responsible for its transport through the cell (St-Arnaud, 2008). Vitamin D also stimulates the renal reabsorption of calcium thus diminishing losses into the urine. Vitamin D induces the expression of calcium channels in the distal convoluted tubule and the distal connecting tubules. The entry of calcium into the cells, its way through the cell and finally its extrusion from the cell into circulation is very similar to the process in enterocytes (Christakos et al., 2003).

To further aid bone mineralization, vitamin D also affects the absorption of phosphate from the intestine. It stimulates the expression of the Na-P<sub>i</sub> co-transporter on the baso-lateral membrane of the intestinal epithelial cell, thereby increasing phosphate uptake (Dusso et al., 2005; Kochupillai, 2008).

Vitamin D plays a role in the formation and maturation of osteoclasts, the cells that resorb bone. Vitamin D stimulates the expression of a membrane

bound protein, RANKL (RANK ligand) in osteoblasts. It is, as indicated by its name, a ligand for the RANK, a receptor which is situated on the cell surface of osteoclast precursors. When the RANKL binds to RANK, it is stimulated and the osteoclast precursors can start to differentiate into osteoclasts (Caetano-Lopes et al., 2007; Gurlek et al., 2002; Suda et al., 2003).

### *Vitamin D and cancer*

Epidemiological studies have for nearly three decades made a connection between UVB exposure and cancer incidence and mortality. In 1980 Garland and Garland reported an inverse correlation between latitude and incidence of colorectal cancer: they found that the mortality rates for colorectal cancer was highest in the northeast of the United States and lower in the south, southwest and west. They hypothesized that vitamin D would be involved, as intensity of UVB radiation is dependent on latitude and as vitamin D synthesis is dependent on intensity of UVB radiation. Several studies have followed where not only latitude of the place of residence, but also UVB exposure and vitamin D serum levels as well were taken into account. These have been found to be connected in an inverse fashion not only to the incidence of colorectal cancer but to the incidence of breast and prostate cancer as well (Giovannucci, 2007; Pérez-López, 2008; Schwartz, 2005). That vitamin D would protect from cancer is supported by the fact that cancer incidence and mortality is higher in populations that are more likely to suffer from vitamin D deficiency like African-Americans and inhabitants of the Nordic countries and northern states of the United States.

One of the limitations of these studies is that latitude of residence and cancer incidence and mortality show the highest correlation. When cancer incidence is compared to direct UVB exposure (i.e outdoor activity) and vitamin D serum levels the correlations are much weaker. One explanation is that cancer takes years to develop and that current vitamin D levels and UVB exposure do not affect current cancer status. Some studies have indicated that latitude of childhood residence and vitamin D intake during childhood and adolescence are of greater importance in lowering cancer incidence than UVB exposure and vitamin D intake in adulthood (Pérez-López, 2008; Schwartz, 2005).

The epidemiological data connecting vitamin D to cancer are supported by animal data and molecular biology findings, which offer possible mechanisms for vitamin D for protections against cancer.

Studies both *in vitro* and *in vivo* (mutant mice) show that vitamin D has anticancer effects. These are mediated by VDR and affect proliferation, apoptosis and angiogenesis.

Many cells express VDR, which is needed for vitamin D action, and the enzymes that activate (CYP27B1) and inactivate (CYP24) vitamin D. Malignant cells often lose their CYP27B1 activity with increasing

aggressiveness whereas CYP24 expression is elevated under the same conditions (Davis, 2008; Thorne and Campbell, 2008), leading to a total lowering of vitamin D activity in these cells.

Data from different animal studies have shown that VDR knock out mice are more prone to developing tumours, both spontaneously and after exposure to carcinogens, than wildtype mice, indicating that vitamin D and VDR play a role in cancer development. Mice that have been inoculated with tumour cells start to develop cancer. When these mice are given vitamin D tumour growth, progression and angiogenesis (important for tumour growth and metastasizing) are diminished (Davis, 2008; Thorne and Campbell, 2008).

Vitamin D exerts its effects on cell proliferation by affecting the transcription of different cyclins, which in turn are responsible for progression through the cell cycle. It also has an effect on the transcription of proteins involved in DNA damage response, DNA replication and cell cycle arrest. Vitamin D also stimulates apoptosis by inducing pro-apoptotic proteins (such as BAX, BAK and BAD), repressing anti-apoptotic proteins (BCL2 and BCL-X<sub>L</sub>) and down-regulating telomerase activity (Deeb et al., 2007; Dusso et al., 2005) and reactive oxygen species neutralization (Thorne and Campbell, 2008). Vitamin D also inhibits angiogenesis by inhibiting endothelial cell growth, migration and tube formation. The mechanism for these observations is not clear, but in SW480-ADH cells (from human colon tumour) vitamin D induces the anti-angiogenic factor thrombospondin 1, whereas in certain prostate cancer cells inhibition of angiogenesis is reached through inhibition of interleukin-8, which stimulates angiogenesis (Deeb et al., 2007).

#### *Vitamin D and the immune system*

As is the case with certain cancers, epidemiological data suggest that the incidence of autoimmune diseases is linked to geography, which in turn suggest vitamin D involvement in the diseases. The incidence of diabetes type 1, rheumatoid arthritis (RA), multiple sclerosis (MS) and inflammatory bowel disease increases with the distance from the equator (Adorini, 2002). Patients suffering from MS, inflammatory bowel disease and RA often have low serum levels of vitamin D (Zittermann, 2003).

The connection between autoimmunity and vitamin D is further strengthened by animal data. Administration of vitamin D either prevents onset or ameliorates symptoms of several autoimmune diseases (like RA, MS and diabetes) in animal models. Vitamin D administration also protects transplanted organs from rejection in animals (Adorini, 2002; DeLuca and Cantorna, 2001).

When VDR was found to be expressed in cells of the immune system it gave an indication of vitamin D being involved in immune response regulation. VDR is expressed in monocytes, macrophages, dendritic cells

and T cells. Macrophages even express CYP27B1 and CYP24A1, indicating an important role for vitamin D in their function. Interestingly the CYP27B1 found in macrophages is not regulated in the same way as is the renal form of the enzyme – it is for instance not feedback regulated by 1,25-dihydroxyvitamin D (van Etten and Mathieu, 2005).

Vitamin D affects the function and maturation of T-lymphocytes on several levels. Immature Th0-cells can form either Th1-cells or Th2-cells, depending on the cytokines and mode of activation they are exposed to. Th1 and Th2 secrete different cytokines and can through their specific cytokine production inhibit each other.

Antigen presenting cells (APC) capture antigens and presents it on its major histocompatibility complex class II (MHC II) to T-cell precursors(Th0). The APC and Th0 form a physical connection: the MHC II on the APC combines with the Tcell receptor on the Th0. This is the beginning of the activation of the Tcell precursor, but is not enough for full activation. That requires further contact between APC and Th0, which is achieved by interaction between proteins on the surface of the APC (e.g. CD80, CD86, CD40) and those on the surface of the Tcell (CD28 and CD40L). APCs also secrete cytokines, like IL-2 and IL-12, which stimulate Th1 development. IL-12 stimulates the expression of type 1 cytokines (e.g. IL-2, interferon  $\gamma$  and tumour necrosis factor  $\alpha$ ) which further stimulate Th1 development (Myers et al., 2006). Dendritic cells (DC) are a form of antigen presenting cell, that have important functions in the regulation of the immune system. Immature dendritic cells promote tolerance whereas mature ones activate Th0-cells into Th1 cells by, among other things, secretion of IL-12 (Adorini, 2002; Kamen and Aranow, 2008).

Th1-cells have proinflammatory properties and are connected to autoimmunity and organ rejection. An elevated Th1 activity is often found in connection with autoimmune diseases.

Th2 cells on the other hand have more regulatory properties. Th2 secretes cytokines (e.g. IL-4, IL-5, IL-6, IL-9, IL-10, IL-13), out of which IL-4 directly inhibits Th1 development and stimulates Th2 formation (Zella and DeLuca, 2003).

Vitamin D suppresses the formation of Th1-cells and favours the maturation of Th2-cells. This leads to an elevated level of tolerance and diminishes autoreactivity of the immune system. This is reached through several different ways. Vitamin D inhibits the production of IL-12, the cytokine which stimulates Th1 maturation and suppresses formation of Th2. Vitamin D also inhibits the expression of MHC class II and surface receptors (e.g. CD40, CD80 and CD86) on APC and DC, thus inhibiting their potential of activating Th1 cells. Vitamin D also enhances the expression of IL-10, a cytokine which opposes the action of IL-12 (van Etten and Mathieu, 2005), further diminishing production of Th1 cells.

## **Vitamin D mode of action**

### *Nuclear receptors*

Vitamin D exerts its various effects on the organism by affecting gene transcription. Both calcitriol and calcidiol are ligands for the vitamin D receptor (VDR), but the affinity of calcidiol towards VDR is much lower – only 1/600-1/700 of that of calcitriol (Tuohimaa et al., 2005). VDR is a member of the steroid nuclear receptor family. Together with the retinoid X receptor (RXR) it forms a heterodimer, which functions as a transcription factor. The VDR-RXR-complex recognizes specific sequences on the DNA called vitamin D response elements (VDRE). The consensus sequence of a VDRE is a hexameric direct repeat of 5'-RGKTCA-3' where R=A or G and K=G or T (Matilainen et al., 2005). The two hexameric halfsites are divided by a three nucleotide spacer (Umesono et al., 1991). The VDR-RXR-complex occupies the VDRE in a specific manner: VDR attaches to the 3' halfsite whereas RXR attaches to the 5' one (DeLuca, 2004), but there are also reports of RXR attaching to the 3'-half-site (Shaffer and Gewirth, 2004).

The active forms of vitamin D can either activate or repress the transcription of a gene. In the case of transcriptional activation, a conformational change of the VDR takes place on ligand binding. This exposes parts of the VDR which are capable of binding coactivator proteins. These in turn can either have chromatin remodeling properties (through histone acetylation) themselves (Christakos et al., 2003; Dusso et al., 2005; Kato et al., 2007; Sutton and MacDonald, 2003) or they can recruit other proteins that are capable of histone acetylation (Malloy et al., 1999). Their actions open up and expose DNA, making transcription possible.

Vitamin D is also able to suppress the transcription of genes. When the VDR-RXR-complex binds to a negative VDRE, corepressors are recruited. The corepressors either have histone deacetylation properties or recruit histone deacetylators (Malloy et al., 1999). Histone deacetylation inhibits chromatin exposure and transcription.

### *Nongenomic effects of vitamin D*

Vitamin D also has so called rapid or nongenomic actions. These reactions appear quickly – within a few seconds or up to one hour – on administration. The effects appear too quickly to be transcriptional effects, which take a few hours to emerge, hence the name nongenomic. The rapid absorption of calcium from the intestine on vitamin D exposure is one of the better known nongenomic effects of vitamin D.

These observations have led to the hypothesis that vitamin D not only exerts its effects through the nuclear receptor VDR, but that it may have a membrane bound receptor. There are a number of possible candidates for a membrane associated receptor which would mediate the nongenomic effects

of vitamin D. There are reports of a membrane bound form of VDR being responsible for the rapid effects. That VDR would be involved with the rapid effects is supported by the finding that VDR knock-out mice lack vitamin D rapid effects (Norman et al., 2002). The same is seen in some patients suffering from HVDDR, who have a non-functional mutated VDR (Nguyen et al., 2004; Norman, 2006). Others report the existence of membrane bound receptors which transmit the rapid actions of vitamin D but are not related to the VDR, like the 1,25D3-MARRS receptor from chick duodenum and annexin II (Dusso et al., 2005; Hendy and Goltzman, 2005). The exact nature of the both the vitamin D membrane receptor and the nongenomic actions themselves remains unclear.

## The cytochrome P450 enzymes

The cytochrome P450 enzymes form a large superfamily of enzymes. They can be found in virtually all organisms from bacteria to humans and plants. Upon binding carbon monoxide an absorption peak at 450 nm is observed, which is characteristic for the CYP enzymes and gives rise to their name (Munro et al., 2007; Werck-Reichhart and Feyereisen, 2000).

The number of known CYP enzymes is very large – in early 2008 more than 7000 CYP were named, most of them of animal or plant origin and the number is constantly increasing. The information of cytochrome P450 numbers is constantly being updated on the internet (<http://drnelson.utmem.edu/CytochromeP450.html>).

The cytochrome P450 enzymes are named and divided into families based on their amino acid sequence. Enzymes with a sequence identity of 40% or more belong to the same family, which is denoted by a number. If the sequence identity is 55% or higher, the enzymes belong to the same subfamily, which is denoted by a capital letter (Nelson, 2006; Nelson et al., 1996). The individual enzymes in a subfamily are denoted by an Arabic number. For example CYP2D25 and CYP2R1 belong to the same family, but to different subfamilies. CYP2J2 and CYP2J3 both belong to the same subfamily, but are individual genes in the CYP2J subfamily.

The sequences among the CYP superfamily vary greatly and often there is less than 20% sequence similarity. There are only three amino acids that are conserved among all CYP enzymes. Even though the sequences are very different, all CYP enzymes display a very similar structural fold in a shape of a triangular prism. This shape is characteristic for the CYP enzymes (Munro et al., 2007).

The cytochrome P450 enzymes are monooxygenases and most commonly they introduce oxygen – usually in the form of a hydroxyl group – into the substrate. However, the cytochrome P450 enzymes are capable of catalyzing

other reactions as well such as dealkylation, carbon-carbon bond cleavage, dehydrogenation and epoxidation (Munro et al., 2007).

The introduction of oxygen into a substrate requires first an activation of the oxygen through reduction, which requires electrons. CYP enzymes receive the necessary electrons required for oxygen activation from NADPH. There are a number of ways for the electrons to reach the enzyme and depending on how the electrons are delivered to the catalytic site, the CYP enzymes are divided into different classes.

Class I CYPs receive their electrons from NAD(P)H by ferredoxin reductase and ferredoxin. Mitochondrial and bacterial CYPs belong to this class. Class II CYPs require a P450-reductase and NADPH for electron transport. Microsomal CYPs belong to this class. Class III CYPs do not need an exogenous electron source at all, as they are self sufficient in that regard. Class IV enzymes receive their electrons directly from NADH and do not have an electron carrier chain (Hlavica et al., 2003; Werck-Reichhart and Feyereisen, 2000).

## Vitamin D 25-hydroxylases

In mammals there are a number of cytochrome P450 enzymes capable of 25-hydroxylating vitamin D and there are at least two 25-hydroxylases present in mammals – one in the microsomal fraction and one in mitochondria. The mitochondrial enzyme CYP27A1 is shared by many species. The microsomal 25-hydroxylases on the other hand are more diverse: CYP3A4, CYP2R1 and CYP2J2 in humans CYP2D25 in pigs and CYP2J3 and CYP2C11 in rats (Prosser and Jones, 2004).

### The quest for the vitamin D 25-hydroxylase

The first studies on vitamin D 25-hydroxylation were done on rats. The liver microsomal fraction was shown to harbour the greatest 25-hydroxylating capacity (Bhattacharyya and DeLuca, 1974). Later a study on perfused rat liver showed kinetics, which indicated that there are two different 25-hydroxylases in rat liver: a high affinity, low capacity enzyme and a low affinity, high capacity enzyme (Fukushima et al., 1976). Further studies ensued, where a 25-hydroxylase in rat liver mitochondria was described (Björkhem and Holmberg, 1978; Björkhem et al., 1980), but others were only able to prove 25-hydroxylation in the microsomal fraction (Madhok and DeLuca, 1979). The rat microsomal vitamin D 25-hydroxylase turned out to be male specific (Andersson and Jörnvall, 1986; Hayashi et al., 1986; Hayashi et al., 1988), which was rather surprising as microsomes from female rats were capable of vitamin D-hydroxylation (Hayashi et al., 1988) and as the serum levels of calcidiol were similar in male and female rats

(Dahlbäck and Wikvall, 1987), proving that vitamin D activation is not sex specific in rats. Later the vitamin D 25-hydroxylation was found to be of the same magnitude in both male and female rats, provided the substrate concentration was low (in the nanomolar range) (Thierry-Palmer et al., 1995). It turned out that there is a microsomal 25-hydroxylase in rats which is expressed in both male and female rats, CYP2J3 (Aiba et al., 2006; Yamasaki et al., 2004). The male-specific microsomal rat 25-hydroxylase was later identified as CYP2C11 (Rahmaniyan et al., 2005).

The mitochondrial 25-hydroxylase was cloned from rabbit (Andersson et al., 1989), rat (Usui et al., 1990) human (Cali and Russell, 1991) and pig (Postlind et al., 2000) and identified as CYP27A1. It was considered to be the principal 25-hydroxylase as it is well conserved among animals and as it is not sex specific. But the scientific community was not altogether convinced of its importance as a vitamin D 25-hydroxylase in a physiological setting. Both animals (knock-out mice) (Rosen et al., 1998) and humans with mutations in the CYP27A1 show severe disturbances in cholesterol metabolism, but normal calcidiol levels (Cali et al., 1991; Federrico and Dotti, 2003; Moghadisian et al., 2002; Verrips et al., 2000). CYP27A1 is not capable of 25-hydroxylating vitamin D<sub>2</sub> (Guo et al., 1993; Holmberg et al., 1986), but 25-hydroxyvitamin D<sub>2</sub> is formed *in vivo* (Holick et al., 2008; Jones et al., 1998). The K<sub>m</sub> for 25-hydroxylation of vitamin D<sub>3</sub> is also high –10 μM (Björkhem et al., 1980)- which would suggest that CYP27A1 works efficiently only at very high vitamin D concentrations.

## The microsomal porcine vitamin D 25-hydroxylase

The pig has only one microsomal vitamin D 25-hydroxylase: CYP2D25. This together with the fact that the pig's enzymatic make up closely resembles that of humans makes it a good model organism for studying vitamin D metabolism. The plasma levels of calcidiol and calcitriol in pigs are the same as in healthy humans (Rungby et al., 1993).

A vitamin D 25-hydroxylase was isolated from pig liver microsomes. The enzyme displayed similar distribution in both male and female pig (Axen et al., 1992) and was capable of 25-hydroxylating both vitamin D<sub>2</sub> and vitamin D<sub>3</sub> (Axen et al., 1994). The enzyme was later cloned and identified as CYP2D25 (Postlind et al., 1997). The K<sub>m</sub> of the 25-hydroxylating capacity of CYP2D25 toward vitamin D<sub>3</sub> was determined to be 95 nM, a concentration believed to be close to the physiological one. The vitamin D 25-hydroxylating capacity of the human CYP2D6 was also studied. It turned out, that it was not a vitamin D 25-hydroxylase, despite belonging to the same subfamily as the porcine vitamin D 25-hydroxylase (Hosseinpour and Wikvall, 2000).

## The human microsomal vitamin D 25-hydroxylases

There are a number of microsomal CYP 450-enzymes in humans capable of 25-hydroxylating vitamin D. Those enzymes reported of being capable of 25-hydroxylating vitamin D are CYP3A4, CYP2J2 and CYP2R1.

CYP2R1 was orphanized in 2003. CYP2R1 is a microsomal enzyme, it is highly conserved from mouse to human to fish, which is a characteristic of enzymes that have endogenous substrates. It is also capable of 25-hydroxylating both vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, which is characteristic of the microsomal vitamin D 25-hydroxylase (Cheng et al., 2003). Further evidence proving the importance of CYP2R1 as a vitamin D 25-hydroxylase emerged a year later. A patient with apparent 25-hydroxylase deficiency, displaying low calcidiol but normal calcitriol levels, was shown to have a homozygous mutation in the CYP2R1 gene, which rendered it unfunctional towards vitamin D<sub>3</sub>. The patient did not show mutations in any other possible vitamin D 25-hydroxylases (CYP27A1, CYP2D6 and CYP3A4)(Cheng et al., 2004; Lin et al., 2003). The enzyme kinetic properties of CYP2R1 have been determined in a yeast expression system. The K<sub>m</sub> for the 25-hydroxylation of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> were both in the micromolar range, but only one tenth of the K<sub>m</sub> value displayed by CYP27A1 towards vitamin D<sub>3</sub> in the same system (Shinkyō et al., 2004).

CYP3A4 has also been reported to be able to hydroxylate vitamin D in 25-position. It was capable of hydroxylating vitamin D<sub>2</sub>, but had no activity towards vitamin D<sub>3</sub> (Gupta et al., 2004), which speaks against it being the most important 25-hydroxylase. No enzyme kinetic data was presented.

When the rat microsomal 25-hydroxylase turned out to be CYP2J3, the possibility of the human CYP2J2 being able to perform 25-hydroxylation of vitamin D became apparent. CYP2J2 was capable of hydroxylating both vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, but showed higher activity towards vitamin D<sub>2</sub>, unlike the rat CYP2J3. The K<sub>m</sub> values were in the micromolar range towards both vitamin D<sub>2</sub> and vitamin D<sub>3</sub> (Aiba et al., 2006).

## Regulation of the vitamin D 25-hydroxylases

Early studies *in vivo* indicated that treatment with calcitriol suppressed the 25-hydroxylating capacity of rat liver microsomes (Bhattacharyya and DeLuca, 1974; Madhok and DeLuca, 1979; Reinholz and DeLuca, 1998). CYP27A1 mRNA in both fetal intestine and in rats have also been shown to be downregulated by calcitriol (Theodoropoulos et al., 2003a; Theodoropoulos et al., 2003b). Even though the studies are few, the regulation of both expression level and enzyme activity of vitamin D 25-hydroxylases by calcitriol has been previously reported.

## Drugs with deleterious effects on bone health

A number of drugs have a negative effect on bone health. The drugs may give rise to increased bone turn over and some drugs give rise to osteomalacia, a disease which results from vitamin D deficiency. The vitamin D deficiency in turn leads to impaired bone mineralization.

Anti-epileptic drugs like phenobarbital, phenytoin, and carbamazepine have long been associated with an increased risk for fractures. Treatment is also often associated with decreased calcidiol levels and decrease in bone mineral density (Petty et al., 2007).

The antibiotic rifampicin, which is used to treat tuberculosis is also connected to increased bone turn over and decreased serum calcidiol levels.

Osteoporosis, osteomalacia and vitamin D deficiency has been reported in connection to antiretroviral therapy (Cozzolino et al., 2003; Gyllensten et al., 2006). In vitro studies have shown that antiretroviral compounds have an inhibitory effect on vitamin D metabolism. Both the activating  $1\alpha$ - and 25-hydroxylations were inhibited by antiretrovirals as was the 24-hydroxylation, which is the first step towards the inactivation of vitamin D (Cozzolino et al., 2003).

All the abovementioned drugs are capable of activating the nuclear receptor PXR. PXR is closely related to the VDR and can recognize and activate VDREs, suggesting that PXR ligands are capable of affecting genes that are under the transcriptional control of VDR (Pascussi et al., 2005).

# Aims of the present investigation

The overall aim of the present investigation was to determine the possible regulation of vitamin D 25-hydroxylase expression and their enzymatic activity by hormones and xenobiotics known to affect bone health.

The specific aims were:

- To sequence the promoter of CYP2D25 and determine whether the promoter could be regulated by the active vitamin D metabolites calcidiol and calcitriol
- To determine the effects of phenobarbital on the expression of porcine CYP2D25 and CYP27A1 and the effect of phenobarbital on the promoter of CYP2D25
- To study the expression of human vitamin D 25-hydroxylases in dermal fibroblasts and whether they are capable of measurable vitamin D 25-hydroxylation
- To study the effects of calcitriol, phenobarbital and efavirenz on the expression and activity of the human vitamin D 25-hydroxylases in fibroblasts and prostate cancer LNCaP cells

# Experimental procedures

## Materials

The expression plasmid for the human VDR was a gift from Dr Leonard Freedman, The Merck laboratories (West Point, PA). The expression plasmid for the human RXR $\alpha$  was a generous gift from Dr Ronald M. Evans, Howard Hughes Medical Institute, The Salk Institute for Biological Studies (San Diego, CA). The expression plasmids for the human PXR and CAR were generously provided by Dr. Masahiko Negishi, Laboratory of Reproductive and Developmental Toxicology, NIEHS, NIH, Research Triangle Park, NC.

1 $\alpha$ -Hydroxyvitamin D was a kind gift from Dr Lise Binderup, Leo Pharma, Denmark. Efavirenz was kindly provided by Dr Filip Josephson, Karolinska Institutet, Sweden and phenobarbital was a kind gift from Dr Lucia Lazarova, Dept of Pharmacy, Uppsala University. HepG2 (HB-8065), LNCaP and BJ cells were purchased from the ATCC. Patient dermal fibroblasts were provided by Dept of women's and children's health, Uppsala University.

All remaining materials were purchased from commercial sources.

## Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin and streptomycin and 1% (v/v) non-essential amino acids.

BJ and patient dermal fibroblasts were cultured in Minimal Essential Medium (MEM) with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotic and antimycotic, 1% (v/v) non-essential amino acids and 1% (v/v) sodium pyruvate.

LNCaP-cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and antibiotics.

Medium was changed every 72 hours. When cells were 90% confluent, they were lifted by trypsin/EDTA and passaged.

## Transient transfection

In experiments to study the effects of 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D and phenobarbital on the promoter of CYP2D25, HepG2 cells were transiently transfected with expression vectors for VDR, RXR, PXR and CAR together with the luciferase reporter construct containing a 700 bp portion of the porcine CYP2D25 promoter and a pCMV  $\beta$ -galactosidase plasmid (in order to control for transfection efficiency). Control experiments were performed with corresponding empty vectors. The transfections were carried out using the Superfect Transfection reagent (Qiagen). Luciferase and  $\beta$ -galactosidase activities were assayed as previously described (Ellfolk et al., 2006). Luciferase reporter activity was expressed as relative light units (RLU) divided by  $\beta$ -galactosidase activity (expressed as absorbance at 420 nm). Luciferase activity was measured using TD-20/20 Luminometer, Turner Designs. The  $\beta$ -galactosidase reagent contains 100xMG-solution: ONPG (4 mg/ml):0,1 M sodium phosphate buffer (ratio, 1:22:67).

## Semi-quantitative RT-PCR

Semi-quantitative RT-PCR analysis to examine the expression of relative mRNA levels was performed using GAPDH as internal control. Using mRNA specific primers the expression of human microsomal vitamin D 25-hydroxylases CYP2J2, CYP2R1, CYP3A4 and CYP27A1 and the nuclear receptors CAR, PXR and VDR were examined. The PCR reactions were performed using a MiniCycler (MJ Research Inc.) with thermal cycler conditions optimized for each PCR transcript. The PCR transcripts were separated on a 2% ethidium bromide gel.

## Quantitation of CYP2R1, CYP27A1 and CYP2J2 mRNA

Total RNA was isolated from BJ, patient derived fibroblasts, and LNCaP with RNeasy total RNA Mini isolation Kit (Qiagen) and the Rnase Free Dnase Set (Qiagen).

PCR amplification was performed on a Bio-Rad ICycler with the gene-specific TaqMan probes and primers for human CYP27A1 and CYP2J2 designed by Applied Biosystems (article no: Hs00168003\_m1 and Hs00356035\_m1, respectively). Eukaryotic 18S rRNA (Applied Biosystems) was used as endogenous control. CYP2R1 was amplified using SYBR Green technique. The primers used are described in the PrimerBank database (<http://pga.mgh.harvard.edu/primerbank/index.html>). The forward primer is the forward primer of Primer Pair 1, with the PrimerBank ID

33591222a1. The reverse primer is the reverse primer of Primer Pair 3, with the Primerbank ID 33591222a3. GAPDH was used as endogenous control.

## Assay of vitamin D 25-hydroxylase activity

Endogenous vitamin D 25-hydroxylase activity in fibroblasts and LNCaP cells was measured by incubation with  $1\alpha$ -hydroxyvitamin D<sub>3</sub> dissolved in DMSO. As dermal fibroblasts contain both CYP2R1 and CYP27A1 fibroblasts were also incubated with  $7\alpha$ -hydroxy-4-cholestene-3-one (to control CYP27A1 activity specifically). After incubation with substrate for 24 or 48 hours, cells were harvested and the medium was analysed for 1, 25-dihydroxyvitamin D<sub>3</sub> and  $7\alpha,27$ -dihydroxy-4-cholestene-3-one, respectively.

Incubations with medium from cell cultures were extracted with ethyl acetate and the products formed were analysed by straight phase HPLC (Column: LiChrosphere Si 60, 5 $\mu$ m (Merck, Rahway, NJ), detector: 2151 Variable Wavelength Monitor (LKB)). Vitamin D derivatives were analysed at 265 nm and cholesterol metabolites at 240 nm. The mobile phase was hexane:isopropanol 90:10 for vitamin D metabolites and hexane:isopropanol 92:8 for cholesterol metabolites.

In order to confirm the identity of the suspected 1,25-dihydroxyvitamin D<sub>3</sub> seen on straight phase HPLC, the mobile phase was collected 2 minutes around the expected retention time of the product. The mobile phase was then evaporated using N<sub>2</sub>-gas and analysed on reverse phase HPLC (column: LiChrospher 100, detector: 655A Variable Wavelength monitor (Merck-Hitachi)) at wavelength 265 nm. The mobile phase used was methanol:water 85:15.

## Statistical analysis

Analysis of statistical significance was performed using one-way ANOVA, Mann-Whitney U-test or Student's T-test. P values <0,05 were considered statistically significant.

# Results and discussion

## Isolation and properties of the CYP2D25 promoter (Paper I)

CYP2D25 is the porcine microsomal vitamin D 25-hydroxylase (Postlind et al., 1997). Its catalytic properties were previously described (Hosseinpour and Wikvall, 2000), but the possible transcriptional regulation of expression of the gene was not known. The vitamin D 25-hydroxylases are generally believed to be constitutively expressed, based on observations made in connection with vitamin D intoxication. Vitamin D intoxication leads to elevated levels of 25-hydroxyvitamin D (calcidiol), whereas the level of  $1\alpha,25$ -dihydroxyvitamin D (calcitriol) hardly changes (Jones, 2008).

In order to study the transcriptional regulation of the CYP2D25 gene, a 700 bp portion upstream of the start site of its promoter was studied. It was sequenced and computer analyzed for putative response elements (figure 3). The analysis of the promoter revealed several putative response elements, most interestingly a VDRE at 220 bp upstream of the transcription start site. This indicates that the transcription of CYP2D25 may be regulated by vitamin D metabolites with affinity to the VDR.

To be able to study the effects of different compounds on the transcription of CYP2D25, a luciferase reporter plasmid was constructed. The aforementioned 700 bp portion of the promoter was fused into a pGL3 basic plasmid, so that the CYP2D25 promoter was driving the expression of the luciferase gene. This plasmid was then transfected into HepG2 cells.

Vitamin D metabolites require the presence of VDR to be able to affect gene transcription. Both  $1\alpha,25$ -dihydroxyvitamin D (calcitriol) and 25-hydroxyvitamin D (calcidiol) have affinity to the VDR and should therefore be able to affect the transcription of genes that have a VDRE in their promoter. In order to function as a transcription factor VDR requires RXR as partner.

Therefore, to study the effects of  $1\alpha,25$ -dihydroxyvitamin D and 25-hydroxyvitamin D on the promoter of CYP2D25, HepG2 cells were transfected with the promoter-luciferase plasmid together with expression plasmids for human VDR and RXR or their empty vectors. The transfected cells were then treated for 24 hours either with  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub> or ethanol, which was used as vehicle.

AP-1

-702 GT CGACGGCCCG GGGGTCAGGT -681

-680 CTGGGCAGCC ACTGAGAAGT AGAGGGTATG TGTGCGTACG -641

-640 TGTGGTACAT ATGTGTTGAT GTGTGGGTTT GTGGGTGTGT -601

AP-1

-600 AAATGTGTGC GTGTGAGTAA GCAGTGTGTG GGTGGTTTAT -561

GR

-560 GAGTGTGTGA CTGTGTCTGT GTTCAGTGTT CTAATGAAAT -521

-520 GAGGGGATGC CAAGACCTTT ATCCCCATG ATGGTCCCTC -481

-480 TGGTCACGAC GATCTCCCTC CAGCACTGGG CAGGTGCCAC -441

PR

-440 TGAAAAACCT GGGCCACTCA GGAGGCAGCA GGACAAGGGT -401

GR

-400 ATGTTCTGGG GCTGAGGCTG TGGCCACATC AGGGAGTGTG -361

AP-1

-360 AGCCCTAAGT GACAAGAGTG GCCAGAGTCAA GTGTAAAGAC -321

-320 GTCTGATTCT TTGGAAGTTT TACAGAGGTG GGAATCCGGC -281

GR

-280 CAGGGGTGAA GGAGGACTTC TTGTGTCCTT GCGGCAAGCA -241

VDRE

-240 CATGGAGAGG GCAGGAGGGA CAGTCACCAT ACCCACACCT -201

-200 GACTTTCCCG GGCCCCTCCC CCACCCTTGC ACCCTCTGGC -161

-160 AGCCCAGTCT CCCTGCAGGT TGGCTCAAGC AGAGGGCAAA -121

TBP

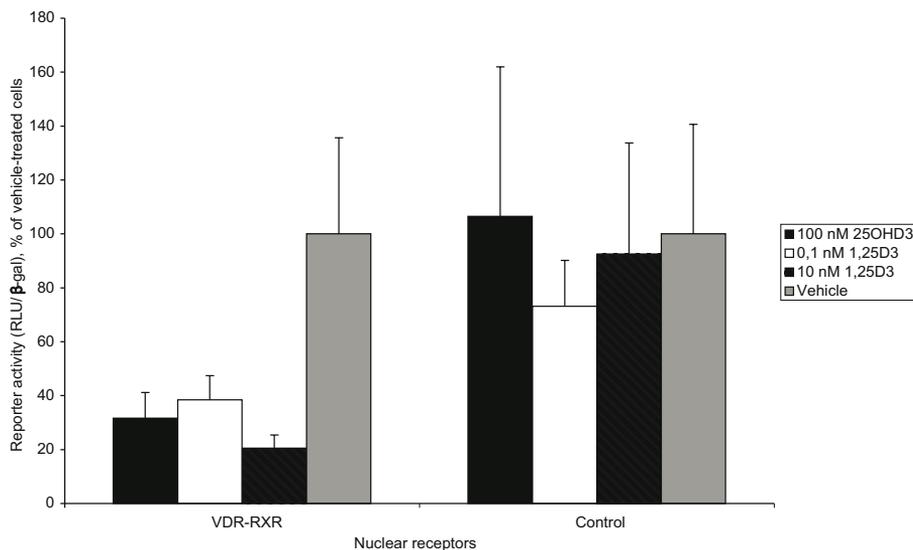
-120 GACCAGTCTC GGCT CCTTT ATAAAATGGG CCTAGGCTGG -81

-80 TCAAGCCTTC AGAGTGCTCT GCATGGCCTG GTCTTAGGTG -41

-40 CTTGGTGCGG TGAGGAGCCC AGTTGTGCAC TGAGGCAGCC -1

+1 ATGGGTCTGC TGA CTGGGG +20

*Figure 3.* The sequence of the CYP2D25 promoter. The putative VDRE half-sites are indicated by underlined bold letters. Other putative regulatory sequences are underlined. (GR: glucocorticoid receptor, PR: progesterone receptor, AP-1: activator protein 1).



*Figure 4.* Effects of vitamin D metabolites on the activity of the CYP2D25 promoter. 25OHD3: 25-hydroxyvitamin D<sub>3</sub>, 1,25D3: 1,25-dihydroxyvitamin D<sub>3</sub>.

The results showed that the luciferase activity was suppressed by approximately 70% in cells treated with either vitamin D metabolite provided that both VDR and RXR were present. In their absence, vitamin D had no effect on the promoter (figure 4).

## The effect of phenobarbital on CYP2D25 (Paper II)

A number of drugs – phenobarbital amongst them- have side effects that give rise to bone diseases like osteomalacia and increased bone turnover. The mechanism is unclear, but changes in vitamin D metabolism is suspected. Phenobarbital in particular is a well known CYP 450 inducer and as both vitamin D activation and breakdown is catalyzed by cytochrome P450 enzymes it is not inconceivable, that phenobarbital would be capable of altering vitamin D metabolism. Patients receiving phenobarbital have also been reported to have low serum levels of 25-hydroxyvitamin D (calcidiol). Recently, phenobarbital has been shown to induce CYP24, the enzyme responsible for vitamin D inactivation, in the presence of the nuclear receptor PXR (Pascucci et al., 2005).

In order to study the effects of phenobarbital on vitamin D 25-hydroxylation primary porcine hepatocytes were treated with phenobarbital and the levels of CYP27A1 and CYP2D25 mRNA were determined with Northern blot. The levels of CYP27A1 mRNA diminished during treatment, whereas the mRNA level of CYP2D25 was induced. The vitamin D 25-hydroxylating capacity of the treated hepatocytes was also determined and

phenobarbital inhibited the 25-hydroxylation of vitamin D by 70%. In order to study the effects of phenobarbital on the 25-hydroxylases themselves, purified CYP2D25 and CYP27A1 from pig were used and phenobarbital was added to the incubation mixture. Phenobarbital inhibited the vitamin D 25-hydroxylase activity of both CYP2D25 and CYP27A1.

Phenobarbital stimulates the transcription of CYP24 (Moreau et al., 2007; Pascussi et al., 2005), and as the mRNA levels of CYP2D25 were elevated by phenobarbital treatment, the effect of phenobarbital on the transcription of the gene was of interest. The CYP2D25-promoter-luciferase reporter plasmid described previously (Ellfolk et al., 2006) was transfected into HepG2 cells. Phenobarbital activates both PXR and CAR (Moreau et al., 2007) and thus cells were transfected with expression plasmids of RXR and either PXR or CAR as well. In control experiments cells were transfected with the empty vectors. The transfected cells were treated with phenobarbital for 24 hours. The transcriptional activity of the cells diminished with 70% when cells transfected with PXR were treated with phenobarbital. In the presence of CAR, phenobarbital managed to suppress the transcriptional activity by 40% (figure 5).

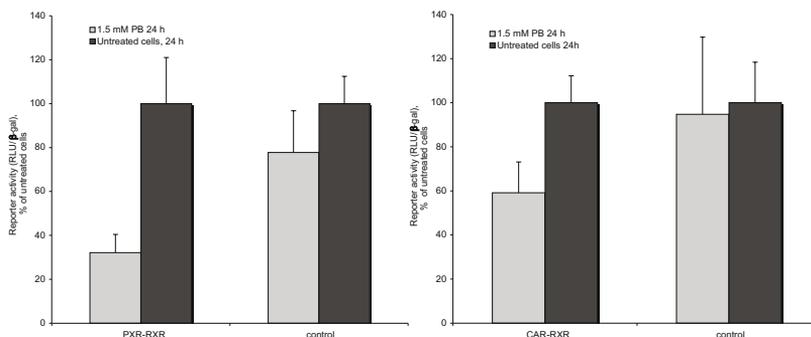


Figure 5. The effects of phenobarbital (PB) on the promoter of CYP2D25 in the presence of PXR (left) and CAR (right).

The heterodimers PXR-RXR and CAR-RXR recognize and bind VDRE. PXR, CAR and VDR belong to the same family of nuclear receptors (Moreau et al., 2007) and the DNA-binding domains of PXR and VDR show 60% sequence similarity (Pascussi et al., 2005). It is therefore not surprising that PXR and CAR have the same effect on a gene as VDR, even though the finding that phenobarbital suppresses transcription is rather unusual as most reports are of phenobarbital as an CYP inducing agent (Czekaj, 2000; Waxman, 1999; Willson and Kliewer, 2002).

The effect of phenobarbital on the mRNA levels of CYP2D25 does not correspond to its effects on enzymatic activity and transcriptional levels. Both transcription and enzymatic activity were diminished in response to the drug. The elevated mRNA levels in response to phenobarbital might be

explained by post-transcriptional effects that may stabilize formed mRNA. The posttranscriptional stabilization of the murine Cyp2a5 by phenobarbital has been reported (Aida and Negishi, 1991).

In summary, phenobarbital seems to have an inhibitory effect on vitamin D bioactivation, and together with the effects on elevated CYP24 levels might offer an explanation to the low 25-hydroxyvitamin D levels and bone deterioration seen after long term phenobarbital treatment.

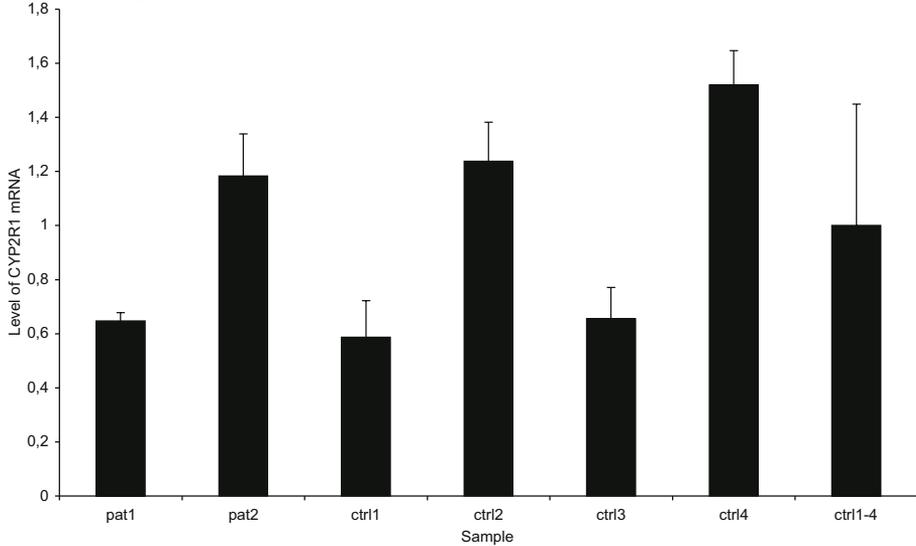
## The vitamin D 25-hydroxylase expression and activity in human dermal fibroblasts (Paper III)

Vitamin D is activated in two steps – first a 25-hydroxylation in the liver which is followed by a  $1\alpha$ -hydroxylation in the kidney. However, the vitamin D activating enzymes are expressed in many tissues and it would at least in theory be possible to determine the vitamin D 25-hydroxylating activity of human subjects in cells that were easier to retrieve than liver biopsies. Vantieghem and co-workers have reported the expression of vitamin D 25-hydroxylases in dermal fibroblasts (Vantieghem et al., 2006). The aim of this study was to determine if dermal fibroblasts could be used in order to measure the vitamin D 25-hydroxylase activity in human subjects.

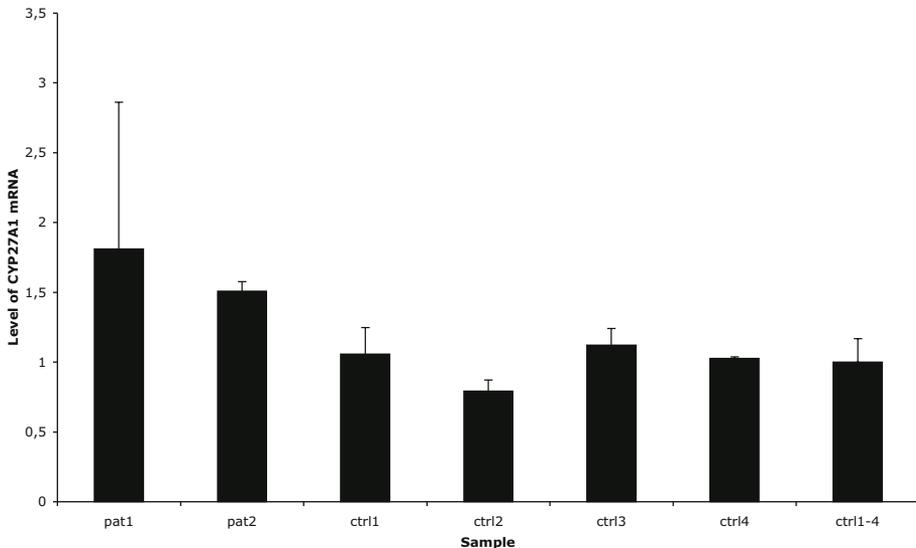
Using semiquantitative RT-PCR the vitamin D 25-hydroxylase expression in a dermal fibroblast cell line, BJ, was studied. The cells were found to express CYP2R1 and CYP27A1 – two well known human enzymes with vitamin D 25-hydroxylating activity. After the 25-hydroxylase expression pattern in fibroblasts was known, the capability of the cells to 25-hydroxylate vitamin D was determined. BJ cells and dermal fibroblasts from six different human subjects two of whom displayed a disturbed vitamin D status and four with normal vitamin D status, were incubated with  $1\alpha$ -hydroxyvitamin D<sub>3</sub> to determine the vitamin D 25-hydroxylating activity of the cells. This activity is mediated by two different enzymes in the fibroblasts – CYP2R1 and CYP27A1. To be able to study the activity of CYP27A1 alone, the cells were also incubated with  $7\alpha$ -hydroxy-4-cholestene-3-one, which is a substrate for CYP27A1. The relative amounts of CYP2R1 and CYP27A1 mRNA in the samples were determined using real-time RT-PCR.

The results showed that fibroblasts were capable of measurable vitamin D 25-hydroxylation and were also capable of metabolizing  $7\alpha$ -hydroxy-4-cholestene-3-one. This indicates that CYP27A1 was functional in all samples. The results indicate that dermal fibroblasts can be used to study 25-hydroxylase activity in human subjects.

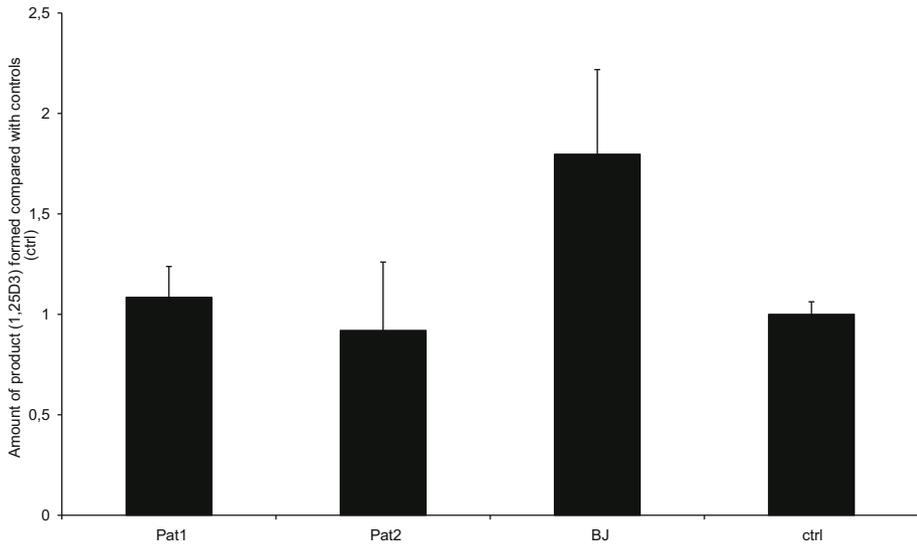
An interesting finding was that the levels of CYP2R1 mRNA showed large inter-individual variation (figure 6). The CYP27A1 levels were not as variable (figure 7).



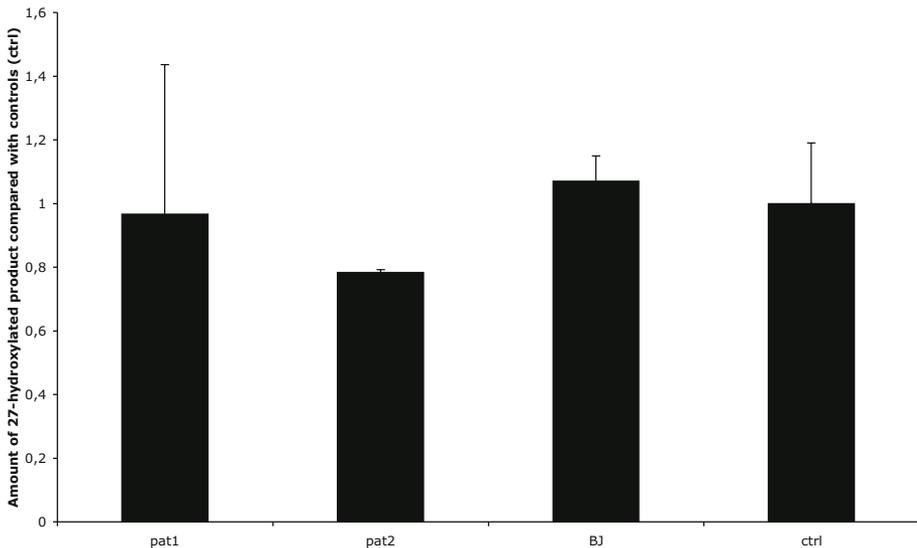
*Figure 6.* The level of CYP2R1 mRNA in human fibroblasts. Pat1 and pat2 denote two subjects with disturbed vitamin D status, whereas ctrl1-ctrl4 denote subjects with normal vitamin D status. The mRNA levels show great interindividual variability.



*Figure 7.* The level of CYP27A1 mRNA in human fibroblasts. Pat1 and pat2 denote two subjects with disturbed vitamin D status, whereas ctrl1-ctrl4 denote subjects with normal vitamin D status.



*Figure 8.* Vitamin D 25-hydroxylating capacity in human fibroblasts. Ctrl denotes a subject with normal vitamin D status, BJ the human fibroblast cell line BJ and pat1 and pat2 two subjects with disturbed vitamin D status. The 25-hydroxylating capacity was similar in all subject samples (pat1, pat2 and ctrl).



*Figure 9.* Capability of human fibroblasts to hydroxylate  $7\alpha$ -hydroxy-4-cholestene-3-one at carbon 27. The hydroxylation is performed by CYP27A1. The test shows no difference in CYP27A1 activity toward  $7\alpha$ -hydroxy-4-cholestene-3-one. Ctrl denotes a subject with normal vitamin D status, BJ the human fibroblast cell line BJ and pat1 and pat2 two subjects with disturbed vitamin D status.

Using these methods a clear reason to the disturbed vitamin D status displayed clinically in two of the subjects studied could not be determined.

Their vitamin D 25-hydroxylating capacity (figure 8) and levels of 25-hydroxylases fell within the normal variation of the subjects with normal vitamin D status. The low serum 25-hydroxyvitamin D<sub>3</sub> levels might be the result of an increased metabolism of 25-hydroxyvitamin D<sub>3</sub> to inactive products such as 24,25-dihydroxyvitamin D<sub>3</sub> or 25,26-dihydroxyvitamin D<sub>3</sub>. It cannot be excluded that the low 25-hydroxyvitamin D<sub>3</sub> levels are the result of a dysfunctional CYP2R1. It may be that the contribution of CYP2R1 to the vitamin D 25-hydroxylation is masked by the actions of CYP27A1. The incubation with 7 $\alpha$ -hydroxy-4-cholestene-3-one showed that the CYP27A1 function did not vary greatly between subjects (figure 9).

## Regulation of vitamin D<sub>3</sub> 25-hydroxylases in dermal fibroblast and prostate cancer LNCaP cells (Paper IV)

Earlier experiments showed that both vitamin D metabolites and phenobarbital, a drug affecting bone health, were capable of affecting the porcine vitamin D 25-hydroxylase, CYP25. We wanted to determine whether the active vitamin D metabolite calcitriol, phenobarbital and efavirenz, an antiretroviral drug causing bone deterioration (Gyllensten et al., 2006), could affect the mRNA levels of vitamin D 25-hydroxylases in dermal fibroblasts (BJ cells) and prostate cancer LNCaP cells. The effects of calcitriol and efavirenz on the enzymatic activity was also of interest.

First the 25-hydroxylase expression pattern of these cells was determined. Fibroblasts (BJ cells) were already known to express CYP2R1 and CYP27A1 (paper III). LNCaP cells were found to express two putative vitamin D 25-hydroxylases – CYP2R1 and CYP2J2. The expression of nuclear receptors needed for calcitriol, phenobarbital and efavirenz effect in the cells was also determined. VDR was expressed in both cell lines, PXR was expressed only in BJ cells and CAR was not expressed in either cell line. This indicates that calcitriol would be able to affect the expression of vitamin D sensitive genes in both cell lines and that only genes expressed in BJ cells would respond to phenobarbital and efavirenz, as the effect of these drugs are mediated by PXR (Hariparsad et al., 2004; Pascussi et al., 2005).

The effects of calcitriol, phenobarbital and efavirenz on the expression levels of the enzymes was determined by real-time PCR. Efavirenz and phenobarbital suppressed the expression of CYP2R1 in fibroblasts but not in LNCaP cells, which can be explained by the difference in PXR expression between the cells. CYP2J2 was slightly suppressed by efavirenz and phenobarbital whereas CYP27A1 was not affected by either treatment. Calcitriol suppressed the expression of CYP2R1 in both fibroblasts and LNCaP cells, but had no clear effect on either CYP2J2 or CYP27A1 (table 1).

Table 1. Effect of calcitriol (1,25D3), efavirenz (EFZ) and phenobarbital (PB) on the expression levels of CYP2J2, CYP2R1 and CYP27A1 mRNA in LNCaP and BJ cells. ↓ denotes suppression and ↔ no effect.

Treatment	LNCaP		BJ	
	CYP2J2	CYP2R1	CYP2R1	CYP27A1
1,25D3	↔	↓	↓	↔
EFZ	↔	↔	↓	↔
PB	↓	↔	↓	↔

In order to determine the effect of the drugs on the cellular 25-hydroxylating activity, the cells were treated for 24 hours with either calcitriol (LNCaP) or either calcitriol or efavirenz (fibroblasts) before receiving substrate (1 $\alpha$ -hydroxyvitamin D<sub>3</sub>). The concentrations were in the physiological range (calcitriol: 0,1 nM) (Lou et al., 2003) or the therapeutic window (efavirenz: 2,5 mg/ml) (Almond et al., 2005; Marzolini et al., 2001). Efavirenz significantly lowered the vitamin D 25-hydroxylating activity of fibroblasts. The 25-hydroxylating activity in fibroblast was suppressed by calcitriol, but it was not statistically significant (figure 10).

In LNCaP cells no product of incubation (1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>) could be determined. Instead loss of substrate was used as an indicator of metabolism. Cells treated with calcitriol prior to adding of substrate had 17% of the substrate left after 24 hours of incubation. In the cells treated with vehicle only 6% of the substrate remained after the incubation period. The difference is statistically significant and indicates that calcitriol does suppress vitamin D activation in LNCaP cells (figure 11).

Earlier transfection experiments have shown that vitamin D 25-hydroxylases may be transcriptionally regulated by vitamin D metabolites and phenobarbital. The question arose, whether these drugs could affect the enzymes in a more physiological setting i.e. whole cells. The mRNA levels were indeed affected by drug treatment in cells, provided that the required nuclear receptors were expressed in the cells as well. The drugs also turned out to affect the enzymatic activity of treated cells as well, further strengthening the evidence for regulation of vitamin D 25-hydroxylase activity.

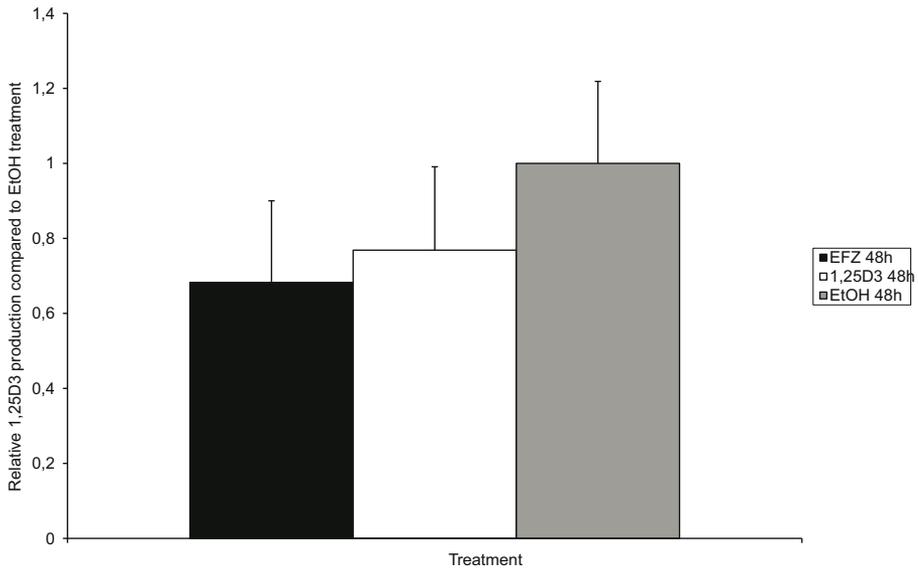


Figure 10. The effect of efavirenz (EFZ) or calcitriol (1,25D3) on the 25-hydroxylating capacity of human fibroblast BJ cells.

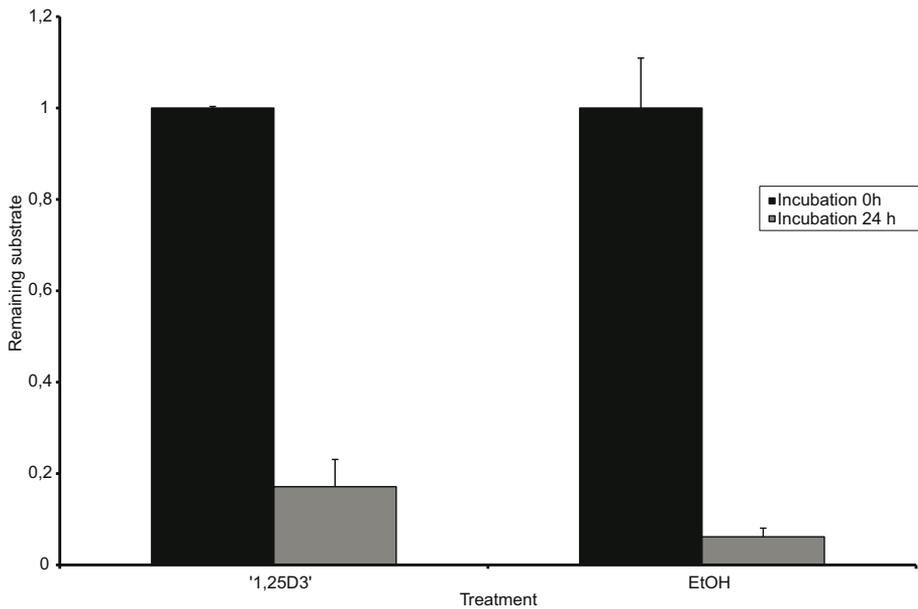


Figure 11. Amount of substrate ( $1\alpha$ -hydroxyvitamin  $D_3$ ) remaining after 24 hours of incubation in LNCaP cells receiving different treatments – either calcitriol (1,25D3) or ethanol (EtOH) 24 hours prior to the addition of substrate.

## Summary and conclusions

The last step in vitamin D bioactivation,  $1\alpha$ -hydroxylation, is strictly regulated. The levels of CYP27B1 are regulated by for instance PTH and calcitriol. Because calcitriol, the product of CYP27B1, is very potent its levels need to be under strict control. That 25-hydroxylation, the first step in the bioactivation of vitamin D, would be completely unregulated seems unlikely considering that calcidiol has affinity for the VDR and is because of that capable of producing biological effect. Calcidiol has also been reported to be able to function as a hormone in prostate cancer cells (Lou et al., 2003; Schwartz et al., 2004).

Sequencing the 700bp of the proximal promoter of CYP2D25, the porcine microsomal vitamin D 25-hydroxylase, revealed a number of interesting putative response elements. Most interestingly it contained a putative VDRE. This indicated that CYP2D25 at least in theory may be regulated by VDR and its ligands. When a luciferase reporter plasmid containing the promoter fragment was transfected into HepG2 cells and the cells were treated with the active vitamin D metabolites calcidiol and calcitriol, the promoter was suppressed. This required that both VDR and RXR were overexpressed in the cells.

Phenobarbital is a drug that when given for long periods of time leads to an increased risk for fracture and in some cases osteomalacia, a disease connected to vitamin D deficiency. Phenobarbital stimulates the expression of CYP24, the enzyme responsible for vitamin D degradation. Transfection studies with the CYP2D25 promoter-luciferase plasmid showed that the promoter was suppressed by phenobarbital in the presence of the nuclear receptors PXR and CAR. Studies with porcine hepatocytes showed that the 25-hydroxylating capacity was diminished when treated with phenobarbital.

Vitamin D activating enzymes can be found in many tissues besides liver and kidney. Dermal fibroblasts are easy to retrieve and it would be valuable if vitamin D 25-hydroxylase activity in individuals could be studied using dermal fibroblasts. RT-PCR showed that human dermal fibroblasts express two 25-hydroxylases: CYP2R1 and CYP27A1. Incubation experiments showed that the fibroblasts were capable of measurable 25-hydroxylation. This indicates that dermal fibroblasts can be used to study 25-hydroxylase activity in human subjects.

The promoter of porcine vitamin D 25-hydroxylase was suppressed by vitamin D metabolites and phenobarbital in transfection experiments when

the necessary nuclear receptors were overexpressed. The question was whether human 25-hydroxylases could be regulated by these substances in whole cells. Two different cell lines were used, their 25-hydroxylase expression pattern was determined and the effect of calcitriol, phenobarbital and efavirenz on both mRNA level and 25-hydroxylating capacity. Provided that the required nuclear receptors were expressed, the substances suppressed the expression of 25-hydroxylase mRNA. The substances also had an effect on the enzyme activity.

In conclusion, the results show that vitamin D 25-hydroxylases can be regulated by both endogenous and xenobiotic compounds. Thus the case for metabolic regulation of the vitamin D 25-hydroxylases is strengthened.

## Svensk sammanfattning / Summary in Swedish

Vitamin D är ett fettlösligt vitamin, som har många uppgifter i kroppen. Det behövs för att upprätthålla kalciumbalansen i blodet, vilket är viktigt bl.a annat för att musklerna skall kunna arbeta. Kalcium är också en viktig beståndsdel i vårt skelett och det är det ämne som gör skelettet hårt. D-vitaminbrist leder till skelettsjukdomar som rakit och benskörhet.

Vitamin Ds verkan på skelettet har varit känt länge. För ett par årtionden sedan visade det sig att vitamin D påverkar även vår generella hälsa. Personer med låga halter vitamin D drabbas i högre utsträckning än andra av bröst-, tjocktarms- och prostatacancer. Även autoimmuna sjukdomar som MS och reumatoid artrit är vanligare och av mer allvarlig grad hos personer med låga vitamin D halter i blodet. Det har visat sig att vitamin D påverkar cellers sätt att växa och att det även påverkar våra immunceller så att de inte angriper kroppens egna celler.

Människor kan få vitamin D i sig på två olika sätt, antingen via födan eller genom att tillverka det själv. Vitamin D-rik mat är ovanlig; bra källor för vitamin D är bl.a. fet fisk (som t.ex. lax och makrill) och äggula. Det är svårt att upprätthålla tillräckliga vitamin D-nivåer enbart genom att äta mångsidigt. Därför är kroppens egen produktion väldigt viktig. Vitamin D bildas i huden då den utsätts för solljus och speciellt dess ultraviolettera strålar.

Det räcker inte att enbart få i sig tillräckliga mängder vitamin D. För att vitamin D skall kunna ha effekt, måste det omvandlas av kroppen till aktivare former. Personer som inte kan aktivera vitamin D får symptom på D-vitaminbrist. Aktiveringen av vitamin D sker i två steg. Först omvandlas vitamin D i levern av enzymer som kallas 25-hydroxylaser till kalcidiol, som sedan i njurarna av 1-hydroxylaset (som även kallas CYP27B1) omvandlas till kalcitriol. Kalcitriol är det aktiva D-vitaminet och det är ett mycket potent ämne. Det är viktigt att inte ha för höga halter av det och därför är det andra aktiveringssteget starkt styrt av blodets kalciumhalt och mängden kalcitriol. När mängden kalcium och kalcitriol i blodet är tillräckligt hög, slutar aktiveringen av vitamin D.

Det första aktiveringssteget är däremot en rätt okänd process. Den sker främst i levern, men vilka enzym 25-hydroxylaserna är har inte varit känt. Hos människa finns minst fyra olika enzymer som klarar av att utföra det första aktiveringssteget: CYP27A1, som finns mitokondrierna, och CYP2R1, CYP2J2 och CYP3A4, vilka finns i den celldel som kallas det endoplasmatiska retiklet d.v.s den mikrosomala fraktionen. Därför kallas de

tre sista för mikrosomala enzymer. Eftersom själva enzymet varit okänt har det naturligtvis varit svårt att bestämma vad det är som styr dess funktion.

För att börja studera styrningen av det första aktiveringssteget, använde vi oss av grisens 25-hydroxylas: CYP2D25. Grisen har bara ett mikrosomalt 25-hydroxylas. Det visade sig att promotorn, som styr i vilken utsträckning enzymet kommer att tillverkas, påverkades av kalcitriol. I närvaro av kalcitriol var promotorn mindre aktiv, vilket leder till att mindre enzym tillverkas vilket i sin tur leder till att aktiveringen av D-vitamin går långsammare. Vissa läkemedel ger upphov till skelettsjukdomar och D-vitaminbrist. Fenobarbital, som används mot epilepsi, är ett sådant läkemedel. Det visade sig att även fenobarbital hämmade promotorns funktion, vilket tyder på att fenobarbital kan försämra aktiveringen av vitamin D.

Vitamin D aktiverande enzymer finns inte bara i levern och njurarna, utan även i andra organ. Det skulle åtminstone teoretiskt vara möjligt att studera någons vitamin D aktiverande förmåga genom att studera celler från andra organ än lever och njure. Fibroblaster är ett slags stödjeceller som finns i många organ, bl.a. i huden. Det är förhållandevis enkelt att ta fibroblastprover från huden. Det visade sig att två vitamin D 25-hydroxylaser (CYP27A1 och CYP2R1) fanns i fibroblaster. Det visade sig även att fibroblasterna klarade av att 25-hydroxylera ett vitamin D-derivat, vilket innebär att de 25-hydroxylaser som fanns i fibroblasterna kunde fungera. Detta betyder att fibroblaster ur hudprover kan användas till att studera vitamin D-aktiveringen hos människa.

Sättet på vilket vitamin D-aktiveringen styrs i människa har inte varit känt tidigare. Efter att fibroblaster visade sig innehålla två av de enzymer som utför det första aktiveringssteget – CYP27A1 och CYP2R1 – blev det möjligt att studera hur dessa enzymer påverkas av olika ämnen. Det visade sig att även prostatacancer cellerna LNCaP innehöll två olika 25-hydroxylaser CYP2R1 och CYP2J2. Dessa celler behandlades sedan med ämnen som skulle kunna påverka vitamin D aktivering: kalcitriol och två olika läkemedel som kanske kan påverka vitamin D omsättning – fenobarbital och efavirenz. Mängden av CYP2R1 och CYP2J2 minskade i de celler som blivit behandlade med kalcitriol eller läkemedel. Cellernas vitamin D 25-hydroxyliserande förmåga minskade också efter behandling med kalcitriol eller läkemedel.

De studier som gjorts visar att 25-hydroxyleringen av D-vitamin påverkas av både aktivt vitamin D, d.v.s. kalcitriol, och läkemedel som är kända för att ge upphov till skelettnedbrytning.

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