Cytochrome P450 Enzymes in the Metabolism of Vitamin D₃

BY

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ABSTRACT


A cytochrome P450 enzyme in pig kidney that catalyzes the hydroxylations of vitamin D₃ and C₂₇-sterols was cloned. DNA sequence analysis of the cDNA revealed that the enzyme belongs to the CYP27 family. The recombinant kidney CYP27A enzyme catalyzed the 25-hydroxylation of vitamin D₃ and the 27-oxygenation of C₂₇-sterols. It was shown that human embryonic kidney cells express CYP27A mRNA and are able to catalyze the same reactions. Microsomal vitamin D₃ 25-hydroxylase (CYP2D25), purified from pig liver, converted vitamin D₃ into 25-hydroxyvitamin D₃ in substrate concentrations which are within the physiological range. The enzyme also converted tolterodine, a substrate for CYP2D6, into its 5-hydroxymethyl metabolite. RT-PCR experiments revealed that CYP2D25 mRNA is expressed not only in liver and kidney but also in other organs. Experiments with human liver microsomes and recombinant human CYP2D6 indicate that the microsomal 25-hydroxylation of vitamin D₃ in human liver is catalyzed by an enzyme different from CYP2D6. Five residues in SRS-3 of CYP2D25 were simultaneously mutated to the equivalent residues in CYP2D6, an enzyme not active in 25-hydroxylation. Both wild-type and mutated CYP2D25 were expressed in the Saccharomyces cerevisiae W(R) strain. The 25-hydroxylase activity of recombinant mutant CYP2D25 was completely lost whereas the activity toward tolterodine remained unaffected. These results indicate that residues in SRS-3 of CYP2D25 are important determinants for its function in vitamin D₃ metabolism.

A cDNA homologous with the hepatic CYP2D25 was cloned from pig kidney. The enzyme purified from pig kidney and the recombinant enzyme expressed in COS cells catalyzed 25-hydroxylation of vitamin D₃ and, in addition, 1α-hydroxylation of 25-hydroxyvitamin D₃. Immunohistochemistry experiments indicate that CYP2D25 is expressed almost exclusively in the cells of cortical proximal tubules. The expression of CYP2D25 in kidney, but not in liver, was much higher in the adult pig than in the newborn. The results imply that CYP2D25 has a biological role in kidney.

Results from experiments with inhibitors in primary cultures of porcine hepatocytes suggest that both CYP2D25 and CYP27A1 contribute to the total 25-hydroxylation in hepatocytes and are equally important in the bioactivation of vitamin D₃. Phenobarbital treatment increased the CYP2D25 mRNA levels but did not affect the CYP27A1 mRNA levels. The rate of 25-hydroxylation by phenobarbital-treated hepatocytes was markedly reduced. These results show that primary cultures of porcine hepatocytes are suitable as a model to study the metabolism of vitamin D₃ and the regulation of the CYP enzymes involved in the 25-hydroxylation of vitamin D₃.

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27-Oxygenation of C27-sterols and 25-hydroxylation of vitamin D3 in kidney: cloning, structure and expression of pig kidney CYP27A

II. Hosseinpour, F., and Wikvall, K.
Porcine microsomal vitamin D3 25-hydroxylase (CYP2D25). Catalytic properties, tissue distribution, and comparison with human CYP2D6

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The importance of residues in substrate recognition site 3 for the catalytic function of CYP2D25 (vitamin D 25-hydroxylase)

IV. Hosseinpour, F., Norlin, M., and Wikvall, K.
Kidney microsomal 25- and 1α-hydroxylase in vitamin D metabolism: catalytic properties, molecular cloning, cellular localization and expression during development.

V. Hosseinpour, F., and Wikvall, K.
25-Hydroxylation of vitamin D3 in primary cultures of porcine hepatocytes
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NOMENCLATURE

The following trivial names and abbreviations are used:

Cholecalciferol, vitamin D₃;
Ergocalciferol, vitamin D₂;
CYP, cytochrome P₄₅₀;
CTX, cerebrotendinous xanthomatosis;
PTH, parathyroid hormone;
IGF-1, insulin-like growth factor-1;
TNFα, tumor necrosis factor α;
IL-6, interleukin-6;
PMA, phorbol 12-myristate 13-acetate;
DMSO, dimethylsulfoxide;
HPLC, high performance liquid chromatography;
RT-PCR, reverse transcriptase-polymerase chain reaction;
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
SRS-3, substrate recognition site 3.
INTRODUCTION AND BACKGROUND

It is likely that the earliest life-forms that evolved in the fertile environment of the oceans over 1 billion years ago were photosynthesizing not only carbohydrates but also vitamin D (1). There is evidence that phytoplankton and zooplankton, which have existed on the earth for over 500 million years, produced relatively large quantities of provitamin D that, on exposure to sunlight, is converted to previtamin D. Studies have shown that most plants and animals have the capacity to produce previtamin D when exposed to sunlight (1,2). A bone-deforming disease commonly known as rickets has been traced back to the ancient history. In 1919, Huldschinsky proved the importance of ultraviolet light in treatment of rickets (3). Vitamin D2 or ergocalciferol from the irradiated plant sterol ergosterol was the first antirachitic substance which was identified by Askew et al in 1931(4). Continuing studies of antirachitic substances in fish liver and other foods led to the structural identification of vitamin D3 (cholecalciferol) by Windaus et al. (5). Mellanby (6,7) reported during the same period that rickets could be produced in dogs by feeding them oatmeal and he could cure the disease by adding cod-liver oil to their diet. For many years vitamin D2 was used as the major source of synthetic form of vitamin D for prevention and treatment of rickets in man. The reason was ease and lower cost of production of vitamin D2 compared with vitamin D3.

The role of some metabolite forms of vitamin D, and not vitamin D itself, in the physiological effect was revealed in 1952, when Carlsson observed a lag period of more than eight hours before one could observe an effect of vitamin D (8). It is now well understood that contrary to its original classification as a vitamin, the active form of vitamin D is in fact a steroid hormone whose biological actions are mediated primarily through its effects on gene transcription (9).

Bioactivation of vitamin D

The fat-soluble vitamin D occurs mainly in two forms: ergocalciferol (activated ergosterol, vitamin D2), found in irradiated yeast; and cholecalciferol (activated 7-dehydrocholesterol, vitamin D3), formed in human skin by exposure to sunlight (ultraviolet radiation) and found chiefly in fish, milk and egg yolk (Fig. 1).
Vitamin D is a prohormone which is metabolically activated to 1α,25-dihydroxyvitamin D₃, that act as hormone. In the skin, previtamin D₃ is synthesized photochemically from 7-dehydrocholesterol and is slowly isomerized to vitamin D₃, which is removed to the circulation by vitamin D-binding protein. In the liver, vitamin D₃ is converted to 25-hydroxyvitamin D₃, the major circulating form. In the kidneys, it is further hydroxylated to the active form, 1α,25-dihydroxyvitamin D₃ (1α,25-dihydroxycholecalciferol, calcitriol, vitamin D hormone) (Fig. 2).

Fig. 2. Photosynthesis and metabolism of Vitamin D₃

I, 7-Dehydrocholesterol; II, Previtamin D₃; III, Vitamin D₃; IV, Vitamin D₃; V, 25-Hydroxyvitamin D₃; VI, 1α,25-Dihydroxyvitamin D₃; VII, 24,25-Dihydroxyvitamin D₃

The main function of the vitamin D hormone is to increase calcium absorption from the intestine and promote normal bone formation and mineralization. One of the principal mechanisms by which 1α,25-dihydroxyvitamin D₃ carries out its biologic effects is through its interaction with a highly specific nuclear receptor protein known as the vitamin D receptor (VDR). It has been purified from intestinal tissue but has been found in several other tissues. Interaction between 1α,25-dihydroxyvitamin D₃ with the nuclear receptor results in the expression of the gene for the calcium-binding protein (calbindin) and several others proteins. The net result is that 1α,25-dihydroxyvitamin D₃ increase the efficiency of small intestine to absorb dietary calcium and phosphorus (9-13). The critical 1α-hydroxylation of 25-hydroxyvitamin D₃ is strongly stimulated by parathyroid hormone (PTH) and, independently of PTH, by hypophosphatemia (14-15).

In addition to its role in calcium and phosphorus homeostasis 1α,25-dihydroxyvitamin D₃ has effects on the regulation of growth and differentiation of certain specialized cell types.
Regulation of calcium and phosphorus homeostasis

The metabolism of calcium (Ca) and phosphate (PO₄) are intimately related. The regulation of both Ca and PO₄ balance is greatly influenced by circulating levels of parathyroid hormone (PTH), 1α,25-dihydroxyvitamin D₃, and to a lesser extent, the hormone calcitonin. Ca and inorganic PO₄ concentrations are also linked by their ability to chemically react with each other to form calcium phosphate. The calcium ion (Ca²⁺) is of fundamental importance to all biologic systems. Calcium, usually complexed to calmodulin, participate in numerous enzymatic reactions of metabolic importance. It is a vital component in the mechanisms of hormone secretion and hormone action. Calcium is intimately involved in neurotransmission, muscle contraction, mitosis and cell division, fertilization, and blood clotting. Ca²⁺ is the major cation in the crystalline structure of bone and teeth. For these reasons, it is vital that cells be bathed with fluid in which the calcium concentration is kept within narrow limits. The phosphate ion (PO₄⁻) is also of critical importance to all biologic systems and is the major intracellular anion. Phosphate is a component of all intermediates in glucose metabolism. It is part of the structure of all high-energy transfer compounds, such as adenosine triphosphate (ATP); of cofactors such as nicotinic acid dinucleotide; and of lipids, such as phosphatidylcholine. Phosphate functions as a covalent modifier of numerous enzymes. Phosphate also forms part of crystalline structure of bone. As indicated, bone is the major and dynamic reservoir for calcium and phosphate. Calcium deprivation stimulates PTH secretion. The PTH increases urinary phosphate excretion and thereby decreases plasma and renal cortical phosphate concentration. These stimulate the production of 1α,25-dihydroxyvitamin D₃. This hormone raises the plasma calcium concentration back toward normal by increasing the absorption of calcium from the gut. PTH also increases bone resorption and calcium reabsorption from the renal tubular urine. Phosphate deprivation directly stimulates 1α,25-dihydroxyvitamin D₃ production. The latter increases the flux of phosphate into plasma by stimulating bone resorption and phosphate absorption from the gut. 1α,25-Dihydroxyvitamin D₃ itself inhibits its own production as well as the production of parathyroid hormone (10, 11, 16-19) (Fig. 3).
Fig. 3. Control of the renal 1α-hydroxylase and 24-hydroxylase activities. Vitamin D, calcium and phosphate deficiency and secretion of parathyroid hormone stimulate 1α-hydroxylase activity and lead to increased formation of the active metabolite, 1,25-dihydroxyvitamin D. By contrast, 24-hydroxylase activity is stimulated by elevated calcium and phosphate concentrations, and by 1,25-dihydroxyvitamin D.

Cytochrome P450 (CYP) monooxygenase system

The enzymes required for the activation of vitamin D₃ to 1α,25-dihydroxyvitamin D₃ are cytochrome P450 enzymes. Cytochrome P450 was discovered in the 1950s. Initially, cytochrome P450 was a term used to identify a liver microsomal hemoprotein that, when reduced in a carbon monoxide atmosphere, showed a characteristic absorption maximum at 450 nm; thus, pigment 450 or P450 (20-22). These enzymes contain one heme group per molecule of protein and catalyze monooxygenations (together with NADPH and molecular oxygen) (23-26). Cytochromes P450 in eukaryotes exist both in membranes of the endoplasmic reticulum and the mitochondrial inner membrane and are about 500 amino acids long, whereas prokaryotic cytochrome P450 are free-floating in the cell and are about 400 amino acids long (27). The cytochrome P450 superfamily of heme-thiolate enzymes is known currently to comprise over 500 members which have been classified into families and subfamilies based on their amino acid sequence identity. Animals have currently 69 families (28-29). Members of the same gene family are defined as usually having ≤ 40% sequence identity with a P450 protein from any other family.
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Mammalian sequences within the same subfamily are > 55% identical. CYP is used to characterize the respective P450 as a hemoprotein. The first arabic number defines the gene family, the following letter the subfamily, and the second number the individual enzyme, e.g., CYP1A1 for cytochrome P4501A1. The functions of P450s are very broad. In mammals they are critical for xenobiotic/drug metabolism (e.g., drugs and toxic materials), blood hemostasis, cholesterol biosynthesis, steroidogenesis (e.g., glucocorticoids, cortisol, estrogens and androgens), arachidonic acid metabolism, bile acid biosynthesis, and vitamin D₃ metabolism (30-33). Some cytochrome P450 enzymes in mammals show age- and sex-related expression (34) and are sensitive to variations in the pathophysiological status (35) of the animal or individual (35-36). Different classes of compounds like phenobarbital, polycyclic aromatic hydrocarbons and ethanol can induce certain types of P450 enzymes (37). The liver contains most of the P450 enzymes in the body but other types of tissues such as kidney, lung, intestine, adrenal, brain and olfactory portion of the nose, contain cytochrome P450 enzymes (30, 38-41).

Mechanism of cytochrome P450-dependent hydroxylation

Cytochromes P450 are monooxygenases. This refers to the way molecular oxygen is incorporated into the product. In the hydroxylation, one atom of oxygen is added to the substrate and the other contributes to forming a water molecule. This process requires the donation of two electrons sequentially from NADPH and an electron donor. The donor is different depending on the location of the P450. In the endoplasmic reticulum (ER) membrane, NADPH-cytochrome P450 reductase is the usual electron donor, though cytochrome b₅ can also participate (43). In the mitochondria, ferredoxin (adrenodoxin) and ferredoxin reductase (adrenodoxin reductase) form a short electron transfer chain to supply the electrons from NADPH (44) (Fig. 4). The cytochromes P450 catalyze the mono-oxygenation of a variety of organic molecules in the following multistep mechanism.

\[
\begin{align*}
\text{Fe}^{3+} + \text{RH} & \rightarrow \text{Fe}^{2+} \text{RH} \quad (1) \\
\text{Fe}^{3+} + e^- & \rightarrow \text{Fe}^{2+} \text{RH} \quad (2) \\
\text{Fe}^{2+} + \text{RH} + \text{O}_2 & \rightarrow \text{Fe}^{3+} \text{RH–}_2 \text{O}_2 \quad (3) \\
\text{Fe}^{2+} + \text{RH–}_2 \text{O}_2 + e^- & \rightarrow \text{Fe}^{3+} + \text{H}_2\text{O} + \text{ROH} \quad (4)
\end{align*}
\]
First, the substrate (RH) binds over the surface of the single heme group directly adjacent to the O$_2$-binding site (step 1). Next, the heme iron is reduced from the ferric (Fe$^{3+}$) to the ferrous (Fe$^{2+}$) state (step 2), and O$_2$ binds to the heme iron (step 3). Finally, a second electron is transferred to the P450, resulting in O – O bond cleavage and substrate hydroxylation (step 4). Electrons are delivered to the P450 by the flavin-containing reductase which catalyzes the oxidation of NADPH with the subsequent delivery of electrons from the reductase to the P450.

**Fig. 4. Mechanism of microsomal (A) and mitochondrial (B) cytochrome P450-dependent hydroxylations**

**Previous work on 25- hydroxylation of vitamin D$_3$**

In 1969, Ponchon and Deluca (45) demonstrated that vitamin D does not circulate for long in the blood stream but, instead, is immediately taken up by adipose tissue for storage or by liver for further metabolism. In humans, tissue storage of vitamin D can last for months or even years. Labeled vitamin D in plasma is cleared within 60 min from all sources by the liver. It takes three to four hours before the radioactivity appears in plasma again and this time primarily as 25-hydroxyvitamin D$_3$. Early data suggested that liver is the only significant site of 25-hydroxylation in vivo (46). There were, however also reports of intestinal and renal extracts containing this activity (47). Research, focused on purification of the major hepatic enzyme activity. Over the years, there has been some controversy over whether 25-hydroxylation is carried out by one enzyme or two and whether this cytochrome P450-based enzyme is found in the microsomal or mitochondrial fraction of liver.

**Microsomal vitamin D$_3$ 25-hydroxylation**

In 1979, Madhok and Deluca (48) and Björkhem et al. (49) reported that a rat liver microsomal system requiring NADPH, molecular oxygen, a flavoprotein and a cytochrome P450 fraction was capable of 25-hydroxylation of vitamin D$_3$, but the rat cytochrome P450 responsible has never been cloned. Vitamin D 25-hydroxylase activity had been found also in partially purified cytochrome P450 from rabbit and bovine liver microsomes (50, 51). In 1983, Andersson et al. (52) purified an apparently homogeneous liver microsomal vitamin D$_3$ 25-hydroxylase from male
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Rats. The purified fraction was also active in 25-hydroxylation of 5ß-cholestan-3α,7α,12α-triol, an intermediate in the conversion of cholesterol to cholic acid, and in 16α-hydroxylation of testosterone, but did not 25-hydroxylate vitamin D$_2$. This enzyme was not found in liver microsomes of female rats. In 1988, Hayashi et al. (53) found 25-hydroxylase activity towards vitamin D$_3$ in liver microsomes of female rats, but the activity was lower than in male rats (about 20% of that in males). It was suggested that different enzymes were responsible for 25-hydroxylation of vitamin D$_3$ in male and female rats. 25-Hydroxyvitamin D$_3$ levels were shown to be the same in both male and female rats (54). Okuda and coworkers (55, 56) and Andersson and Jörnvall (57) reported that the 25-hydroxylating microsomal P450 from male rat liver had the same NH$_2$-terminal amino acid sequence as CYP2C11. This P450 is a constitutive P450 known to exist in liver microsomes of male rats but not in the females. Further studies with recombinant expressed CYP2C11 in Saccharomyces cerevisiae showed that the expressed protein was active in 16α-hydroxylation of testosterone but not in 25-hydroxylation of vitamin D$_3$. This result indicated that the 16α- and 25-hydroxylase activities originated from different gene products (58).

A microsomal cytochrome P450 active in 25-hydroxylation of vitamin D$_3$ was purified to apparent homogeneity from pig kidney by Postlind et al. (59). Proteins with the same apparent M$_r$ in kidney and liver microsomes from male and female pigs were recognized by a monoclonal antibody raised against the kidney microsomal vitamin D$_3$ 25-hydroxylase. In 1992-1994, Axén et al. (60-61) purified and characterized the pig liver microsomal 25-hydroxylase. The enzyme had an NH$_2$-terminal sequence different from that of CYP2C11 and was capable of 25-hydroxylation of both vitamin D$_2$ and D$_3$. There was no sex difference in the amount of this 25-hydroxylase in liver. In 1997, the porcine vitamin D$_3$ 25-hydroxylase was cloned and characterized by Postlind et al. (62). The enzyme was designated CYP2D25. Porcine CYP2D25 cDNA consists of 1652 bp, and the protein consists of 500 amino acids. The cDNA sequence indicated 83% identity with human CYP2D6, an enzyme involved in the metabolism of debrisoquine and other drugs.

Mitochondrial vitamin D$_3$ 25-hydroxylation

Cytochrome P450 with 25-hydroxylase activity has been demonstrated also in liver mitochondria of different species and the mitochondrial vitamin D$_3$ 25-hydroxylase has been purified to apparent homogeneity (63). Mitochondrial 25-hydroxylation of vitamin D$_3$ was demonstrated in solubilized rat liver mitochondria by Björkhem and Holmberg in 1978 (64). Further studies showed that partially purified cytochrome P450 from rat liver mitochondria catalyzed, besides the vitamin D$_3$ 25-hydroxylation, also 27-hydroxylation of 5ß-cholestan-3α,7α,12α-triol. It was reported that phenobarbital treatment of rats increased the mitochondrial 25-hydroxylation of vitamin D$_3$, whereas the 27-hydroxylation decreased (65). A rachitogenic diet caused a marked stimulation of 25-hydroxylation of vitamin D$_3$ activity but had little effect on the 27-hydroxylation of the C$_{27}$-sterol. It was consid-
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...that these differences in “inducibility” suggested the existence of two species of P450 in liver mitochondria, one or more involved in sterol 27-hydroxylation. Oftebro et al. (66) detected 25-hydroxylase activity towards vitamin D in liver mitochondria from patients with the inherited lipid storage disease cerebrotendinous xanthomatosis (CTX), while no 27-hydroxylase activity was detectable, suggesting at least two mitochondrial enzymes being involved in 25-hydroxylation of vitamin D₃.

In 1988, Dahlbäck and Wikvall (63) and Masumoto et al. (67) purified liver mitochondrial vitamin D₃ 25-hydroxylase to apparent homogeneity from rabbit and rat, respectively. Both preparations catalyzed also 27-hydroxylation and showed the same apparent Mᵦ, similar cytochrome P450 content and similar spectral properties. Dahlbäck prepared a monoclonal antibody against the purified rabbit liver enzyme. The antibody inhibited sterol 27-hydroxylation but did not inhibit vitamin D₃ 25-hydroxylation. She suggested that the two hydroxylase activities could be due to separate enzymes (68).

The NH₂-terminal sequence of the rabbit liver mitochondrial cytochrome P450 was determined (68). From the knowledge of this sequence, Andersson et al. (69) isolated a cDNA encoding the enzyme. The enzyme was designated CYP27. COS cells transfected with this cDNA 27-hydroxylated 5β-cholestane-3α,7α,12α-triol. Dahlbäck showed that COS cells transfected with CYP27 cDNA also catalyzed 25-hydroxylation of vitamin D₃ (70).

Usui et al. (71) isolated a cDNA for the rat liver CYP27. COS cells transfected with the cDNA were able to catalyze both sterol 27-hydroxylation and vitamin D₃ 25-hydroxylation. In 1990, Cali and Russell (72) isolated a cDNA for the human liver CYP27.

In 1994, Axén et al. (73) reported that CYP27 of pig and human catalyzes not only 27-hydroxylation of C₂₇-sterols and 25-hydroxylation of vitamin D₃, but also 1α-hydroxylation of 25-hydroxyvitamin D₃. The 1α-hydroxylase activity was much lower compared to the other two activities. CYP27 mRNA is widely distributed, it is found not only in liver but also in e.g. kidney, lung, and duodenum (69).

In summary, the 25-hydroxylation of vitamin D₃ is catalyzed by cytochrome P450-dependent systems in both the mitochondrial and microsomal fractions of liver. The relative importance of the two 25-hydroxylation systems in vivo is not known. Saarem et al. (74) reported that only a mitochondrial form of the 25-hydroxylase is present in human liver and that human liver microsomes do not possess 25-hydroxylase activity. On this basis, some authors (64,74) have questioned the physiological significance of the microsomal enzyme. However, 25-hydroxylation by human liver microsomes was later reported by others (75). The physiological role of the mitochondrial CYP27A as a vitamin D₃ 25-hydroxylase has been questioned by some authors because of the finding that some patients with cerebrotendinous xanthomatosis, lacking CYP27A enzyme do not have markedly reduced circulating 25-hydroxyvitamin D₃ levels (76).

DeLuca et al. (77,78) suggested that the microsomal 25-hydroxylation of vitamin...
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D₃ is more important than the mitochondrial 25-hydroxylation under physiological conditions. This assumption was based on the reported $K_m$ values for microsomal and mitochondrial 25-hydroxylation, which indicated that the former is more specific in 25-hydroxylation reaction.

**Previous work on 1α-hydroxylation of 25-hydroxyvitamin D₃**

It was known that patients with severe renal failure had a resistance to vitamin D₃. In 1970, Fraser and Kodicek (79) reported that 25-hydroxyvitamin D₃ was converted to a biologically active metabolite by the homogenate of chicken kidney. This result indicated for the first time the relationship between the kidney and vitamin D metabolism. In 1972, Semmler et al. (80) synthesized 1α,25-dihydroxyvitamin D₃ chemically and showed that it was identical with the biologically formed active compound. This metabolite showed 10 times more activity on a molar basis than vitamin D in healing rickets in rats, stimulating mobilization of calcium from bone, and increasing intestinal calcium reabsorption (13,14). In 1974, Ghazarian et al. (81) demonstrated 1α-hydroxylation of 25-hydroxyvitamin D₃ in a reconstituted system containing a solubilized mitochondrial cytochrome P450 fraction from chicken kidney and adrenodoxin and adrenodoxin reductase from bovine adrenals. In 1978, Saarem et al. (82) reported that the 1α-hydroxylase system in mammals is similar to that found in chicken kidney mitochondria. Several laboratories tried to purified a specific mitochondrial 1α-hydroxylase from kidney but so far without success (11,40,83). In 1994, Axén et al. (73) reported that mitochondrial CYP27A was able to 1α-hydroxylate 25-hydroxyvitamin D₃ to some extent. Because CYP27A is expressed also in kidney it was suggested that CYP27A could be a renal 1α-hydroxylase (84). In the second half of 1997, four independent groups using different approaches reported the cloning of a mouse, rat and human kidney 25-hydroxyvitamin D 1α-hydroxylase (85-88). A cDNA for human keratinocyte 1α-hydroxylase was also isolated (89). The cDNA sequences showed a high degree of identity and similarity to CYP27A and the 1α-hydroxylase was referred to as CYP27B (87). CYP27B is expressed predominantly in the kidney and at lower levels in lung and pancreas (88). The detected expression levels of keratinocyte 1α-hydroxylase mRNA in human adult kidney and fetal kidney, brain, and testis was less than 5% of the level of its expression in cultured neonatal keratinocytes (89). In mouse and rat, CYP27B mRNA levels were decreased by treatment with 1α,25-dihydroxyvitamin D₃ and increased by treatment with parathyroid hormone (85-87,90). Treatment of rats with 1α,25-dihydroxyvitamin D₃ has also been shown to suppress the levels of mRNA for CYP27A in kidney (91).

In 1995, Axén (92) described a microsomal 25-hydroxyvitamin D₃ 1α-hydroxylating enzyme in pig kidney. The activity and immunodetectable levels of the renal microsomal 1α-hydroxylase were reduced in piglets suffering from pseudo vitamin D-deficiency rickets type I (93).
24- and 26-Hydroxylation

The discovery of 24,25-dihydroxyvitamin D₃ predated even the identification of 1α,25-dihydroxyvitamin D₃ (94). 24,25-Dihydroxyvitamin D₃ is the major circulating dihydroxy metabolite of vitamin D₃. The 24-hydroxylase is a mitochondrial cytochrome P450 enzyme (95). It has been cloned and is referred to as CYP24 (96,97). The kidney is considered to be the primary site for 24-hydroxylation but extrarenal formation of 24,25-dihydroxyvitamin D₃ has also been reported (98). The physiological role of 24,25-dihydroxyvitamin D₃ in calcium and bone metabolism is a matter of some controversy. Some authors have proposed that 24,25-dihydroxyvitamin D₃ production represents a means to inactivate circulating 25-hydroxyvitamin D₃ and thus regulate production of 1α,25-dihydroxyvitamin D₃. In this view, 24,25-dihydroxyvitamin D₃ is considered a catabolite of 25-hydroxyvitamin D₃. Some authors consider that it is important for mineralization of bone matrix as massive doses of 24,25-dihydroxyvitamin D₃ stimulate bone formation without inducing hypercalcemia (99-100).

26-Hydroxylation of 25-hydroxyvitamin D₃ has also been found in kidney homogenate from several animal species. In 1970, Suda et al. (101) reported the identification of 25,26-dihydroxyvitamin D₃ as a vitamin D₃ metabolite in pig plasma. In 1989, a mitochondrial 26-hydroxylating cytochrome P450 was partially purified from pig kidney by Postlind et al. (102). The 25,26-dihydroxyvitamin D₃ is apparently biologically inert and only participate in a catabolic pathway.

Bioactivation of vitamin D₂

Vitamin D₂ is the major naturally occurring form of the vitamin in plants. Shortly after discovery of vitamin D₃ and vitamin D₂, it seemed apparent that vitamin D₂ and vitamin D₃ had similar biological activity in most mammals. However, it was recognized that the response of chicks and other fowls to vitamin D were markedly dependent upon the nature of the side chain in the vitamin molecule. Vitamin D₂ differs from vitamin D₃ only in a methyl group in the 24 position and a double bond in the 22, 23 position. It was demonstrated at an early date that vitamin D₂ was considerably more effective in the chick as an antirachitic agent as is vitamin D₃ (103). New world monkeys, pigs and cows discriminated against vitamin D₂ in favor of vitamin D₃ (104). In contrast to this, rats discriminated against a cholecalciferol metabolite in favor of an ergocalciferol metabolite. However, the nature of discrimination against vitamin D₂ or D₃ was not known. It was not clear whether vitamin D₂ was rapidly degraded and excreted or whether it was not to act in the vitamin D-dependent systems. In 1988, Hoy et al. (105) reported that chicks discriminate against vitamin D₂ as a result of enhanced clearance of the vitamin D₂ metabolites 25-hydroxyvitamin D₂ and 1α,25-dihydroxyvitamin D₂. Vitamin D₂ undergoes generally the same metabolism pathway as vitamin D₃. In 1969, Suda et al. (106) isolated and identified 25-hydroxyvitamin D₂ from mammalian blood. The active form of vitamin D₂, 1α, 25-dihydroxyvitamin D₂, was isolated and identified by
Jones et al. in 1975 (107). Vitamin D$_2$ and vitamin D$_3$ are believed to be equally potent in humans. Both 1α-hydroxyvitamin D$_2$ and 1α-hydroxyvitamin D$_3$ are 25-hydroxylated by the liver into the corresponding active 1α,25-dihydroxylated sterols (76).
AIMS OF THE PRESENT INVESTIGATION

The aims of the present investigation were

→ to characterize the mitochondrial vitamin D₃ 25-hydroxylase in pig kidney

→ to study the catalytical properties and tissue distribution of pig microsomal vitamin D₃ 25-hydroxylase (CYP2D25)

→ to study amino acid residues in CYP2D25 of importance for vitamin D₃ 25-hydroxylation

→ to characterize the microsomal vitamin D₃ 25-hydroxylase in pig kidney

→ to study vitamin D₃ metabolism in pig primary hepatocytes
EXPERIMENTAL PROCEDURES

Materials

Unlabeled vitamin D₃, vitamin D₂, sulfaphenazole, troleandomycin, quinidine, cyclosporin A, 2-phenylcyclopropylamine were from Sigma and 25-hydroxyvitamin D₃ was obtained from Solvay (Duphar, the Netherlands). 1α-Hydroxyvitamin D₃ was generously donated by Leo Pharma (Copenhagen, Denmark). 25-Hydroxy[23,24(n)-³H]vitamin D₃ (100 Ci/mmol), [α-³²P]CTP (3000 Ci/mmol), the megaprime DNA labeling system, [guanidine-¹⁴C]debrisoquine and restriction endonucleases were purchased from Amersham Pharmacia Biotech. All primers were purchased from Interactiva. Unlabeled and ¹⁴C-labeled tolterodine were donated by Dr. Hans Postlid, Pharmacia & Upjohn (Stockholm, Sweden). The adrenodoxin expression vector, pBadX, was provided by Professor David Russell (Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). The pTrc99AP450c27 expression vector was provided by Dr. Irina Pikuleva (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN, USA). Human embryonic kidney 293 cells (ATCC CRL 1573) were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The expression vector V60 (pYeDP60) and Saccharomyces cerevisiae strain W(R) were kind gifts from Drs. Denis Pompon and Philip Urban, Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette. Remaining compounds were purchased from commercial sources. All chemicals were of analytical grade.

Animals

Pig liver, kidney, lung, heart, muscle, thymus, spleen, intestine, adrenal and brain and bovine adrenals were from the local slaughter house. Livers and kidneys from pigs aged from a few days to three months were obtained from the Funbo-Lövsta Research Centre, Department of Animal Breeding and Genetics, Swedish University of Animal Sciences, Ultuna. All male pigs were castrated except those five days old or younger.
Purification of pig liver and kidney microsomal vitamin D₃ 25-hydroxylase (CYP2D25)

Purification of pig microsomal vitamin D₃ 25-hydroxylase was performed as described by Axén et al. (60). The purification procedure is summarized below:

Pig liver microsomes
                                               ↓
Sodium cholate solubilization
                                               ↓
Polyethylene glycol precipitation
                                               ↓
Octylamine-Sepharose chromatography
                                               ↓
Hydroxylapatite chromatography
                                               ↓
High performance Q-Sepharose chromatography
                                               ↓
High performance S-Sepharose chromatography
                                               ↓
Hydroxylapatite chromatography

High performance S-Sepharose chromatography was carried out with a stepwise gradient with the following concentration of sodium acetate in the equilibration buffer: 0 mM, 25 mM and 150 mM. The highest activity and highest specific cytochrome P450 content per mg of protein was found in the 25 mM sodium acetate eluate.

Purification of microsomal cytochrome P450 extracts enriched in vitamin D₃ 25-hydroxylase from kidneys of pigs of varying ages

The procedure for the purification of pig kidney P450 extract was the same as described by Axén et al. (92). The purification procedure is summarized below:

Microsomes
                                               ↓
Sodium cholate solubilization
                                               ↓
Polyethylene glycol fractionation
                                               ↓
Octylamine-Sepharose chromatography
                                               ↓
Hydroxylapatite chromatography
Cytochrome P450 Enzymes in the Metabolism of Vitamin D3

Incubation procedures and analysis of incubation mixtures with vitamin D3, 1α-hydroxyvitamin D3, 25-hydroxyvitamin D3 and vitamin D2

Substrates were incubated at 37 °C for 10-60 min with 0.025-0.2 nmol of purified or partially purified cytochrome P450 from pig kidney and liver, NADPH-cytochrome P450 reductase or ferrodoxin and ferrodoxin reductase and NADPH in a final volume of 1 ml of 50 mM Tris acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Microsomes (1 mg) from insect cells transfected with human CYP2D6 (SUPERSOMES™) were incubated with substrates as described above but without addition of NADPH-cytochrome P450 reductase.

In the inhibition experiments various P450 inhibitors, dissolved in methanol or acetone, were added to the reaction mixtures together with vitamin D3 in order to study their effect on 25-hydroxylation. All incubations were terminated and extracted with 5 ml trichloroethane/methanol (2:1, v/v). The organic phases were dried with N₂ and dissolved in 100 µl mobile phase and submitted to straight and reverse phase HPLC. Mobile phase for straight phase HPLC for 25-hydroxyvitamin D₃ and 25-hydroxyvitamin D₂ analysis was hexane/propan-2-ol (49:1, v/v) and for 1α,25-dihydroxyvitamin D₃ analysis hexan/propan-2-ol (9:1, v/v). The mobile phases for reverse phase HPLC were methanol/water (23:2, v/v) and (17:3, v/v), respectively. Straight phase analysis was performed with a LiChrosorb Si 60 column and reverse phase analysis with a LiChrosorb RP-18. For details in retention times for 25-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃, see paper III. Incubations with radiolabeled debrisoquine, tolterodine and bufuralol were carried out and analyzed as described in papers II and III.

Cloning of the pig kidney CYP27A1

Pig kidney cDNA was prepared from kidney poly (A)⁺ RNA according to the manufacturer’s instructions (Invitrogen), ligated into λZAP II arms and packed to reveal the library. The pig kidney cDNA library was subjected to several rounds of screening with a ³²P-labeled rabbit liver CYP27A cDNA insert, subcloned into pBluescript SK- and sequence analysis. Isolation of the 5'-end of the CYP27A cDNA which included the mitochondrial leader sequence was performed using the 5'-RACE system according to the protocol supplied by manufacturer (Life Technologies).

Cloning of the pig kidney CYP2D25

A 1568 bp cDNA sequence was amplified from pig kidney RNA by using sense and antisense oligonucleotide primers designed for the hepatic CYP2D25 in a RT-PCR reaction. The amplified PCR product ligated in a pBluescript II KS plasmid and the full-length nucleotide sequence analysis was performed as described in paper IV.
Expression of pig kidney CYP27A1 and CYP2D25 cDNA in simian COS cells

COS cells were grown in Dulbecco’s Modified Eagle’s medium, supplemented with 10% fetal calf serum and antibiotics, on different size of culture dishes in a humidified incubator at 37°C in a atmosphere of 95% air and 5% CO₂. For CYP2D25 expression, cells were transfected by electroporation with pcDNA4/HisMax vector, containing cDNA encoding CYP2D25. COS cells electroporated without vector were used as negative control. Following transfection, the cells were cultured and unlabeled 1α-hydroxyvitamin D₃ and 25-hydroxyvitamin D₃, respectively, were added to the culture medium and incubated for 48 h. Following incubation, medium and cells were harvested separately. The cells were suspended in 50 mM Tris-acetate buffer and homogenized. Cells and medium were extracted with trichloroethane/methanol (2:1, v/v) and the organic phase was analyzed by straight phase and reverse phase HPLC for 25-and 1α-hydroxylated products as described above.

For CYP27A expression, pSVL vector harboring pig CYP27A cDNA together with pBadX plasmid encoding bovine adrenodoxin were transfected into COS-M6 cells by the DEAE-dextran method. The control cells were transfected with vector containing no insert.

The transfected cells were incubated with vitamin D₃, 1α-hydroxyvitamin D₃, 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, respectively. Product formation with vitamin D₃ and 1α-hydroxyvitamin D₃ was analyzed as described above. Incubations with 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol were extracted with acidified ether, and the organic phase was analyzed by TLC and radioactivity scanning (108).

Metabolism of vitamin D₃ and 5β-cholestane-3α,7α-diol by human embryonic kidney cells (293 cells)

Human embryonic kidney cells (293 cells) were grown and assayed for 25-hydroxylation of vitamin D₃ and 1α-hydroxyvitamin D₃ and 27-hydroxylation of 3H-labeled 5β-cholestane-3α,7α-diol by addition of the substrates, dissolved in dimethylsulfoxide (0.3% final concentration) as described above.

Expression of wild-type CYP2D25 and mutant CYP2D25 in yeast

The wild-type CYP2D25 and mutant CYP2D25 cDNA were subcloned into the yeast expression vector V60 (pYeDP60). The plasmids were transformed by the lithium acetate method into the Saccharomyces cerevisiae strain W(R), which overexpresses NADPH-cytochrome P450 reductase under a galactose-inducible promotor (109). Clones were selected by growing the yeast in adenine- and uracil-deficient medium. The yeast cells were first grown to high density with glucose as the main energy source, thereafter, galactose was added to induce expression. Glass beads were used to disrupt the cells and microsomes were isolated by centrifugation as described in paper III.
Cytochrome P450 Enzymes in the Metabolism of Vitamin D

Expression of human liver CYP27A1 in E. Coli

Glycerol stocks of pTrc27H transformed JM 105 cells (73) and pTrc99AP450c27 transformed DH5α cells (110) served as inoculum for expression experiments. For purification of CYP27A from pTrc27H transformed JM 105 cells, membranes were isolated from 1 L culture, solubilized by addition of 0.8% sodium cholate and centrifuged at 100,000 × g for 1 h. The supernatant was dialyzed and used as source of human liver CYP27A. Purification of CYP27A from pTrc99AP450c27 transformed DH5α cells was started from 1.5 L culture. Solubilization and centrifugation were carried out as above. Further purification was made using Octylamine-Sepharose and hydroxylapitite chromatographies. The final eluate from the hydroxylapatite column was dialyzed and used as source of human liver CYP27A.

Immunoblot experiments

Western blot experiments were carried out as described by Andersson and Jörnvall (57). The immunoreactive bands were visualized either by alkaline phosphatase detection or by enhanced chemiluminescence (ECL). The monoclonal and polyclonal antibodies against porcine CYP2D25, used in immunoblotting, were produced in this laboratory as described before (61).

Immunohistochemistry

Small pieces of pig kidney and liver were dissected and fixed in phosphate-buffered formaldehyde (4%), dehydrated and imbedded in paraffin (5 μm), sectioned and mounted on chrome alum coated glass slides. Endogenous peroxidase activity was blocked by PBS-T containing 1% H2O2. Nonspecific binding were also blocked and tissue section incubated with polyclonal antibodies directed against porcine hepatic CYP2D25. The sections were then incubated with biotinylated goat anti-rabbit IgG or rabbit anti-mouse IgG secondary antibody followed by incubation with avidin-biotin-horseradish peroxidase complex. Thereafter, they were developed using 3-amino-9-ethylcarbazole. Sections not reacted with primary antibody but otherwise treated as the others were used as negative controls. All sections were counterstained with Mayers hematoxylin.

Isolation of RNA and Northern blot analysis

Total RNA was isolated from adrenal, brain, heart, intestine, kidney, liver, lung, muscle, spleen, thymus and pig primary hepatocytes either with the Rneasy total RNA Midi isolation kit (Qiagen) or by single-step guanidine thiocyanate phenol method (111). Northern blot analysis was carried out as previously described (62). The 32P-labeled cDNA probes used were a 1.6 bp KpnI-XbaI fragment of pig liver microsomal CYP2D25 cDNA (62), a 1.7 kb CYP27A pig kidney cDNA fragment, a 1.8 kb CYP27A human liver cDNA (75), a 1.7 kb fragment of human β-actin cDNA, a 400 bp fragment of rat cyclophilin and a 1.0 kb human glyceraldehyde-3-phosphate dehydrogenase cDNA.
Isolation of genomic DNA and Southern blot analysis

Genomic DNA were isolated from pig kidney by TRIZOL Reagent™ (Life Technologies) according to the manufacturer’s instructions or purchased from Clontech. Southern blot analysis was carried out as described (71,112). Pig genomic DNA was digested with different restriction enzymes, and 10 µg of DNA from each digestion was electrophoresed in agarose, transferred to a nylon membrane, and probed with a 32P-labeled 1.7 kb pig kidney CYP27A cDNA or with a 32P-labeled 1.6 kb KpnI-Xbal fragment pig liver microsomal CYP2D25 cDNA.

Isolation of primary porcine hepatocytes

Primary hepatocytes were isolated from the liver of 3 months old male or female domestic pigs.

The isolation procedure included perfusion of one of the major blood vessels of a small piece of liver by three different perfusion solutions in three steps. At the first step the liver was perfused with a buffer solution containing EGTA. At the second step, perfusion was carried out by a buffer without EGTA. Collagenase type XI (0.05% w/v) was added to the third perfusion solution.

All perfusion solutions were prewarmed to 37 °C and each perfusion was carried out for 20 min.

The isolated primary hepatocytes were cultured in 60 mm Primaria™ culture dishes using Williams medium E, supplemented with 4% foetal calf serum for the first 2h. After 2h the serum supplemented medium was replaced by Williams serum-free medium.

The duplicate dishes of cultured primary hepatocytes were treated with 25-hydroxyvitamin D₃, 1α,25-dihydroxyvitamin D₃, PMA, phenobarbital, IGF-1, TNFα and IL-6 using dimethyl sulfoxide (DMSO) as carrier.

The control dishes of primary hepatocytes were also treated with DMSO. DMSO was also used as carrier in incubations of primary hepatocytes with vitamin D₃, 1α,hydroxyvitamin D₃, tolterodine and 7α-hydroxy-4-cholesten-3-one.

The RNA isolation, Northern blot analysis, extraction of incubation mixtures and analysis of product formation were carried out as described above.

Other methods

NADPH-cytochrome P450 reductase, ferrodoxin and ferrodoxin reductase were prepared as described previously (113,114). Assay of cytochrome P450, NADPH-cytochrome P450 reductase, ferrodoxin, ferrodoxin reductase and protein were determined as described by Omura and Sato (21), Yasukochi and Masters (113), Huang and Kimura (114), Chu and Kimura (115) and Lowry et al. (116), respectively. SDS-PAGE was performed according to Laemmli (117).
RESULTS AND DISCUSSION

Molecular cloning of pig kidney CYP27A1 (paper I)

The CYP27A mRNA is expressed in many tissues and CYP27A was suggested to have important metabolic roles in extrahepatic tissues (69,118). The 25-hydroxylation of vitamin D_3 and 27-hydroxylation of cholesterol in pig kidney had only been characterized by enzymatic analysis of partially purified fractions and by immunological methods (59,102,119). cDNA encoding extrahepatic CYP27A had not been isolated and the cloning of pig CYP27A had not been reported. Therefore, it was considered of interest to clone CYP27A cDNA in pig kidney in order to study the biological role of this enzyme in the kidney.

Screening of a pig kidney library with a 32P-labeled rabbit liver CYP27A cDNA, 5´-RACE PCR, restriction mapping and DNA sequencing resulted in the cloning of a 1848 bp cDNA which encodes the entire amino acid sequence of the pig kidney CYP27A protein. The deduced amino acid sequence included the first 36 amino acids (residues –36 to –1) which closely resembled a mitochondrial signal sequence and the conserved heme-liganded cysteine at residue 443. A polyadenylation signal was found in the 3´-untranslated region of the cDNA. Alignment of the amino acid sequence with the sequences of human, rabbit and rat CYP27A displayed, respectively, 83 %, 81 % and 77 % identity. Northern blot analysis with total RNA isolated from pig kidney and liver and human embryonic kidney cells showed that a CYP27A cDNA probe hybridized to mRNA of approximately 1.8 kb in size in all samples.

In order to compare sequences of CYP27A in pig kidney and liver, RT-PCR experiments were carried out with total RNA isolated from these tissues. The sequence analysis of a 833 bp amplified fragment from the cDNA of both kidney and liver showed that their nucleotide sequences are the same. These results together with the results of the Southern blot analysis of digested pig genomic DNA with 4 different restriction enzymes, strongly indicate that CYP27A in kidney and liver is encoded by a single gene in the pig.

25-hydroxylation of vitamin D_3 and 27-oxygenation of C_27-sterols by recombinantly expressed CYP27A1 in COS-M6 cells and by human embryonic kidney cells (paper I)

COS-M6 cells transfected with pSVL expression vector, harboring pig kidney CYP27A cDNA, catalyzed 25-hydroxylation of 1α-hydroxyvitamin D_3 at a rate of 105 pmol/mg protein/48 h. The transfected COS cells catalyzed 25-hydroxylation of vitamin D_3 at a rate of 32 pmol/mg protein/48 h. Recombinant pig kidney CYP27A expressed in COS cells catalyzed also the 27-hydroxylation of 5β-cholestan-3α, 7α-diol and 5β-cholestan-3α, 7α,12α-triol and the further oxidation of the 27-hydroxycholesteryl to the corresponding acids (Table 1).
To study whether the CYP27A in human kidney has the same properties as the pig kidney CYP27A, experiments with human embryonic kidney cells were performed. Human embryonic kidney 293 cells converted 5β-cholestane-3α, 7α-diol to the same 27-oxygenated products as the recombinantly expressed kidney CYP27A1. The human cells also catalyzed 25-hydroxylation of 1α-hydroxyvitamin D₃ at a rate of 350 pmol/mg protein/24 h and the 25-hydroxylation of vitamin D₃ at a rate of 220 pmol/mg protein/24 h (Table 2).

<table>
<thead>
<tr>
<th>Product formation (pmol/mg of protein)</th>
<th>pSVL-PK27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₃ 25-hydroxylation</td>
<td>32±8</td>
</tr>
<tr>
<td>1α-Hydroxyvitamin D₃ 25-hydroxylation</td>
<td>105±11</td>
</tr>
<tr>
<td>5β-Cholestane-3α,7α-diol 27-hydroxylation</td>
<td>700±81</td>
</tr>
<tr>
<td>5β-Cholestane-3α,7α,12α-triol 27-hydroxylation</td>
<td>5705±419</td>
</tr>
<tr>
<td>Oxidation to acid</td>
<td>1925±888</td>
</tr>
</tbody>
</table>

Table 1. Hydroxylase activities in COS-M6 cells transfected with pig kidney CYP27A1 cDNA

<table>
<thead>
<tr>
<th>Product formation (pmol/mg of protein)</th>
<th>Reaction measured (24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₃ 25-hydroxylation</td>
<td>220±39</td>
</tr>
<tr>
<td>1α-Hydroxyvitamin D₃ 25-hydroxylation</td>
<td>350±130</td>
</tr>
<tr>
<td>5β-Cholestane-3α,7α-diol 27-hydroxylation</td>
<td>2000±644</td>
</tr>
</tbody>
</table>

Table 2. Endogenous hydroxylase activities in human embryonic kidney cells

There are conflicting reports about the role of hepatic CYP27A as a physiological vitamin D₃ 25-hydroxylase. However, the results of a recent study on vitamin D₃ hydroxylation in piglets suffering from rickets suggest that CYP27A is important for 25-hydroxyvitamin D₃ production in the pig (93). Studies with hepatectomized rats and patients with biliary cirrhosis, indicate that liver is not the sole site for the 25-hydroxylation of vitamin D₃ (46,120). Measurement of the concentration of vitamin D₃ in various tissues have shown that vitamin D₃ is present in kidney in almost the same concentration as that found in liver (121). The present study demonstrates the presence in kidney of mitochondrial CYP27A that has the capacity to convert vitamin D₃ into 25-hydroxyvitamin D₃. This finding together with results on microsomal 25-hydroxlation of vitamin D₃ by CYP2D25 in pig kidney (see paper IV).
support a role for the kidney in the production of 25-hydroxyvitamin D₃. Since the kidney is the major site for the 1α-hydroxylation that convert 25-hydroxyvitamin D₃ into 1α,25-dihydroxyvitamin D₃, the kidney would then be capable of the complete conversion of vitamin D₃ into its hormonal form.

The recombinant kidney CYP27A enzyme and human embryonic kidney cells, which expressed CYP27A, also carried out the 27-hydroxylation of C₂₇-sterols and the further oxidation into C₂₇-acids. These findings, together with previous work describing partially purified 27-hydroxylase from kidney, indicate that the kidney may play a hitherto unrecognized role in the metabolism of C₂₇-sterols.

Biochemical characterization of porcine microsomal vitamin D₃ 25-hydroxylase (CYP2D25) in pig liver and kidney (papers II and IV)

The catalytic properties of microsomal vitamin D₃ 25-hydroxylase purified from liver and kidney were investigated in papers II and IV. CYP2D25 purified from liver catalyzed 25-hydroxylation of 1α-hydroxyvitamin D₃ (760 pmol/nmol/min), vitamin D₃ (200 pmol/nmol/min) and vitamin D₂ (110 pmol/nmol/min). Previous studies indicated that $K_m$ values for microsomal and mitochondrial 25-hydroxylases purified from pig and rat liver were very high, about 5-10 µM (63,61,52,56). In paper II, it was investigated if microsomal CYP2D25 in pig liver is capable to metabolize vitamin D₃ in physiological concentrations. The experiment with nanomolar concentrations of vitamin D₃ showed that CYP2D25 was able to 25-hydroxylate vitamin D₃ in a concentration range of 65-500 nM. The apparent $K_m$ value for 25-hydroxylation by CYP2D25 was about 0.1 µM. The concentration of vitamin D₃ in liver has been estimated to be about 0.1 µM (121).

The inhibition studies with various known cytochrome P450 inhibitors were carried out to investigate the effect of these inhibitors on the microsomal 25-hydroxylation of vitamin D₃. The results revealed that quinidine which is a potent inhibitor for CYP2D6, inhibited the CYP2D25-dependent 25-hydroxylation of vitamin D₃ only by 50% in a concentration of inhibitor up to 125 µM. Cyclosporin A (30 µM), which is considered as a specific inhibitor of mitochondrial CYP27A (122) inhibited the microsomal CYP2D25-dependent 25-hydroxylation of vitamin D₃ by 45%. The 25-hydroxylation by CYP27A was inhibited by 60%. Tolterodine which was shown to be a substrate for CYP2D25 (see paper II) inhibited the microsomal 25-hydroxlation of vitamin D₃ by more than 80% at a 50 µM concentration but had no effect on the mitochondrial CYP27A-dependent 25-hydroxylation of vitamin D₃.

The primary structure of CYP2D25 shows 77% identity with that of human CYP2D6. Several experiments were carried out in order to compare catalytical properties and immunological discrepancy between CYP2D25 and CYP2D6. Tolterodine a known substrate for CYP2D6, was incubated with CYP2D25 and was found to be efficiently converted to its 5-hydroxymethyl metabolite. Debrisoquine, another substrate for CYP2D6, could not be metabolized in an efficient way by CYP2D25.
To study the immunological similarity between CYP2D6 and CYP2D25, Western blot experiments were carried out using antibodies against CYP2D25 or CYP2D6. Purified CYP2D25 and human liver microsomal protein from different donors were subjected to reactions with respective antibodies. The antibody against CYP2D6 did not react with purified CYP2D25 protein while it recognized protein in the human liver microsomal samples containing CYP2D6.

In order to investigate the possible correlation between the 25-hydroxylase activity and CYP2D6 in human liver microsomes, incubations were carried out with 1α-hydroxyvitamin D$_3$ as substrate and human liver microsomes from nine different donors. The microsome samples contained different amounts of CYP2D6. The 25-hydroxylase activity in different microsome samples showed no correlation with the amount of CYP2D6 in the microsomes. In another experiment, 1α-hydroxyvitamin D$_3$ was incubated with purified hepatic CYP2D25 or with recombinant CYP2D26, overexpressed in insect microsomes. There was no detectable 25-hydroxylation with recombinant CYP2D6 whereas a time-dependent increase in 25-hydroxylation was carried out by purified CYP2D25 (Fig. 5).

![Graph showing 25-hydroxylated product formation over time](image)

**Fig. 5.** Effect of time on the formation of 25-hydroxylated product in incubations with 1α-hydroxyvitamin D$_3$ and recombinantly expressed CYP2D6 (▲) and purified pig liver microsomal CYP2D25 (■).

Taken together, these results suggest that the microsomal 25-hydroxylation of vitamin D$_3$ in human liver is catalyzed by an enzyme different from CYP2D6. The differences in metabolic activity between CYP2D6 and CYP2D25 indicate that, despite the 77% identity in amino acid sequences, these two enzymes contain structural differences in their substrate recognition sites (SRS) (123). Indeed, the identification and comparison of different SRS-regions in CYP2D6 and CYP2D25 revealed such differences (Fig. 6).
Cytochrome P450 Enzymes in the Metabolism of Vitamin D₃

Renal microsomal vitamin D₃ 25-hydroxylase could be purified with the same procedure as described for hepatic microsomal vitamin D₃ hydroxylase. Incubations of the purified kidney enzyme with vitamin D₃, 1α-hydroxyvitamin D₃, tolterodine and different inhibitors were performed (paper IV). The purified renal enzyme acted in the same manner as the purified hepatic CYP2D25. Incubations with 25-hydroxyvitamin D₃ showed that the renal microsomal 25-hydroxylase can metabolize also 25-hydroxyvitamin D₃ to 1α,25-dihydroxyvitamin D₃ which is the most physiologically potent form of vitamin D₃.

Molecular cloning, sequence analysis and expression of pig kidney CYP2D25 (paper IV)

Using total RNA isolated from pig kidney and oligonucleotide primers designed for the hepatic CYP2D25 in RT-PCR experiments, a pig kidney cDNA was isolated. The sequence analysis indicated that the isolated cDNA is identical with the hepatic CYP2D25 cDNA. COS cells were used as hosts for harboring and expression of isolated pig kidney cDNA which was ligated into a pcDNA4/HisMax expression vector. The protein expressed by transfected COS cells was investigated.
by Western blot analysis and enzymatic activity assay. In the Western blot experiment, an antibody against hepatic CYP2D25 recognized a single protein band of the same apparent M as for purified hepatic CYP2D25. The catalytical activity analysis showed that COS cells harboring isolated pig kidney cDNA were able to carry out 25-hydroxylation of 1α-hydroxyvitamin D₃ and also 1α-hydroxylation of 25-hydroxyvitamin D₃.

**Tissue distribution of CYP2D25 and genomic Southern blot analysis (papers II and IV)**

Northern blot and RT-PCR experiments were used to study the expression of CYP2D25 in different pig tissues. Total RNA isolated from adrenal, brain, heart, intestine, kidney, liver, lung, muscle, spleen and thymus were examined. The results from the Northern blot experiments indicate the expression of CYP2D25 mRNA only in liver and kidney. However, the more sensitive RT-PCR technique revealed CYP2D25 mRNA expression in all tissues examined (Fig. 7).

**Fig. 7. RT-PCR analysis of CYP2D25 mRNA from various porcine tissues.**

Lane 1, intestine; lane 2, thymus; lane 3, spleen; lane 4, muscle; lane 5, heart; lane 6, brain; lane 7, lung; lane 8, adrenal; lane 9, liver; lane 10, kidney

Previous studies on mitochondrial vitamin D₃ metabolizing cytochrome P450 enzymes have suggested that these enzymes could be expressed differently in several regions and cell types in the kidney (124-127). In order to locate the CYP2D25 expression in pig kidney, immunohistochemical analysis was carried with kidney tissue and antibodies against hepatic CYP2D25. The results indicate that CYP2D25 is expressed almost exclusively in the proximal tubules of pig kidney.

Small pieces of pig liver were subjected to immunohistochemical experiments with antibodies directed against porcine hepatic CYP2D25. Immunostaining was clearly detected in hepatocytes throughout the liver. These results show that CYP2D25 appears to be expressed exclusively in hepatocytes of the liver.

Southern blot analysis with pig genomic DNA was carried out in order to investigate the number of CYP2D25 genes in the pig genome. Genomic DNA isolated from pig kidney was digested with different restriction enzymes which do cut or not cut the CYP2D25 cDNA. The digested DNA was subjected to Southern blot analysis. The result suggested the existence of only one CYP2D25 gene in the pig genome.
Developmental changes in the expression of CYP2D25 in pig kidney and liver (paper IV)

The developmental expression of members of the CYP2D subfamily has been studied in rat liver (128-129). The results indicate that CYP2D enzymes are expressed and regulated differently during development in rat liver. The only CYP2D isoform which has been found in humans, CYP2D6, appears not to be regulated (130).

There is only limited information whether vitamin D₃-metabolizing enzymes such as CYP24, CYP27A and CYP27B are subject to developmental regulation in kidney. In a recent report, the expression of CYP27B was found to be higher in kidneys of the adult mouse than in the fetus (131). Paper IV describes the investigation of developmental variation of the 25-hydroxylating and 1α-hydroxylating CYP2D25 in pig.

CYP2D25 levels in kidneys and livers from pigs of different ages

The 25-hydroxylation of 1α-hydroxyvitamin D₃ and 1α-hydroxylation of 25-hydroxyvitamin D₃ were measured in partially purified microsomal cytochrome P450 extract from kidneys of newborn (≤ five days) and six months old pigs. The catalytic activity assay showed that the rates of both 25-hydroxylation of 1α-hydroxyvitamin D₃ and 1α-hydroxylation of 25-hydroxyvitamin D₃ were about 10-fold lower in the newborn pigs in comparison with six months old pigs. The CYP2D25 mRNA level was not detectable in the kidneys of newborn and two weeks old piglets. CYP2D25 mRNA was expressed at the same levels in kidneys of three months and six months old pigs (Fig. 8). Western blotting with an antibody directed against CYP2D25 showed that the expression of CYP2D25 protein in kidney microsomes from pig of different ages followed the same pattern as described in the Northern blot experiments. In contrast, CYP2D25 mRNA in pig liver did not follow the age-dependent changes which was observed in kidneys. The results from Western and Northern blot experiments indicated no changes in levels of protein or mRNA expression in livers from pigs of different ages.

The previous studies with rat liver indicated that CYP2D enzymes are differently regulated during development (128-129). This is in contrast to the results on CYP2D25 in pig liver. The present study shows a tissue-specific developmental regulation of CYP2D25 which could suggest the involvement of hormonal factors in the regulation of the CYP2D25 gene.
Fig. 8. Northern blot analysis of RNA from kidneys and livers of newborn and 6 months old pigs with CYP2D25 and cyclophilin cDNA probes. Lanes 1 and 2, RNA from livers of 6 months old pigs; lanes 3 and 4, RNA from livers of newborn pigs; lanes 5 and 6, RNA from kidneys of 6 months old pigs; lanes 7 and 8, RNA from kidneys of newborn pigs.

Expression of wild-type CYP2D25 and mutant CYP2D25 in yeast (paper III)

In paper III, the substrate recognition sites in CYP2D25 and CYP2D6 have been identified and compared. The comparison revealed that the largest differences in sequence exist in the SRS-3 region. Therefore the SRS-3 region in CYP2D25 was subjected to site-directed mutagenesis as shown in Fig. 9. In order to investigate the importance of residues in this substrate recognition site for the catalytic function of CYP2D25, the enzyme was expressed in yeast cells. The expression vector V60 (pYedP60) and Saccharomyces cerevisiae strain W(R), which overexpresses NADPH-cytochrome P450 reductase, were used to express CYP2D25. Both wild-type and mutated CYP2D25 cDNA, ligated into the expression vector were transfected in yeast cells and cultured. The isolated microsomes from cells transfected with wild-type and mutant CYP2D25 were subjected to analysis of the CO-difference spectrum and Western blot analysis. Both wild-type and mutant CYP2D25 were expressed at similar levels as detected with CO-difference spectrum and Western blot analysis.
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Fig. 9. Amino acids sequence in the SRS-3 region of wild-type CYP2D25, mutant CYP2D25, and CYP2D6. The region is 241A-250F in CYP2D6 (1, 4). The arrows indicate the amino acids which have been subjected to mutation.

Catalytical properties of wild-type and mutant CYP2D25 (paper III)

1α-Hydroxyvitamin D₃ was incubated with wild-type and mutant CYP2D25. The HPLC analysis showed that wild-type CYP2D25 was able to convert 1α-hydroxyvitamin D₃ to 1α,25-dihydroxyvitamin D₃. The rate of 25-hydroxylation was about 1.8 nmol/nmol/min. In contrast, incubation with mutant CYP2D25 did not lead to any detectable 1α,25-dihydroxyvitamin D₃ product formation. As mentioned before, the site-directed mutagenesis of SRS-3 in CYP2D25 resulted in a cDNA which was more homologous to CYP2D6 cDNA than wild-type CYP2D25 cDNA. It was considered of interest to measure the catalytical activity of these two types of CYP2D25 with tolterodine, which is a substrate for both CYP2D25 and CYP2D6. The results from incubation of wild-type and mutant CYP2D25 microsomes with tolterodine indicate clearly that both wild-type and mutant CYP2D25 were able to form the 5-hydroxymethyl metabolite efficiently.

The catalytical activity of CYP2D25 in bufuralol 1′-hydroxylation, which is generally used as model reaction for CYP2D6, was also studied (132). Both wild-type and mutant CYP2D25 were capable to carry out 1′-hydroxylation of bufuralol. The \( K_m \) value for bufuralol 1′-hydroxylation by wild-type and mutant CYP2D25 was 1.4 and 1.0, respectively. The lack of 25-hydroxylase activity in the mutant CYP2D25 indicate that one or several residues in the SRS-3 region are important for the function of CYP2D25 in vitamin D₃ metabolism.

Studies on the role of CYP2D25 and CYP27A1 in vitamin D₃ 25-hydroxylation by primary cultures of porcine hepatocytes (paper V)

The availability of inhibitors for CYP2D25 and CYP27A1, respectively, would provide a tool for studies on the roles of these enzymes in the hepatic 25-hydroxylation. It has previously been shown that tolterodine dose-dependently inhibits the 25-hydroxylation of vitamin D₃ by purified CYP2D25 but not the 25-hydroxylation by purified CYP27A1 (paper II). 7α-Hydroxy-4-cholesten-3-one, a substrate for CYP27A1 (108), was incubated with purified pig liver CYP2D25 and with recombinant human CYP27A1. The results showed that 7α-hydroxy-4-cholesten-3-one is a substrate only for CYP27A1 and not for CYP2D25. In a separate experiment, purified CYP2D25 or CYP27A1 was incubated with vitamin D₃ and various concentration of 7α-hydroxy-4-cholesten-3-one (0-50 µM). The results showed that 7α-hydroxy-4-cholesten-3-one had a concentration-dependent inhibitory effect on the rate of 25-hydroxylation by CYP27A1 but did not inhibit the 25-hydroxylation
by CYP2D25. The results indicate that tolterodine and 7α-hydroxy-4-cholesten-3-one would be suitable as selective inhibitors of mitochondrial and microsomal 25-hydroxylation in hepatocyte experiments.

In an attempt to study the relative contribution of CYP2D25 and CYP27A1 in the total 25-hydroxylation of vitamin D₃, the primary hepatocytes were incubated with vitamin D₃ in presence of the inhibitors tolterodine or 7α-hydroxy-4-cholesten-3-one. Both tolterodine and 7α-hydroxy-4-cholesten-3-one decreased the rate of 25-hydroxylation of vitamin D₃ in primary hepatocytes. Tolterodine, in concentrations of 4, 12 and 16 µM, decreased the rate of 25-hydroxylation to 70, 58 and 43%, respectively. 7α-Hydroxy-4-cholesten-3-one, in concentrations of 4 and 12 µM, decreased the rate of 25-hydroxylation to 83 and 60% compared with that by control hepatocytes incubated without addition of inhibitor. In other experiments, primary hepatocytes were incubated with 1α-hydroxyvitamin D₃ in presence of tolterodine or 7α-hydroxy-4-cholesten-3-one. After 12 h incubation, tolterodine decreased the rate of 25-hydroxylation to 38% of that by control hepatocytes without addition of tolterodine and 7α-hydroxy-4-cholesten-3-one decreased the rate of 25-hydroxylation to 21% of that by control hepatocytes. Thus, both tolterodine and 7α-hydroxy-4-cholesten-3-one inhibited the rate of 25-hydroxylation of vitamin D₃ to about the same extent in primary cultures of porcine hepatocytes. These results indicate the importance of both the microsomal and mitochondrial 25-hydroxylation in hepatic vitamin D metabolism.

Studies on the regulation of CYP2D25 and CYP27A1 (paper V)

The availability of inhibitors for CYP2D25 and CYP27A1, respectively, would provide a tool for studies on the roles of these enzymes in the hepatic 25-hydroxylation. It is generally considered that the hepatic 25-hydroxylation is not highly regulated. However, some previous reports have indicated that 25-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃ can suppress the 25-hydroxylase activity in liver (90,133). In the present experiments, the treatment of primary pig hepatocytes with different concentrations (25-500 µM) of 25-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃ for 6, 12, 24 and 48 h did not have any significant effect on the CYP27A1 and CYP2D25 mRNA levels as analyzed in Northern blot experiments. These results suggest that the hepatic 25-hydroxylation is not regulated by 25-hydroxyvitamin D₃ or 1α,25-dihydroxyvitamin D₃.

Phorbol 12-myristate 13-acetate (PMA) is known as a protein kinase C stimulator. The pig primary hepatocytes were treated with PMA for 6, 12, 24, and 48 h. Northern blot analysis showed that PMA downregulated the expression of CYP2D25 mRNA in a time-dependent fashion and the highest repression occurred at 48 h of treatment. CYP27A1 mRNA was also successively repressed with time by PMA up to 24 h of treatment. However, after 48 h of treatment the CYP27A1 mRNA level was the same as in the untreated control hepatocytes. The results indicate that protein kinase C may be involved in the regulation and activity of hepatic 25-hydroxylation.
Pig primary hepatocytes were also treated with phenobarbital, an anticonvulsant drug, which has been reported to influence vitamin D metabolism (134). The hepatocytes were treated for 6, 12, 24 and 48 h. Northern blot analysis showed that treatment with phenobarbital for 24 and 48 h resulted in a 3 and 4-fold increase in CYP2D25 mRNA level. CYP27A1 mRNA level did not change after 24 h of treatment whereas the CYP27A1 mRNA level decreased by nearly 60% after 48 h treatment with phenobarbital.

Hepatocytes were also treated with the cytokines IGF-1, TNFα, and IL-6. IGF-1 resulted in a 2-fold increase in CYP2D25 after 24 and 48 h. IL-6 increased the CYP2D25 mRNA level only slightly after 24 h treatment. Treatment with TNFα did not result in any changes in CYP2D25 and CYP27A1 mRNA levels (Fig. 10).

Fig. 10. Effects of phenobarbital, IGF-1, TNFα and IL-6 on the expression of CYP2D25 mRNA and CYP27A1 mRNA in cultured porcine hepatocytes. (A) 1, 2: untreated hepatocytes (24 h); 3, 4: phenobarbital treatment for 24 h; 5, 6: IGF treatment for 24 h; 7, 8: TNF treatment for 24 h; 9, 10: IL-6 treatment for 24 h. (B) hepatocytes treatment as described above but for 48 h. Hybridization signals were quantified by NIH Image (1.62) program. The results are given as percent of the band intensity for respective mRNA in untreated hepatocytes after normalization to the expression of GAPDH mRNA. CYP2D25 mRNA, 24 h treatment (A), CYP2D25 mRNA, 48 h treatment (B), CYP27A1 mRNA, 48 h treatment (B). The data are given as the means of three experiments ± S.D. 1, untreated hepatocytes; 2, phenobarbital treatment; 3, IGF-1 treatment; 4, TNFα treatment; 5, IL-6 treatment.
In a separate set of experiments the pig primary hepatocytes were incubated with 1-hydroxyvitamin D₃ in the presence of phenobarbital, PMA, IGF-I, and TNF for 48 h. TNF, PMA and phenobarbital inhibited the rate of 25-hydroxylation by 46, 70 and 89\%, respectively. The anticonvulsant drug phenobarbital is well-known generally for its upregulating effects on the expression of mRNA for certain cytochromes P450 (135). Long term administration of phenytoin to patients has been reported to result in osteomalacia and rickets (136-138). It has also been reported that phenobarbital inhibits the vitamin D₃ 25-hydroxylation in liver mitochondria and microsomes (139-140). The results in paper V show that phenobarbital upregulated CYP2D25 expression, downregulated CYP27A expression and markedly decreased the rate of 25-hydroxylation by the hepatocytes. It is tempting to suggest that phenobarbital inhibits the CYP2D25-mediated 25-hydroxylation of vitamin D₃ on the enzyme level, e. g. by competing with vitamin D₃.

In conclusion, the results of paper V show that primary cultures of pig hepatocytes are suitable as a model to study the metabolism of vitamin D₃ and the regulation of CYP enzymes involved in 25-hydroxylation of vitamin D₃.
SUMMARY AND CONCLUSIONS

Cytochrome P450 enzymes catalyzing hydroxylations in the metabolism and bio-activation of vitamin D₃ were studied.

A cytochrome P450 enzyme in pig kidney that catalyzes the hydroxylations of vitamin D₃ and C₂₇-sterols was cloned. DNA sequence analysis of the cDNA revealed that the enzyme belongs to the CYP27 family. The mature pig kidney CYP27A1 protein contains 498 amino acids and the first 36 amino acids have many hallmarks of a mitochondrial signal sequence. Simian COS cells transfected with the renal CYP27A cDNA catalyzed the 25-hydroxylation of vitamin D₃ and the 27-oxygenation of C₂₇-sterols. Furthermore human embryonic kidney cells were found to express CYP27A mRNA and catalyze 25-hydroxylation of vitamin D₃ and 27-oxygenation of C₂₇-sterols. These results suggest that this enzyme is involved in the renal metabolism of vitamin D₃ and that the kidney plays a role in the metabolism of cholesterol and other C₂₇-sterols.

Microsomal vitamin D₃ 25-hydroxylase (CYP2D25), purified from pig liver, converted vitamin D₃ into 25-hydroxyvitamin D₃ in substrate concentrations which are within the physiological range. The enzyme 25-hydroxylated vitamin D₃, 1α-hydroxyvitamin D₃, and vitamin D₂ and also converted tolterodine, a substrate for CYP2D6, into its 5-hydroxymethyl metabolite. The primary structure of the CYP2D25 shows 77% identity with that of human CYP2D6. Reverse transcription-polymerase chain reaction experiments revealed that CYP2D25 mRNA is expressed not only in liver and kidney but also in other organs. Experiments with human liver microsomes and recombinantly expressed CYP2D6 indicate that the microsonal 25-hydroxylation of vitamin D₃ in human liver is catalyzed by an enzyme different from CYP2D6.

The site-directed mutagenesis technique was used to study the role of substrate recognition site 3 (SRS-3) for the catalytic specificity of CYP2D25. Five residues in SRS-3 of CYP2D25 were simultaneously mutated to the equivalent residues in CYP2D6, an enzyme not active in 25-hydroxylation. Both wild-type and mutated CYP2D25 were expressed in the Saccharomyces cerevisiae W(R) strain. The 25-hydroxylase activity of recombinant mutant CYP2D25 was completely lost whereas the activity toward tolterodine remained unaffected. These results indicate that residues in SRS-3 of CYP2D25 are important determinants for its function in vitamin D₃ metabolism.

A cDNA encoding a protein of 500 amino acids was cloned from pig kidney. Both the DNA sequence and the deduced peptide sequence of the renal enzyme are homologous with those of the hepatic CYP2D25. The enzyme purified from pig kidney and the recombinant enzyme expressed in COS cells catalyzed 25-hydroxylation of vitamin D₃ and 1α-hydroxyvitamin D₃ and, in addition, 1α-hydroxylation of 25-hydroxyvitamin D₃. Immunohistochemistry experiments indicate that CYP2D25 is expressed almost exclusively in the cells of cortical proximal tubules. The expression of CYP2D25 in kidney, but not in liver, was much higher in the adult pig than in the newborn. These results imply that CYP2D25 has
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a biological role in kidney.

The regulation and relative roles of the CYP2D25 and CYP27A1 in 25-hydroxylation of vitamin D₃ were examined in primary cultures of porcine hepatocytes.

Results from experiments with inhibitors suggest that both CYP2D25 and CYP27A1 contribute to the total 25-hydroxylation in hepatocytes and are important in the bioactivation of vitamin D₃. Phorbol 12-myristate 13-acetate down-regulated the expression of both CYP2D25 and CYP27A1 as well as the 25-hydroxylase activity of the hepatocytes. Phenobarbital treatment increased the CYP2D25 mRNA levels but did not affect or decreased the CYP27A1 mRNA levels. The rate of 25-hydroxylation by phenobarbital-treated hepatocytes was markedly reduced. These results show that primary cultures of porcine hepatocytes are suitable as a model to study the metabolism of vitamin D₃ and the regulation of the CYP enzymes involved in the 25-hydroxylation of vitamin D₃.
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