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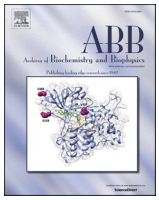

Review article

# Enzymatic activation in vitamin D signaling – Past, present and future

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ABSTRACT

Vitamin D signaling is important in regulating calcium homeostasis essential for bone health but also displays other functions in cells of several tissues. Disturbed vitamin D signaling is linked to a large number of diseases. The multiple cytochrome P450 (CYP) enzymes catalyzing the different hydroxylations in bioactivation of vitamin $D_3$ are crucial for vitamin D signaling and function. This review is focused on the progress achieved in identification of the bioactivating enzymes and their genes in production of 1α,25-dihydroxyvitamin $D_3$ and other active metabolites. Results obtained on species- and tissue-specific expression, catalytic reactions, substrate specificity, enzyme kinetics, and consequences of gene mutations are evaluated. Matters of incomplete understanding regarding the physiological roles of some vitamin D hydroxylases are critically discussed and the authors will give their view of the importance of each enzyme for vitamin D signaling. Roles of different vitamin D receptors and an alternative bioactivation pathway, leading to 20-hydroxylated vitamin $D_3$ metabolites, are also discussed. Considerable progress has been achieved in knowledge of the vitamin $D_3$ bioactivating enzymes. Nevertheless, several intriguing areas deserve further attention to understand the pleiotropic and diverse activities elicited by vitamin D signaling and the mechanisms of enzymatic activation necessary for vitamin D-induced responses.

## 1. Introduction

Vitamin D has a central role in bone health and the major and classical function is regulation of calcium and phosphate homeostasis [1]. Vitamin D deficiency causes rickets among children and also precipitates and exacerbates osteoporosis among adults and causes the painful bone disease osteomalacia. In addition, vitamin D also has other physiological roles [2–5]. Vitamin D occurs in two forms, vitamin $D_2$ (ergocalciferol) and vitamin $D_3$ (cholecalciferol) as shown in Fig. 1. Vitamin $D_2$ is present in plants and is only ingested with vegetable food or as a supplement. Vitamin $D_3$, which is the quantitatively most important form of vitamin D in the animal kingdom, is ingested with animal food, e.g. dairy products and fat fishes. However, only a small proportion of circulating vitamin $D_3$ is derived from the diet. The major part of vitamin $D_3$ is synthesized non-enzymatically in the skin (the epidermis) from 7-dehydrocholesterol on exposure to ultraviolet light (UVB) from the sun [6] (Fig. 2). The photons cleave the B-ring of the steroid skeleton producing pre-vitamin D which subsequently undergoes thermal isomerization to the seco-steroid vitamin $D_3$. Vitamin $D_3$ is then removed from the epidermis into the circulation by binding to the vitamin D binding protein (DBP). Vitamin $D_3$ itself is a biologically inert prohormone and must be metabolically activated by hydroxylations to achieve biological activity. Several hydroxylating CYP enzymes may participate in the bioactivation pathway (Fig. 3). Vitamin $D_3$ is initially hydroxylated to 25-hydroxyvitamin $D_3$ (calcidiol or calcifediol) and then into its hormonal form, 1α,25-dihydroxyvitamin $D_3$ (calcitriol). Vitamin $D_2$ is bioactivated in a similar way, although the hydroxylating enzymes may differ (Fig. 3). The activated hormone is a ligand for the cellular vitamin D receptor (VDR) in target tissues which will result in biological responses [3,4,7,8]. The highly efficient hormone 1α,25-dihydroxyvitamin $D_3$ has not only a function in regulating calcium homeostasis essential for bone health but also in modulation of cell proliferation and differentiation. Several other intriguing roles have been reported, such as regulation of the immune system, brain and fetal development, and hormone secretion. Other suggested roles for 1α,25-dihydroxyvitamin $D_3$ include functions in hair follicle cycling, blood pressure regulation,

*Abbreviations:* CTX, cerebrotendinous xanthomatosis; CYP, cytochrome P450; FGF23, fibroblast growth factor 23; MARRS, membrane-associated rapid response steroid-binding receptor; PDIA3, protein disulfide isomerase A3; PTH, parathyroid hormone; PVDR, pseudo-vitamin D deficiency rickets; RXR, retinoid X receptor; VDDR, vitamin D-dependent rickets; VDR, vitamin D receptor; VDRE, VDR-responsive elements.

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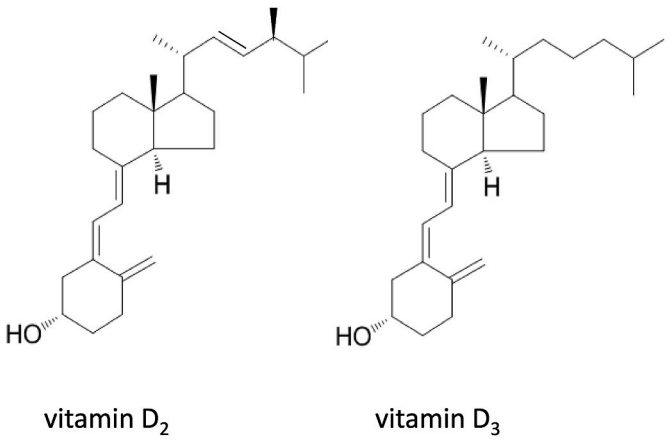


**Fig. 1. Vitamin $D_2$ (ergocalciferol) and $D_3$ (cholecalciferol).** Vitamin $D_3$ is synthesized in the skin and is also found in animal food (e.g. fatty fishes, dairy products). Vitamin $D_2$ is present in plants and is only ingested with vegetable food or as a supplement. Vitamin $D_2$ is metabolically activated in a similar way as vitamin $D_3$ although the enzymes may differ.

and mammary gland development [5,9–13]. Also, the enzymatically formed metabolites 25-hydroxyvitamin $D_3$ [14,15] and 24,25-dihydroxyvitamin $D_3$ [16] are ascribed various functions in vitamin D signaling pathways. Links between the functions and/or levels of 25-dihydroxyvitamin $D_3$ have been found for multiple diseases including development of cancer and neurodegenerative dysfunctions [2,10,11,17,18]. In addition to the well-established activation pathway involving 25- and 1-hydroxylations, an alternative pathway, starting with 20-hydroxylation of vitamin $D_3$, has been reported. The non-classical 20-hydroxylated vitamin $D_3$ metabolites are also ascribed functions in vitamin D signaling pathways [19]. For examples of reviews describing various aspects of vitamin D activation and functions, see [1–6,8–13,19–21].

The multiple cytochrome P450 enzymes catalyzing the different hydroxylations in bioactivation and metabolism of vitamin $D_3$ are crucial for vitamin D signaling and vitamin D function (Fig. 3). The present article aims to provide a more detailed overview on the progress in research on the different bioactivating vitamin D hydroxylases and their genes in production of 1α,25-dihydroxyvitamin $D_3$ and other active metabolites. The roles of reported enzymes are critically discussed and the authors will give their view of the importance of each enzyme for vitamin D signaling. A major section is devoted to the properties and roles of the multiple enzymes catalyzing 25-hydroxylation of vitamin D. In a separate section, reports on different receptors in vitamin D signaling are addressed.

## 2. Reactions in the production and metabolism of 1α,25-dihydroxyvitamin $D_3$

The circulating active 1α,25-dihydroxyvitamin $D_3$ hormone is produced by reactions that occur mainly in liver and kidney. The bioactivation is initiated by 25-hydroxylation in liver followed by 1α-hydroxylation in the proximal convoluted tubule of the kidney (Fig. 3). The formed 1α,25-dihydroxyvitamin $D_3$ hormone (also called calcitriol) is then transported through the circulation to its target organs where it interacts with receptor(s) and induces biological activities. 25-Hydroxyvitamin $D_3$ (calcidiol or calcifediol), produced by the first activating step, is the major circulating vitamin $D_3$ metabolite and is generally used as indicator of vitamin D status. The vitamin $D_3$ metabolites are transported bound to the vitamin D binding protein through the bloodstream.

The hormone 1α,25-dihydroxyvitamin $D_3$ is inactivated by catabolism via 24-hydroxylation in kidney and other tissues [22]. 25-Hydroxyvitamin $D_3$ is also metabolized by 24-hydroxylation (Fig. 3). CYP24A1 is the major 24-hydroxylase in kidney and most other tissues and cells. CYP3A4 catalyzes 24-hydroxylation preferentially in intestine and liver. Further metabolism of the 24-hydroxylated metabolites in several steps leads to formation of 1α,25-dihydroxyvitamin $D_3$-26,23-lactone or calcitroic acid which can be excreted in the bile [23].

CYP24A1 catalyzes all reactions in the formation of calcitroic acid and the 26,23-lactone [24–28]. Although 24-hydroxylation is generally considered important for elimination/excretion of the active vitamin D hormone, some studies indicate that 24-hydroxylated vitamin D metabolites also might induce cellular responses [16,29].

The circulating 1α,25-dihydroxyvitamin $D_3$ has mainly endocrine functions, e.g. in regulation of intestinal calcium absorption and maintenance of bone health. The biologically potent 1α,25-dihydroxyvitamin $D_3$ is produced in small quantities and there is an exact control mechanism for its formation and inactivation. The renal production and inactivation of 1α,25-dihydroxyvitamin $D_3$ is regulated by the need for calcium and phosphorus and involves transcriptional regulation of the 1α- and 24-hydroxylating enzymes. The most important regulators of the 1α- and 24-hydroxylases in the kidney are 1α,25-dihydroxyvitamin $D_3$ itself, parathyroid hormone and fibroblast growth factor-23 (FGF23).

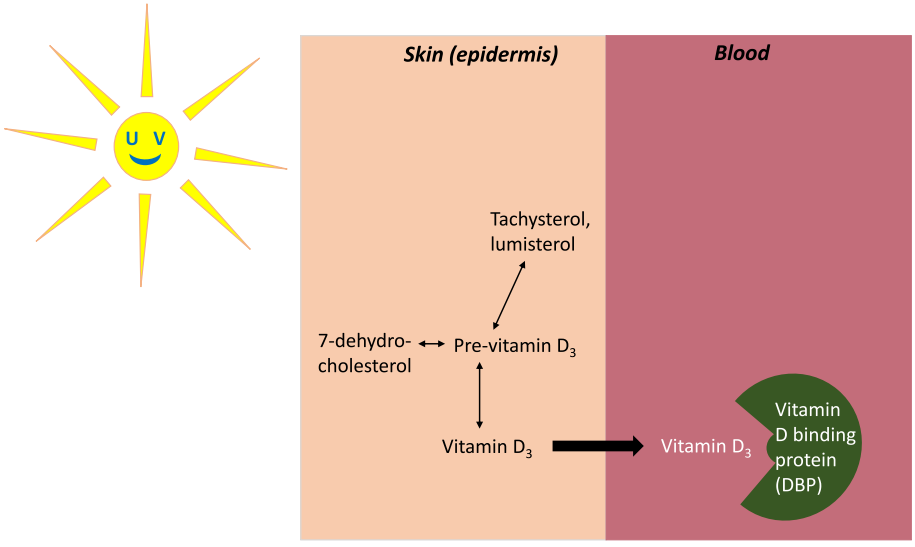


**Fig. 2. UVB-mediated synthesis of vitamin $D_3$ in the skin.** Vitamin $D_3$ is nonenzymatically formed from 7-dehydrocholesterol in the epidermis under the influence of UVB-radiation in sunlight.

FGF23 is produced in bone cells and is necessary for regulating the phosphate levels within the body (phosphate homeostasis) [20,30].

Even if the circulating endocrine 1α,25-dihydroxyvitamin D is mainly produced by reactions in liver and kidney, the hormone can also be formed and catabolized locally in various extrarenal tissues and cells where it functions in an autocrine or paracrine manner. Extrarenally produced 1α,25-dihydroxyvitamin $D_3$ has for example been reported to inhibit cell proliferation and stimulate cell differentiation in prostate, breast and colon cancers, to be involved in regulation of the immune system, and to have a role in brain development and function [9,18,31].

## 3. Genes and enzymes in the formation and inactivation of 1α,25-dihydroxyvitamin $D_3$

The important genes and enzymes required for the 25-, 1α- and 24-hydroxylations in bioactivation and metabolism of vitamin D belong to the cytochrome P450 superfamily. Cytochromes P450 (CYP) are heme proteins catalyzing monooxygenase reactions, i.e. insertion of one atom of atmospheric oxygen into the substrate resulting in hydroxylation. The vitamin D hydroxylases are located in mitochondria and also in the endoplasmic reticulum (microsomes). Fig. 4 shows the electron transport chains and protein components of the vitamin D hydroxylases and other cytochrome P450 enzymes. For a review describing cytochromes P450, see Ref. [32].

Projects on isolation and purification of the 25-, 1α-, and 24-hydroxylases and characterization of their catalytic properties and partial amino acid sequences were initiated in the 1980′s independently by the laboratories of Wikvall in Sweden [33–35] and Okuda in Japan [36]. For review, see Refs. [37,38]. Subsequently, progress was made in cloning of the vitamin D hydroxylases by several groups [38–45].

At least nine mammalian CYP450 enzymes have been identified as capable of being active in the formation and metabolism of 1α,25-dihydroxyvitamin $D_3$. These enzymes include CYP2C11, CYP2D25, CYP2J2, CYP2J3, CYP2R1, CYP3A4, CYP24A1, CYP27A1, and CYP27B1. Some of these are found in many species whereas others are species-specific enzymes. Among these, the 25-hydroxylases CYP27A1, CYP2R1, CYP3A4, CYP2J2, the 24-hydroxylases CYP24A1, CYP3A4 and the 1α-hydroxylase CYP27B1 are all present in humans. However, CYP2C11, CYP2J3, and CYP2D25 are species-specific 25-hydroxylases in rat and pig, respectively. CYP2C11 is a male-specific microsomal vitamin $D_3$ 25-hydroxylase in rat liver [46,47], The microsomal CYP2J3 is reported to be the principal 25-hydroxylase in the rat and is present in

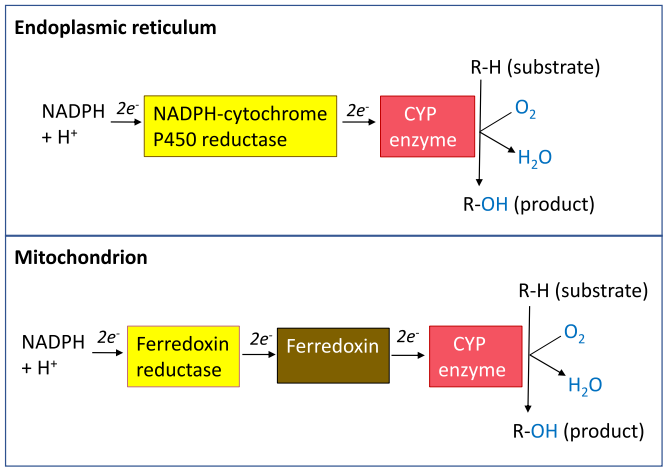


**Fig. 4. Electron transport chains and protein components of the vitamin D hydroxylases.** (A) In the endoplasmic reticulum, NADPH is oxidized by the flavoprotein NADPH-P450 reductase (also known as P450 oxidoreductase, POR) and the two electrons from NADPH are then transferred sequentially to the respective microsomal P450 (CYP2R1, CYP2D25, CYP2C11, CYP2J2, CYP2J3, CYP3A4 discussed in this review). (B) In the mitochondrion, NADPH is oxidized by the flavoprotein ferredoxin reductase, which transfers the electrons to the iron-sulfur protein ferredoxin. The two electrons from NADPH are then transferred to the respective mitochondrial P450 in the inner membrane (CYP27A1, CYP27B1, CYP24A1, CYP11A1 discussed in this review).

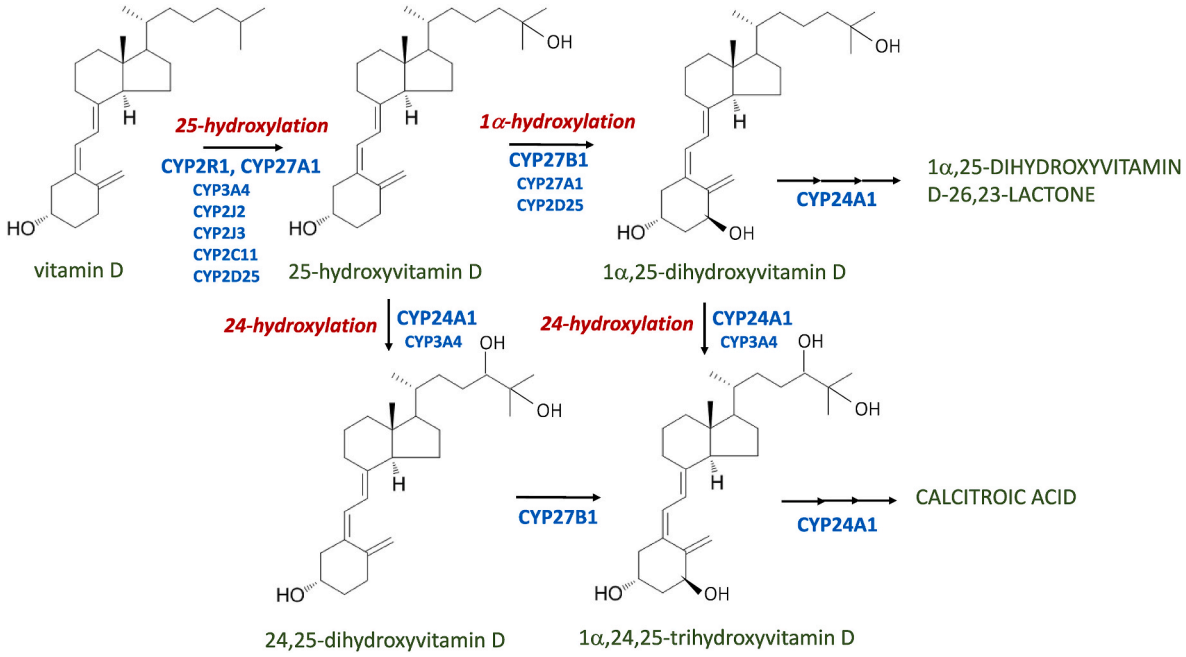


**Fig. 3. Reactions in the formation and inactivation of 1α,25-dihydroxyvitamin D.** The figure summarizes enzymatic formation and inactivation of 1α,25-dihydroxyvitamin D and is intended as an overview. Thus, properties such as species specificity, localization, affinity for vitamin $D_2$ or $D_3$ and physiological importance during various conditions may vary for the enzymes shown. 25-Hydroxylation: Some of the 25-hydroxylating CYP enzymes shown in the figure are species specific i.e. CYP2C11 (rat), CYPJ3 (rat) and CYP2D25 (pig). 24-Hydroxylation (inactivating): CYP24A1 is the major 24-hydroxylase in kidney and most other tissues and cells. CYP3A4 catalyzes 24-hydroxylation preferentially in intestine and liver. CYP24A1 catalyzes all reactions in the formation of calcitroic acid and the 26,23-lactone [24–28]. For more information, see text.

both sexes [48]. CYP2D25 is an efficient microsomal porcine vitamin D 25-hydroxylase with a $K_m$ in the physiological range of vitamin $D_3$, active towards both vitamin $D_2$ and vitamin $D_3$. It is expressed in pig liver and kidney. However, the corresponding CYP2D enzyme in humans, CYP2D6, does not show vitamin D 25-hydroxylase activity [49,50].

The information available on different mammalian vitamin D hydroxylases will be described in detail in sections 5 and 6 and is briefly summarized in Tables 1 and 2.

## 4. An alternative activation pathway producing non-classical vitamin D hydroxy metabolites

The general concept that vitamin $D_3$ is only activated through 25- and 1α-hydroxylations has been challenged. An interesting alternative pathway for activation of vitamin $D_3$ and $D_2$ in some tissues was first proposed by Estabrook and collaborators in 2003 [51] and has been further explored by Slominski and Tuckey and collaborators [19,52] This alternative activation pathway is initiated by the steroidogenic enzyme CYP11A1 and involves formation of non-classical vitamin D metabolites, such as 20-and 22-hydroxylated vitamin D derivatives (Fig. 5). The initial CYP11A1-mediated 20-, and 22-hydroxylations of the side chain are followed by further metabolism via 1-, 24-, 25-, or 26-hydroxylations catalyzed by the regular vitamin D enzymes. The metabolites in these pathways are reported to be biologically active. Some of them inhibit cell proliferation but are lacking calcemic activity making them interesting candidates for anti-cancer treatment [19].

The mitochondrial CYP11A1, known as cholesterol side-chain cleavage enzyme (P450scc), is required in the production of $C_{19}$- and $C_{21}$-steroids such as sex hormones and glucocorticoid hormones, for

**Table 1**
Vitamin D 25-Hydroxylating cytochrome P450 enzymes.

| | Species | Tissues/Cells | Catalytic reactions and substrate specificity | Apparent $K_m$ for vitamin $D_3$ (recombinant enzyme) | Effects of disease | Mouse models (gene knock out) |
|---|---|---|---|---|---|---|
| CYP2R1 (see also section 5.2, 5.5, 5.6 and 5.7) | Many species including mouse and human | Primarily in liver and testis | **25-hydroxylation**: Vitamin $D_3$, vitamin $D_2$, 20(OH)-vitamin $D_3$ | 0.45–4.4 μM | Mutations found in rare forms of vitamin D-dependent rickets with low levels of 25(OH)-vitamin $D_3$. | Reduced, but not abolished, circulating 25(OH)-vitamin $D_3$ levels. KO mice were healthy. |
| **CYP27A1** (see also section 5.1, 5.5, 5.6 and 5.7) | Many species e.g. rabbit, rat, mouse, pig, human | Liver and most extrahepatic tissues and cells | **25-hydroxylation**: Vitamin $D_3$ (but not vitamin $D_2$), 1α(OH)-vitamin $D_3$, 20(OH)-vitamin $D_3$ **Hydroxylations of vitamin D metabolites in other positions:** Vitamin $D_2$ (C-24), 25 (OH)-vitamin $D_3$, (C-1α). **27-hydroxylation in bile acid biosynthesis:** cholesterol and other $C_{27}$-sterols | 3.2 μM | Mutations found in patients with CTX[a], a rare cholesterol-related disease with neurological symptoms and (in some patients) decreased bone mass, increased risk of fractures and low serum 25 (OH)-vitamin $D_3$. | Severe disturbances in cholesterol metabolism but different symptoms than in humans. No rickets or reduction of 25(OH)-vitamin $D_3$ levels. |
| **CYP3A4** (see also section 5.3 and 5.5) | Humans | Mainly liver | **25-hydroxylation**: Vitamin $D_2$, 1α(OH)-vitamin $D_2$, 1α(OH)-vitamin $D_3$ Vitamin $D_3$ appears not to be a substrate. CYP3A4 has also been ascribed a role as a **24-hydroxylase** in vitamin D metabolism | | | |
| **CYP2J2** (see also section 5.4 and 5.5) | Humans | Primarily in heart | **25-hydroxylation:** Vitamin $D_2$ > vitamin $D_3$ (low activities) **Other activities:** Major biological role in metabolism of arachidonic acid | 7.7 μM | | |
| **CYP2D25** (see also section 3) | Pig | Liver, kidney | **25-hydroxylation:** Vitamin $D_3$, vitamin $D_2$, 1α(OH)-vitamin $D_3$,1α (OH)-vitamin $D_2$ **1α-hydroxylation:** 25(OH)-vitamin $D_3$ | 0.1 μM | Decreased levels in kidneys, but not in livers, of rachitic piglets with PVDRI[b] | |
| **CYP2C11** (see also section 3) | Rat (male-specific) | Liver | Vitamin $D_3$, 1α(OH)-vitamin $D_3$, (not vitamin $D_2$) **Other activities:** Hydroxylation of testosterone | 5 μM | | |
| **CYP2J3** (see also section 3) | Rat (both males and females) | Liver | **25-hydroxylation:** Vitamin $D_3$ > vitamin $D_2$ | 0.8 μM | | |

[a] CTX, cerebrotendinous xanthomatosis.
[b] PVDRI, pseudo-vitamin D deficiency rickets type I.

**Table 2**
1α-Hydroxylating cytochrome P450 enzymes.

| | Mitochondrial CYP27B1 (see also section 6 and 6.1) | Mitochondrial CYP27A1 (see also section 6 and 6.2) | Microsomal 1α-hydroxylase (CYP2D25) see also section 5 |
|---|---|---|---|
| **Species** | Several species e.g. rat, mouse, pig, human | Many species e.g. rabbit, rat, mouse, pig, human | Pig |
| **Tissue/Cells** | Predominantly kidney. Reported expression also in e.g. keratinocytes, brain, lung, pancreas and testis | Liver and most extrahepatic tissues and cells | Kidney, liver |
| **Catalytic reactions and substrate specificity** | 1α-Hydroxylation of 25-hydroxyvitamin $D_3$, but also of some other vitamin D compounds. A 25-hydroxyl group seems to be required. | **1α-Hydroxylation:** 25-hydroxyvitamin $D_3$. A 25-hydroxyl group seems to be required **25-hydroxylation:** Vitamin $D_3$ **27-hydroxylation:** $C_{27}$-sterols | **1α-Hydroxylation**: 25-hydroxyvitamin $D_3$ **25-hydroxylation:** vitamin $D_3$ |
| **Apparent $K_m$ for 25-hydroxyvitamin $D_3$** | 2.7 μM | 3.5 μM | No information |
| **Regulation in kidney** | Suppressed by 1α,25-dihydroxyvitamin $D_3$; Increased by PTH[a]. The regulation involves FGF23[b] and α-klotho. | Suppressed by 1α,25-dihydroxyvitamin $D_3$ | No information |
| **Effects of disease** | Mutations in the *CYP27B1* gene in patients with PVDRI[c] | Mutations in the *CYP27A1* gene in patients with CTX[d] | Decreased enzyme activity levels in kidneys of rachitic piglets with PVDRI[c] |
| **Mouse models (gene knock out)** | *Cyp27b1* KO mice show rachitic conditions including growth retardation, abnormal bone formation, and low plasma concentrations of Ca, P and 1,25$(OH)_2D_3$. A large dose of 25$(OH)D_3$ is reported to normalize levels of 1,25$(OH)_2D_3$ in the plasma of KO mice. | A large dose of 25$(OH)D_3$ is reported to normalize levels of 1,25$(OH)_2D_3$ in the plasma of *Cyp27b1* KO mice. It has been proposed that Cyp27a1 may produce 1,25$(OH)_2D_3$ in Cyp27b1 KO mice administered with 25$(OH)D_3$ | No information. |

[a] Parathyroid hormone.
[b] Fibroblast growth factor.
[c] CTX, cerebrotendinous xanthomatosis.
[d] PVDR1, pseudo-vitamin D deficiency rickets type I.

review see Nebert et al. [32]. The cholesterol side-chain cleavage enzyme, encoded by the *CYP11A1* gene, is expressed in high levels in steroidogenic cells/tissues, such as adrenal cortex, gonads and placenta, and in lower amounts in other tissues such as skin and brain. The CYP11A1-initiated vitamin D metabolites are detectable in human serum, epidermis and adrenal glands in vivo [53]. CYP11A1-formed vitamin D metabolites have also been reported in incubations with isolated mitochondria from placenta and adrenal glands as well as with cultured cells of different origin. The CYP11A1-mediated bioactivation of vitamin $D_3$ is reviewed in detail elsewhere [19,54].

## 5. Enzymes catalyzing 25-hydroxylation of vitamin $D_3$

As described in section 2, the first activation step is a 25-hydroxylation of vitamin $D_3$ (Fig. 3). The liver has a high capacity to produce 25-hydroxyvitamin $D_3$. The high capacity of liver in 25-hydroxylation could be due to the presence of several hepatic 25-hydroxylating enzymes. Both mitochondrial and microsomal vitamin D 25-hydroxylases are expressed in the liver cell. The multiple hepatic 25-hydroxylating enzymes may cooperate and compensate for each other in vitamin D metabolism under various conditions. This will be discussed further in the present article. 25-Hydroxyvitamin $D_3$ is the major vitamin D metabolite in serum and circulating levels of 25-hydroxyvitamin $D_3$ is a useful marker for human vitamin D status. Early studies suggested that the activity of vitamin $D_3$ 25-hydroxylation in rat liver is regulated [55]. Nowadays, many authors state that hepatic 25-hydroxylation is not under major regulation in humans [56]. 25-Hydroxylation of vitamin D occurs also extrahepatically. Some of the 25-hydroxylases are reported to be under regulation by calcitriol and drugs in extrahepatic cells [57–61]. More information is needed on the regulation of the various vitamin $D_3$ 25-hydroxylases, particularly in extrahepatic cells where vitamin D bioactivation may undergo cell- or tissue-specific regulation due to local needs.

To date, at least five enzymes have been identified as vitamin D 25-hydroxylases in humans, including CYP27A1 (human and other species), CYP2R1 (human and other species), CYP3A4 (human) and CYP2J2 (human). In addition, there are also species-specific 25-hydroxylases, such as CYP2C11 and CYP2J3 in the rat and CYP2D25 in the pig (cf. Section 3 and Table 1).

### 5.1. *CYP27A1 – mitochondrial vitamin $D_3$ 25-hydroxylase*

The mitochondrial vitamin $D_3$ 25-hydroxylase was the first 25-hydroxylase that was purified to apparent homogeneity, characterized and cloned. It was first purified from rabbit liver mitochondria as a $C_{27}$-sterol 27-hydroxylase (the 27-hydroxylase enzyme was previously called 26-hydroxylase) active in bile acid biosynthesis [62] and subsequently found to catalyze also 25-hydroxylation of vitamin $D_3$ [63]. The corresponding rat liver mitochondrial enzyme was then isolated and reported to exhibit similar (or the same) catalytic properties as the rabbit enzyme [36]. This mitochondrial enzyme was cloned from several species, including rabbit [39], rat [64] and human [65]. The enzyme was named CYP27A1. Studies with recombinant CYP27A1 confirmed that it is a multifunctional enzyme, carrying out important reactions required in both cholesterol metabolism and bioactivation of vitamin $D_3$ [66–69]. Further, it was demonstrated that it is widely expressed among species and has a wide tissue distribution, being expressed not only in liver but also in most extrahepatic tissues [66–68,70–73].

CYP27A1 is active in 25-hydroxylation of vitamin $D_3$ but not vitamin $D_2$. Instead, it catalyzes formation of 24- and 27-hydroxylated metabolites of vitamin $D_2$, that have been detected in human serum [67,74]. Purified and recombinant human CYP27A1 were found to also convert 25-hydroxyvitamin $D_3$ into 1α,25-dihydroxyvitamin $D_3$, 25,27-dihydroxyvitamin $D_3$ and 4β,25-dihydroxyvitamin $D_3$ [68,74–76].

CYP27A1 has been reported to play a role in vivo also as a pharmacologically relevant 25-hydroxylase for synthetic 1α-hydroxylated vitamin D analogs. Such derivatives are developed to be used as prodrugs in the treatment of metabolic bone diseases [67,77]. CYP27A1 also participates in the formation of biologically active non-classical vitamin D hydroxy metabolites by hydroxylation at C25 and C26 in

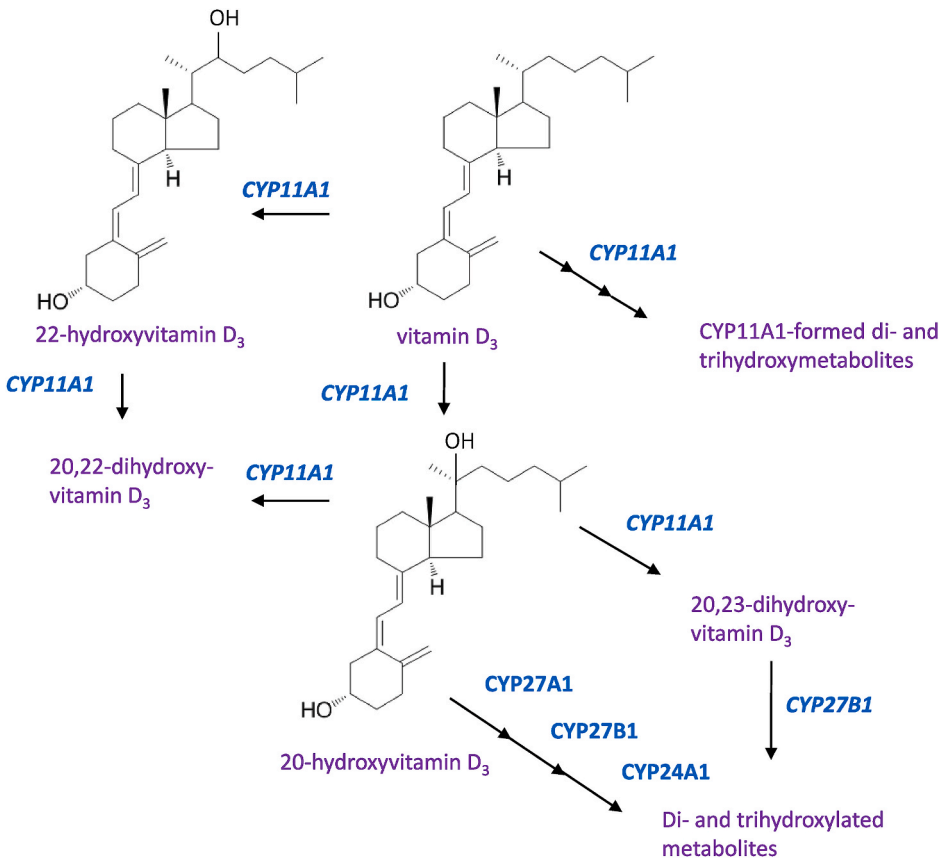


**Fig. 5. Alternative pathways for production of non-classical active vitamin D metabolites.** CYP11A1 initially produces 20-hydroxymetabolites from vitamin D. The CYP11A1-initiated vitamin D metabolites are detectable in e.g. human serum, epidermis and adrenal glands in vivo [53]. These metabolites are reported to be further metabolized by CYP27A1, CYP27B1 and CYP24A1. Modified from Slominski et al. [200].

the pathways starting with CYP11A1-mediated 20-hydroxylation of vitamin D (Fig. 5) [53]. It has also been reported that CYP27A1-mediated hydroxylation of lumisterol 3 (a pre-vitamin $D_3$ photoproduct in the skin) (Fig. 2) produces 25- and 27-hydroxylated lumisterol metabolites, which inhibit melanoma cell proliferation [78]. Recently, CYP11A1 and CYP27A1 were found to participate in the metabolic activation of tachysterol 3, another photoproduct of pre-vitamin $D_3$ (Fig. 2), to biologically active 20-, and 25-hydroxyderivatives, respectively, that act on VDR and other receptors [79].

*CYP27A1* is expressed in most extrahepatic tissues, e.g. in skin and brain [39,61,78,80–84], tissues which do not produce bile acids. This indicates potential autocrine/paracrine roles in local production of 25-hydroxyvitamin $D_3$ affecting cellular gene regulation. CYP27A1 may be an important enzyme in bioactivation of vitamin $D_3$ in extrahepatic cells. For example, CYP27A1 has been reported to be the key 25-hydroxylase in gingival fibroblasts and periodontal ligament cells [85]. The presence of CYP27A1 in these cells has led to the suggestion that CYP27A1 might be involved in periodontal immune defense and to have a role in gingival inflammation and alveolar bone loss [86,87].

Because mitochondrial CYP27A1 was the first vitamin $D_3$ 25-hydroxylase to be purified, enzymatically characterized and cloned, it was initially considered the principal human vitamin $D_3$ 25-hydroxylase. *CYP27A1* is well conserved among animals. The expression of *CYP27A1* in human adult liver was found to vary significantly with season and to correlate with levels of serum 25-hydroxyvitamin D [88]. In a later study, fetal hepatic expression of *CYP27A1* and also of *CYP2R1*, another vitamin D 25-hydroxylase, was reported to be highest in summertime [89]. Intestinal, hepatic and renal *CYP27A1* was found to be downregulated by the active vitamin D hormone, 1α,25-dihydroxyvitamin $D_3$ [57,88,90].

However, results emerged suggesting that CYP27A1 could not be the sole hepatic 25-hydroxylase. A monoclonal antibody, raised against purified rabbit liver mitochondrial 27-hydroxylase (i.e. CYP27A1), inhibited about 80% of the mitochondrial 27-hydroxylase activity but did not markedly inhibit the mitochondrial vitamin $D_3$ 25-hydroxylase activity. This result indicates that liver mitochondria might contain an additional, as yet not defined, vitamin D 25-hydroxylase besides CYP27A1 [91]. Furthermore, our and other laboratories have also characterized several mammalian microsomal vitamin D 25-hydroxylases which will be discussed below. It has been claimed that CYP27A1 could not be the principal vitamin D 25-hydroxylase since recombinant CYP27A1 selectively 25-hydroxylate the endogenously produced vitamin $D_3$ but not the exogenous vitamin $D_2$ [23]. In our view, this property should not be contradictory to the hypothesis that CYP27A1 may be a physiologically important 25-hydroxylase for vitamin $D_3$. Other enzymes are able to 25-hydroxylate vitamin $D_2$, with more or less specificity. For metabolism in general it is not unusual that two (or more) enzymes catalyzing the same reaction might have different substrate specificity.

### 5.2. *CYP2R1 – microsomal vitamin D 25-hydroxylase*

*CYP2R1* was cloned and characterized as a vitamin D 25-hydroxylase by Cheng et al. [40]. These authors screened a cDNA library prepared from liver mRNA of sterol 27-hydroxylase-deficient mice with a ligand activation assay and succeeded to identify CYP2R1 with vitamin D 25-hydroxylase activity. *CYP2R1* is highly conserved from mouse to human and is primarily expressed in liver and testis. In contrast to CYP27A1, it is capable of 25-hydroxylating both vitamin $D_2$ and vitamin $D_3$ [40]. CYP2R1 appears highly specific for hydroxylation of vitamins

$D_3$ and $D_2$ in the 25-position. Similar to CYP27A1, it is also active in 25-hydroxylation of 20-hydroxyvitamin $D_3$, the main product in vitamin $D_3$ bioactivation by the alternative pathway catalyzed by CYP11A1 [92]. However, in contrast to CYP27A1, which also catalyzes 1α-hydroxylation of 25-hydroxyvitamin $D_3$, CYP2R1 showed no detectable 1α-hydroxylation activity [74].

Strong evidence indicating the importance of CYP2R1 as a vitamin D 25-hydroxylase emerged from studies on patients with rare mutations in *CYP2R1,* which rendered it unfunctional towards vitamin $D_3$ and caused 25-hydroxylase deficiency [93,94]. It has been suggested that mutations in *CYP2R1* are responsible for an atypical form of vitamin D-deficiency rickets, which has been classified as vitamin D-dependent rickets type 1 B (VDDR1B) [95]. Although CYP2R1 appears to be the most important human vitamin D 25-hydroxylase, its deficiency is extremely rare. The rare occurrence of the abnormal phenotype and the relatively mild disease of affected individuals suggest compensatory effects of other enzymes, possibly CYP27A1, that may contribute to vitamin $D_3$ 25-hydroxylation in vivo [96].

### 5.3. CYP3A4 – human microsomal vitamin D 25-hydroxylase with broad substrate specificity

CYP3A4, which is abundantly present in human liver, is the major enzyme in metabolism of exogenous compounds, including drugs. This enzyme metabolizes a large number of structurally diverse drugs and other xenobiotic compounds and also some endogenous substances. CYP3A4 has been identified as a 25-hydroxylase in human liver microsomes towards the $C_{27}$-steroid 5β-cholestane-3α,7α,12α-triol, an intermediate in bile acid biosynthesis [97]. CYP3A4 has also been reported to 25-hydroxylate vitamin $D_2$, 1α-hydroxyvitamin $D_2$ and 1α-hydroxyvitamin $D_3$. However, CYP3A4 has no activity towards vitamin $D_3$ [98]. The finding that CYP3A4 does not 25-hydroxylate vitamin $D_3$ speaks against it being a physiologically important biosynthetic 25-hydroxylase. However, it may play a role in 25-hydroxylation of ingested exogenous vitamin $D_2$ and 1α-hydroxyvitamin $D_3$ and possibly other synthetic vitamin D analogs.

### 5.4. CYP2J2

CYP2J3 was characterized as a principal rat microsomal vitamin $D_3$ 25-hydroxylase and the possibility was considered that the corresponding CYP2J2 enzyme in humans might be a 25-hydroxylase [48]. However, CYP2J2 is located primarily in the heart and has less 25-hydroxylase activity than CYP2J3. The $K_m$ values for CYP2J2 are in the micromolar range towards both vitamin $D_2$ and vitamin $D_3$. Rat liver CYP2J3 has higher activity towards vitamin $D_3$ whereas human CYP2J2 was found to have higher activity towards vitamin $D_2$ [99]. The biologic role of human CYP2J2 appears to relate primarily to its metabolism of arachidonic acid for production of cardioprotective epoxyeicosatrienoic acids [100]. Thus, the properties of CYP2J2 suggest that this enzyme is of minor importance as a human vitamin $D_3$ 25-hydroxylase [101].

### 5.5. Roles of the various human 25-hydroxylases in the bioactivation of vitamin $D_3$

Vitamin $D_3$ is endogenously produced in the human body and is essential for the vitamin D status whereas vitamin $D_2$ is an exogenous form of vitamin D and only ingested with vegetable food or as supplement. Among the human vitamin D 25-hydroxylases, CYP2R1 and CYP27A1 activate vitamin $D_3$, whereas CYP3A4 and CYP2J2 activate mainly vitamin $D_2$ and 1α-hydroxyvitamin $D_3$. The latter is a synthetic compound not endogenously produced from vitamin $D_3$. Thus, CYP2R1 and CYP27A1 appear to be the two major human 25-hydroxylases for vitamin $D_3$ whereas CYP3A4 and CYP2J2 may be important in metabolism of ingested exogenous (supplementary) vitamin $D_2$ and 1α-hydroxyvitamin $D_3$. CYP2J2 is now considered primarily as an arachidonic acid metabolizing enzyme [100,101]. It should be mentioned that CYP3A4 appears to play a role in the inactivation of the active 1α,25-dihydroxyvitamin $D_3$ hormone by 24-hydroxylation in liver and intestine [25]. Both *CYP27A1* and *CYP2R1* are well conserved among species from mice to humans and are not sex-specific. Besides in liver, mRNA for the two enzymes is expressed also in many other human tissues/cell types indicating that CYP27A1 and CYP2R1 can mediate local autocrine functions of vitamin $D_3$.

### 5.6. CYP27A1 and CYP2R1 - comparison of their properties in liver and extrahepatic tissues

#### 5.6.1. Enzyme properties and functions

Recombinant human CYP27A1 and CYP2R1 catalyze 25-hydroxylation of vitamin $D_3$ at rates of similar order of magnitude and both enzymes show $K_m$ values in the micromolar range. Reported $K_m$-values for recombinant CYP2R1 vary between 0.45 μM and 4.4 μM [74,102]. $K_m$ for recombinant CYP27A1 was found to be 3.2 μM [72,74]. The 25-hydroxylation of vitamin $D_3$ by recombinant CYP2R1 and CYP27A1 has been studied in different expression systems [74,92]. In one study, where experiments were carried out in a membranous environment, CYP2R1 had a $k_{cat}$ half that of CYP27A1 [92] whereas in another study CYP2R1 had a higher $k_{cat}$ than CYP27A1 [74]. Both studies reported, however, that CYP2R1 showed an overall higher catalytic efficiency (as measured by $k_{cat}/K_m$) than CYP27A1. In a study where vitamin $D_3$ was incorporated into phospholipid vesicles, the reported $k_{cat}$ for 25-hydroxylation by CYP27A1 was found to be higher than by any other human cytochrome P450, including CYP2R1 and CYP2J2. The authors of that study suggested that CYP27A1 might be an important contributor to the synthesis of 25-hydroxyvitamin $D_3$ [103]. It has been suggested that mitochondrial CYP27A1-dependent 25-hydroxylation works most efficiently under conditions with high circulating concentrations of vitamin $D_3$ due to the relatively high $K_m$ for 25-hydroxylation of vitamin $D_3$ [74,104].

CYP2R1 appears specific for vitamin D metabolism catalyzing hydroxylation of vitamins $D_3$ and $D_2$ in the 25-position. It is also active in 25-hydroxylation of 20-hydroxyvitamin $D_3$, the main product of vitamin $D_3$ in an alternative bioactivation pathway initiated by CYP11A1 [92].

In contrast to the apparent high specificity of CYP2R1 for vitamin D metabolism, CYP27A1 has a major role also in another physiologic pathway. It functions in cholesterol metabolism as a $C_{27}$-sterol 27-hydroxylase being essential for both the classical and alternative pathways of hepatic bile acid biosynthesis. CYP27A1 catalyzes side-chain oxidation of cholesterol and other $C_{27}$-sterols in these pathways. It is notable that the $C_{27}$-sterols, acting as substrates for CYP27A1, are present in higher concentrations than that of vitamin $D_3$ in liver.

In vitamin D metabolism, CYP27A1 catalyzes not only 25-hydroxylation of vitamin $D_3$ but also hydroxylations of other vitamin D compounds in other positions (C1α, C4, C24, C26/27), including the exogenous vitamin $D_2$ and several vitamin D analogs. These activities towards exogenous compounds indicate that CYP27A1 may have a function also as a pharmacologically relevant enzyme for metabolism of synthetic vitamin D analogs. For example, the vitamin D analogs 1α-hydroxyvitamin $D_3$ and 1α-hydroxyvitamin $D_2$ are used as prodrugs in the treatment of osteoporosis/metabolic bone disease and the secondary hyperparathyroidism associated with chronic kidney disease-metabolic bone disease (CKD-MBD) [105]. It has been reported that CYP27A1 synthesizes 1α,25-dihydroxyvitamin $D_3$ from 1α-hydroxyvitamin $D_3$ and 1,24S-dihydroxyvitamin $D_2$ from 1α-hydroxyvitamin $D_2$. 24-Hydroxylated metabolites, such as 1,24S-dihydroxyvitamin $D_2$, are also observed in vivo following administration of pharmacological amounts of vitamin $D_2$ compounds [106–108]. These catalytic properties imply that CYP27A1 can contribute to the metabolism of several vitamin D compounds, in addition to the 25-hydroxylation of vitamin $D_3$.

*5.6.2. Consequences of mutations in the CYP2R1 and CYP27A1 genes*

An approach to estimate the role of an enzyme in a metabolic pathway may be to study the biochemical and symptomatic consequences of mutations of its human gene. However, the evaluation may be complicated by compensating effects by other enzymes able to carry out the same or similar reactions. Mutations in the *CYP2R1* gene are very rare but have been found in Nigerian, Saudi Arabian and Sudanese children with rachitis and decreased levels of 25-hydroxyvitamin $D_3$. This finding indicates that CYP2R1 is an important human vitamin $D_3$ 25-hydroxylase [93,94,109–112]. It has been suggested that mutations in the *CYP2R1* gene are responsible for an atypical form of vitamin D-deficiency rickets, which has been classified as vitamin D dependent rickets type 1B (VDDR1B) [95]. Although CYP2R1 appears to be the most important human hepatic vitamin D 25-hydroxylase, CYP2R1 deficiency giving symptomatic disease is extremely rare, suggesting that also other enzymes contribute to vitamin $D_3$ 25-hydroxylation in vivo [96,109]. CYP27A1 is the most likely enzyme that can cooperate and compensate for CYP2R1 in 25-hydroxylation.

Results from studies with humans having mutations in the *CYP27A1* gene have led to discussion on the importance of CYP27A1 as a human hepatic vitamin D 25-hydroxylase. It has been stated that CYP27A1 could not be an important physiological vitamin $D_3$ 25-hydroxylase in humans because CYP27A1 has a major important role in cholesterol metabolism, being essential for bile acid biosynthesis, and because patients with mutations in the *CYP27A1* gene develop the bile acid-related disease cerebrotendinous xanthomatosis (CTX) rather than rickets [23,101]. However, as discussed below, it has been reported that a number of subjects with mutations in the *CYP27A1* gene, causing CTX, also have decreased levels of 25-hydroxyvitamin $D_3$. Furthermore, osteoporosis and low bone mass is common in CTX patients.

A major problem in our attempts to evaluate vitamin D metabolism in CTX patients is that although osteoporosis and low bone mass are frequently reported, only few studies report determination of 25-hydroxyvitamin $D_3$ levels in these subjects. Also, the interpretations of these results are different in different studies. Therefore, the role of CYP27A1 as a human vitamin $D_3$ 25-hydroxylase deserves critical discussion in relation to results with mutations in the *CYP27A1* gene causing the lipid storage disease cerebrotendinous xanthomatosis (CTX).

Humans with CTX show severe disturbances in cholesterol metabolism [113–117]. However, a number of reports also describes development of osteoporosis and low bone mass in these subjects. Berginer et al. [118] originally reported that extensive osteoporosis and increased risk of bone fractures occurred in patients with CTX. The serum levels for 25-hydroxyvitamin $D_3$ were reduced by 50%, compared with healthy subjects, in eleven patients with CTX [118]. Also, other research groups [119–122] reported that patients with CTX suffer from a condition of osteopenia and bone loss. The presence of low bone mass and low 25-hydroxyvitamin D levels in patients with cerebrotendinous xanthomatosis was recently confirmed [123–125].

Okuda et al. [38] suggested that osteoporosis in the patients may be explained by low or null activity of the mitochondrial vitamin $D_3$ 25-hydroxylase, owing to the abnormal enzyme formed from the mutated *CYP27A1* gene. In contrast, some authors have discussed an alternative explanation suggesting that the bone disease in CTX patients would be the result of malabsorption of dietary vitamin D caused by bile-acid insufficiency rather than inadequate 25-hydroxylase enzyme activity [23]. However, this theory has been questioned by other authors. Gupta et al. [126] argue that because the major source of vitamin $D_3$ is synthesized in skin and its metabolites are the major circulating forms, it is unlikely that interference with enterohepatic circulation of vitamin D caused by abnormal bile acid metabolism would contribute to abnormal vitamin D metabolism in patients with CTX. If the bone disease in CTX patients would be the result of malabsorption of dietary vitamin D caused by bile-acid insufficiency rather than inadequate 25-hydroxylase enzyme activity, it would be expected that symptoms also of deficiency of other fat-soluble vitamins would occur. To our knowledge no such symptoms have been reported.

Gupta et al. [126] performed an analysis of *CYP27A1* mutations causing CTX and assessment of 27-hydroxylation of cholesterol and 25-hydroxylation of vitamin D by recombinant mutant CYP27A1 proteins. Three of 15 causative mutations of *CYP27A1* associated with CTX showed the same or lower values in 25-hydroxylation of vitamin $D_3$ and 27-hydroxylation of cholesterol compared to wild type when expressed in *E. coli*. It was suggested that patients with such mutations should be of interest to study with respect to their circulating 25-hydroxyvitamin D levels and whether they are less predisposed to osteoporosis. In most patients with CTX, *CYP27A1* is either not expressed or the enzyme is biologically inactive as regards 25-hydroxylation of vitamin D. It is possible that the expression of other 25-hydroxylase(s), such as CYP2R1, compensates for the lack of active CYP27A1 by increased expression. Life-long deficiency of circulating 25-hydroxyvitamin D may account for the early development of osteoporosis and fractures in some patients with the CTX disorder.

Despite an increasing number of reports on the many different mutations of the *CYP27A1* gene causing CTX, there is only a few studies that document vitamin D metabolite levels in CTX-patients with osteoporosis/osteopenia [118,120,123,124]. Berginer et al. [118] and Federico et al. [120] reported that patients with CTX suffer from a condition of osteopenia and bone loss. Berginer et al. [118] found in their study that eleven patients had reduced 25-hydroxyvitamin D levels. In the study by Federico et al. [120] on nine CTX patients, however, the serum levels of vitamin D metabolites showed no significant changes, except in one case where the 25-hydroxyvitamin D level was lower than normal [120]. Martini et al. [123] found that nine out of eleven CTX patients had decreased 25-hydroxyvitamin D levels. Four patients showed deficiency, five insufficiency, and two patients normal values. Sasamura et al. [124] reported that a CTX-patient who developed osteoporosis before menopause, was found to have markedly decreased serum levels of 25-hydroxyvitamin D. In a review on inborn errors in bile acid biosynthesis, it is mentioned that reduced serum levels of 25-hydroxyvitamin D have been shown in some but not all patients with CTX [127].

The underlying mechanisms for the development of osteoporosis in CTX patients with *CYP27A1* mutations are still controversial. From the results of Gupta et al. [126], in their mutational analysis study on CYP27A1 and 25-hydroxylation of vitamin D, it was concluded that evaluation of vitamin D metabolism and possible osteoporosis in patients with CTX should be considered. Further studies should shed light on the role of CYP27A1 in 25-hydroxyvitamin D production.

*5.6.3. Consequences of disruption of the Cyp27a1 and Cyp2r1 genes in mouse models*

Another approach often used to evaluate an enzyme's role in a metabolic pathway may be to study consequences of disruption of its gene in mice. However, there are species differences between mice and humans that can complicate such evaluations. Some authors have questioned the physiological role of CYP27A1 as a human vitamin $D_3$ 25-hydroxylase because of the finding that mice with disrupted *Cyp27a1* gene show severe disturbances in cholesterol metabolism but do not have markedly reduced circulating 25-hydroxyvitamin $D_3$ levels or rickets [128]. However, the lack of effect on the levels of circulating vitamin $D_3$ metabolites may be due to a compensatory increased activity of the microsomal vitamin $D_3$ 25-hydroxylase under these pathological and unphysiological conditions. Indeed, a marked upregulation of CYP2R1 has been observed in *Cyp 27a1(−/−)* mice and the levels of 25-hydroxyvitamin $D_3$ were increased compared with wild-type mice [129]. It is not clear why *Cyp27a1 (−/−)* mice have an upregulation of CYP2R1. This might indicate that CYP27A1 is involved in the 25-hydroxyvitamin $D_3$ balance in mice in a, so far, unknown way. It is interesting that neither *Cyp2r1-null* mice nor the *Cyp27a1* (−/−) mouse phenotype was observed to include rickets or other bone deformities [129]. This is in contrast to studies with human subjects having *CYP2R1* mutations and rickets [93–95,109,130]. Surprisingly, *Cyp27a1*

knockout mice *(Cyp27a1−/−)* do not present a CTX phenotype despite generating a similar pattern of sterols [131]. Thus, in contrast to studies with mouse models, human subjects with mutation in the *CYP2R1* or *CYP27A1* gene present with rickets and CTX symptoms, respectively. It is apparent that there are species differences between mice and humans with respect to clinical manifestations as a consequence of disruption/mutation of the *CYP2R1* and *CYP27A1* genes that complicates interpretations in studies comparing *Cyp27a1*-deficient mouse models and humans with *CYP27A1* mutations.

An interesting study by Zhu et al. suggests that CYP2R1 may not be the major important 25-hydroxylase [129].These authors examined mice with deleted *Cyp2r1* and/or *Cyp27a1* genes and results were obtained indicating the expression of an additional, yet not identified, major vitamin D 25-hydroxylase. The results showed that *Cyp2r1 (−/−)* mice had about 50% reduction in serum levels of 25-hydroxyvitamin $D_3$ but unchanged levels of 1α,25-dihydroxyvitamin $D_3$. Although deletion of the *Cyp2r1* gene significantly reduced circulating 25-hydroxyvitamin $D_3$ levels, the production of 25-hydroxyvitamin $D_3$ was not abolished and the knockout mice were healthy. This result led the authors to the conclusion that CYP2R1 is a major, but not exclusive, vitamin $D_3$ 25-hydroxylase in mice and they suggested that there is another yet unidentified 25-hydroxylase. Although mice were used in this report and species differences between mice and humans occur, the results are intriguing and may be relevant also for humans. Studies on mice with disruption of a gene is useful in evaluation of the importance of a particular enzyme but it is also known that there are important species differences in the clinical manifestations, e.g. between mice and humans. There are examples from steroid biochemistry that a mutated gene in humans does not show the same clinical manifestations as disruption of the corresponding gene in mouse [132–135]. It is also known that, in contrast to humans, rodents have many sex-specific enzymes [46,136].

### 5.7. Hepatic vs extrahepatic 25-hydroxylation of vitamin $D_3$

#### 5.7.1. Hepatic 25-hydroxylation

The results discussed in the previous sections are consistent with CYP2R1 being more important than CYP27A1 in liver for production of circulating 25-hydroxyvitamin $D_3$ under physiological conditions. It is worth considering that CYP27A1 has a major physiological function in liver as an obligatory enzyme in bile acid biosynthesis metabolizing $C_{27}$-steroids [137]. These substrates compete with vitamin D for the active site of hepatic CYP27A1. However, results from studies with subjects having certain types of mutations in the *CYP27A1* gene leading to reduced 25-hydroxyvitamin $D_3$ concentrations and low bone mass and osteoporosis suggest that CYP27A1 also contributes to vitamin $D_3$ 25-hydroxylation in liver. Thus, even if CYP2R1 is the major 25-hydroxylase at physiologically relevant vitamin D concentrations in liver, CYP27A1 also has the capacity to contribute to the hepatic metabolism of vitamin $D_3$. Further, it seems likely that CYP27A1 also has a role as a pharmacological vitamin D hydroxylase that metabolizes synthetic vitamin D analogs including 1α-hydroxyvitamin $D_3$ and 1α-hydroxyvitamin $D_2$. It is our opinion that both CYP2R1 and CYP27A1 are effective 25-hydroxylases and that the importance of the individual 25-hydroxylases may vary during different conditions and in different tissues and cells.

#### 5.7.2. Extrahepatic 25-hydroxylation

mRNA for CYP27A1 and CYP2R1 are expressed in many extrahepatic tissues and have been reported to be important in vitamin D metabolism in extrahepatic cells. It is probable that CYP27A1 could be more active than CYP2R1 in certain extrahepatic cells where the concentrations of substrates in bile acid synthesis are low or absent. The expression of *CYP27A1* in extrahepatic tissues, which do not produce bile acids, suggests autocrine/paracrine roles in local production of 25-hydroxyvitamin $D_3$ affecting cellular gene regulation [59,61,138]. For example, CYP27A1 has been reported to be the key 25-hydroxylase in gingival fibroblasts and periodontal ligament cells [85] as discussed in section 5.1.

Table 1 summarizes information on vitamin D 25-hydroxylases.

## 6. Enzymes catalyzing 1α-hydroxylation of 25-hydroxyvitamin $D_3$

The final bioactivation step in the production of 1α,25-dihydroxyvitamin $D_3$ is a renal 1α-hydroxylation of 25-hydroxyvitamin $D_3$. This reaction is rate-limiting and tightly regulated in kidney. For many years, efforts to purify a specific mitochondrial 1α-hydroxylase from kidney were made in several laboratories including ours [75,139–141]. The exquisitely low abundance of the enzyme and low enzymatic activity were major reasons for the relatively slow progress in isolation and characterization of the 1α-hydroxylase. As a result, the 1α-hydroxylase activity was also difficult to assay due to the extremely low amounts of 1α-hydroxylated product in renal tissue and also to the low stability of the vitamin D compound.

In the first half of the 1990′s, we reported separation of the cytochromes P450 in pig kidney mitochondria catalyzing 1α- or 24-hydroxylations of 25-hydroxyvitamin $D_3$, respectively [139]. Subsequently, our studies provided evidence that at least some of the 1α-hydroxylase activity might be derived from the mitochondrial vitamin $D_3$ 25-hydroxylase CYP27A1 [57,68,75]. A breakthrough in identification of the 25-hydroxyvitamin D 1α-hydroxylase came in 1997 when several groups reported the cloning of cDNA encoding another 1α-hydroxylating enzyme belonging to the CYP27 family [41–44,142,143]. This 25-hydroxyvitamin $D_3$ 1α-hydroxylase, designated CYP27B1, is now generally considered the physiologically relevant 1α-hydroxylase in kidney.

### 6.1. CYP27B1 - mitochondrial 25-hydroxyvitamin $D_3$ 1α-hydroxylase

In the autumn of 1997, several groups reported the isolation of cDNA encoding mouse, rat and human kidney 25-hydroxyvitamin $D_3$ 1α-hydroxylase. A cDNA for human keratinocyte 1α-hydroxylase was also isolated [41–44,142]. The sequences reported have a high degree of identity, and a high degree of similarity to CYP27A1. In fact, the enzyme constitutes a new subfamily of CYP27, i.e. CYP27B [43]. Studies with recombinant mouse and human CYP27B1, produced in *E. coli*, showed a $K_m$ of 2.7 μM for 25-hydroxyvitamin $D_3$. The mouse and human enzymes were not specific for 25-hydroxyvitamin $D_3$, also e.g. 24,25-dihydroxyvitamin $D_3$ was efficiently 1α-hydroxylated. However, a 25-hydroxyl group is reported to be essential for 1α-hydroxylation [144,145]. The tissue distribution of CYP27B1 is reported to be relatively broad. The mRNA for CYP27B1 is predominantly expressed in the kidney, but also in small amounts in other tissues e.g. lung, pancreas, keratinocytes, brain, and testis [42,142].

The expression of *CYP27B1* is reduced by 1α,25-dihydroxyvitamin $D_3$ and increased by parathyroid hormone [20,41,43,44,146,147]. The regulation involves FGF23 and α-klotho. FGF23 (a protein among the fibroblast growth factors) is a hormone, predominately produced by osteoblasts/osteocytes, whose major functions are to inhibit renal tubular phosphate reabsorption and suppress circulating 1α,25-dihydroxyvitamin $D_3$ levels. FGF23 functions together with α-klotho, which is a transmembrane protein that is highly expressed in the renal distal tubule and acts as an obligate coreceptor for FGF23 [148]. Together, FGF23 and α-klotho, suppress the expression of *CYP27B1* and induce the expression of *CYP24A1* leading to inhibition of the synthesis and stimulation of the catabolism of 1α,25-dihydroxyvitamin $D_3$ [20].

Mutations in the *CYP27B1* gene have been identified in patients with vitamin D 1α-hydroxylase deficiency (also called pseudo vitamin D-

deficiency rickets, type I) which is a genetic form of rickets. The disease is characterized at the biochemical level by low serum calcium and very low 1α,25-dihydroxyvitamin $D_3$ concentrations despite normal concentrations of 25-hydrozyvitamin $D_3$. The patients respond to treatment with physiologic replacement doses of 1α,25-dihydroxyvitamin $D_3$. Many different *CYP27B1* mutations have been reported showing different severity in enzymatic activity [149–151].

For review on the regulation of gene expression, and mutations of human 25-hydroxyvitamin $D_3$ 1α-hydroxylase, the reader is referred to a number of excellent reviews [23,152–155].

### 6.2. Other 25-hydroxyvitamin D 1α-hydroxylases besides CYP27B1

CYP27B1 is considered by many the only 25-hydroxyvitamin $D_3$ 1α-hydroxylase in mammals. However, there are reports indicating that this issue is more complex. Firstly, several laboratories have reported that other enzymes, expressed in kidney and in liver, have the capacity to produce 1α,25-dihydroxyvitamin $D_3$ from 25-hydroxyvitamin $D_3$ [68,72,156–158]. Secondly, reported results on the generation of 1α,25-dihydroxyvitamin $D_3$ in a patient with mutated and non-functional human *CYP27B1* gene and in *Cyp27b1* knockout mice are not consistent with the concept of CYP27B1 being the only 1α-hydroxylase [159,160]. In fact, the results strongly indicate the presence of additional physiological 1α-hydroxylase(s) besides CYP27B1.

As mentioned in the beginning of section 6, results indicating that at least some of the 1α-hydroxylase activity might be derived from the mitochondrial CYP27A1 were early reported [57,68,75]. Recombinant human CYP27A1 expressed in *E. coli* and in mammalian COS cells was found to catalyze 1α-hydroxylation of 25-hydroxyvitamin $D_3$ [68]. This finding indicated that the gene for *CYP27A1*, in addition to 25-hydroxylase activity, also expresses 1α-hydroxylase activity in vitamin D bioactivation. Evidence from purification experiments and inhibition of the 1α-hydroxylase activity in a kidney mitochondrial extract, by an antibody directed against CYP27A1, was presented, indicating a role for CYP27A1 as a mitochondrial 1α-hydroxylase in kidney. Interestingly, treatment of rats with 1α,25-dihydroxyvitamin $D_3$, known to down-regulate renal 1α-hydroxylation, suppressed the levels of mRNA for CYP27A1 in kidney, intestine and liver [57,90,161]. Thus, *CYP27A1* and *CYP27B1* appears to be regulated in a similar way by 1α,25-dihydroxyvitamin $D_3$ in kidney. The ability of recombinant human CYP27A1 to catalyze 1α-hydroxylation of 25-hydroxyvitamin $D_3$ was subsequently confirmed by Pikuleva et al. [158] and Sawada et al. [72]. Pikuleva et al. [158] suggested that the activity of CYP27A1 was too small for a physiological function as a 1α-hydroxylase. However, the results of enzyme kinetic studies by Sakaki, Sawada and coworkers indicate that CYP27A1 may have a physiological role as a 1α-hydroxylase [72,162]. These authors determined the $K_m$ value of CYP27A1 for 25-hydroxyvitamin $D_3$ 1α-hydroxylation and found it to be quite similar to that of CYP27B1, which is considered to be the most important 1α-hydroxylase. Requirement for a 25-hydroxyl group has been reported for 1α-hydroxylation by both CYP27B1 and CYP27A1 in Ref. [72]. Since *CYP27A1* is expressed in many tissues it is possible that CYP27A1 may act as an extrarenal 1α-hydroxylase, in addition to CYP27B1.

Interestingly, Kitanaka et al. [159] reported that a patient with mild symptoms of pseudovitamin D-deficient rickets had nearly normal serum 1α,25-dihydroxyvitamin $D_3$ levels in spite of a mutated and non-functional *CYP27B1* gene [159]. The authors discussed the possibility that enzymes other than CYP27B1 may exert 1α-hydroxylase activity in either renal or extrarenal tissues. They referred to the mitochondrial 25-hydroxylase CYP27A1, which has activity for 1α-hydroxylation [68,158] and also discussed the presence of 1α-hydroxylase activity in microsomes of the kidney and the liver [57,157,163,164]. Sawada et al. and Sakaki et al. [72,162] suggested that the nearly normal 1α,25-dihydroxyvitamin $D_3$ level in the serum of the patient with pseudovitamin D-deficient rickets, reported by Kitanaka et al. [159] could be derived from CYP27A1-dependent 25-hydroxyvitamin $D_3$ 1α-hydroxylase activity. They suggested that the 1α-hydroxylation activity catalyzed by human CYP27A1 should not be physiologically neglected. Although the CYP27A1-dependent 1α-hydroxylase activity may be weak in the normal state, the activity of such a compensatory enzyme may be potentiated in PVDRI patients and produce 1α,25-dihydroxyvitamin $D_3$ [72,162].

In the recent study by Nishikawa et al. [160] using *Cyp27b1* knockout mice, it was found that treatment with 25-hydroxyvitamin $D_3$ resulted in endogenous production of 1α,25-dihydroxyvitamin $D_3$ and rescued their rachitic phenotypes [160]. Also, these authors concluded that the most probable candidate for 1α-hydroxylation in the *Cyp27b1* knockout mice is CYP27A1.

Table 2 summarizes information on 25-hydroxyvitamin $D_3$ 1α-hydroxylating enzymes.

## 7. Vitamin D receptor(s) mediating vitamin D signaling pathways

The bioactivating hydroxylations, catalyzed by the multiple vitamin D hydroxylases described in this review, are crucial for vitamin D signaling. The major pathway leads to production of the hormone 1α,25-dihydroxyvitamin D, which elicits receptor-mediated effects on transcription of target genes. Other 1α,25-dihydroxyvitamin-mediated effects than those on transcription have also been reported (Fig. 6).

The genomic actions of vitamin D are mediated by transcriptional regulation of target genes through the interaction of 1α,25-dihydroxyvitamin D with the nuclear vitamin D receptor (VDR), which is a member of the steroid hormone receptor family. VDR functions as a transcriptional factor which is activated by binding of 1α,25-dihydroxyvitamin $D_3$. The active receptor forms a dimer with RXR (retinoid X receptor) and binds to VDR-responsive elements (VDRE) influencing expression of responsive genes (Fig. 6). VDR thus mediates the so called "genomic effects" of vitamin D. The vitamin D receptor is widely expressed among tissues and it has been assessed that up to 5% of the genome might be regulated by the vitamin D hormone, with more than 900 genes that respond directly [2,153,165,166].

1α,25-Dihydroxyvitamin $D_3$ is the most efficient ligand for the vitamin D receptor (VDR) and is the most potent form of vitamin D. 25-Hydroxyvitamin $D_3$, produced by the first activating step, is the major circulating vitamin D metabolite. The normal serum levels of 25-hydroxyvitamin $D_3$ are almost 1000 times higher than the levels of 1α,25-dihydroxyvitamin $D_3$. 25-Hydroxyvitamin $D_3$ is also a VDR ligand but binds much less potently than the end product 1α,25-dihydroxyvitamin $D_3$ [167,168]. 25-Hydroxyvitamin $D_3$ has previously been considered an inactive prohormone, probably because of the much less efficient binding to VDR. However, the much higher serum levels of 25-hydroxyvitamin $D_3$ means that it still can be a physiologically important metabolite which can activate VDR. 25-Hydroxyvitamin $D_3$ can exert biological effects and has been ascribed gene regulatory properties [15,169]. Evidence has been presented that 25-hydroxyvitamin $D_3$ functions as a hormone and has direct gene regulatory properties in e.g. human prostate cells, skin cells, primary mouse kidney cells, renal tubular cells, and human MCF-7 breast cancer cells [14,15,169–174].

In addition to the effects by 1α,25-dihydroxyvitamin $D_3$ and other vitamin D compounds on gene transcription, there are some cellular vitamin D-mediated responses that are too rapid to be a result of gene regulation, e g the increased influx of calcium in some cells. Some potential explanations for these findings, suggested by various authors, are either that so-called "non-genomic" effects by 1α,25-dihydroxyvitamin $D_3$ are mediated by a separate pool of VDR (mVDR), translocated to the plasma membrane, or that they result from actions by a different, membrane-bound receptor and cell signaling e.g. by MAP (mitogen activated protein) kinases [175–178]. A proposed candidate for such a membrane-bound protein mediating rapid non-genomic effects of 1α,25-dihydroxyvitamin $D_3$ is PDIA3 (protein disulfide isomerase) also called 1α,25D3-MARRS (membrane-associated, rapid response

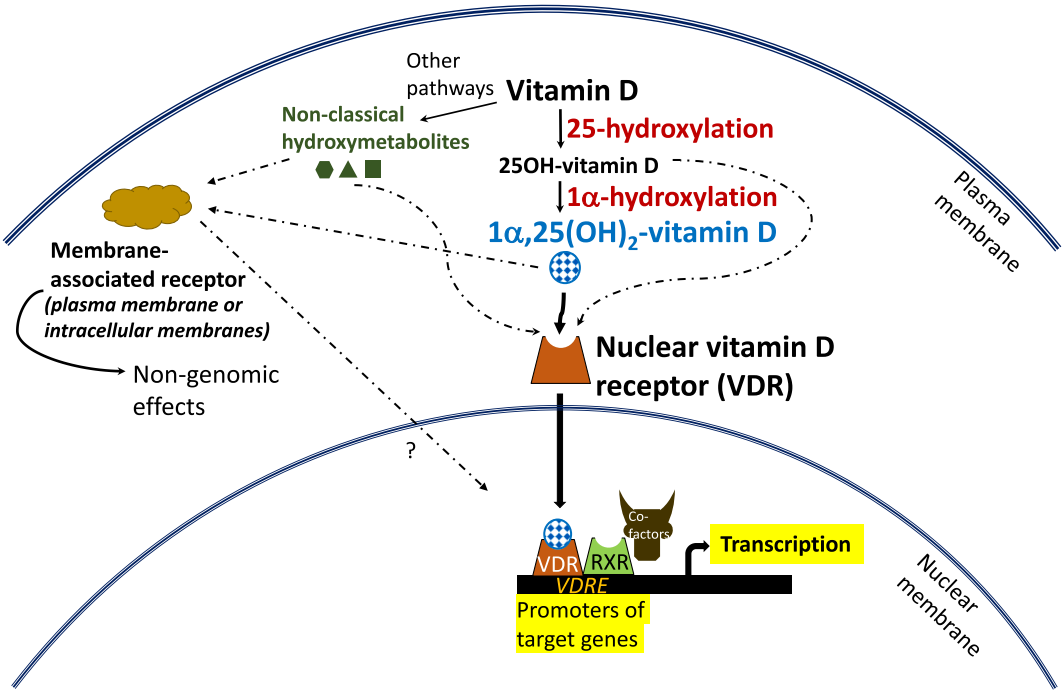


**Fig. 6. Vitamin D signaling.** The bioactivating hydroxylation reactions, catalyzed by the multiple vitamin D hydroxylases described in this review, are crucial for vitamin D signaling. The major pathway leads to production of the hormone 1α,25-dihydroxyvitamin D, which elicits effects on transcription of more than 900 target genes by interaction with the nuclear vitamin D receptor (VDR). Potential involvement of membranous receptor-mediated signaling pathways for non-genomic effects has been reported in many studies but the mechanisms and physiological relevance of such pathways are still unclear. In addition to the effects mediated by 1α,25-dihydroxyvitamin D, non-classical 20-hydroxymetabolites produced in alternative vitamin D-activating pathways may interact with both the nuclear VDR and the membranous receptor. Dashed arrow lines indicate interaction of hydroxylated vitamin D metabolites with receptor(s) that are reported but less well documented.

steroid-binding) [179–186]. This protein, which is reported to be associated with different cellular membranes, including the plasma membrane and the endoplasmic reticulum, is otherwise known for its important role in protein folding. Some of the novel non-classical vitamin D hydroxy-metabolites, formed by CYP11A1, have been reported to interact with both nuclear VDR and the membrane-bound 1α,25D3-MARRS [19]. However, potential interactions with 1α,25-dihydroxyvitamin $D_3$ and/or other hydroxylated vitamin D metabolites, mechanisms of action and physiological role for this protein are not fully understood. The nuclear transcription factor vitamin D receptor (VDR) is the only protein known to bind 1α,25-dihydroxyvitamin $D_3$ with high affinity. For a recent review on vitamin D receptors, the reader is referred to that by Zmijewski and Carlberg (174).

## 8. Extrarenal formation of 1α,25-dihydroxyvitamin D

The kidney is clearly the most important tissue in formation of the multifunctional 1α,25-dihydroxyvitamin $D_3$ hormone. However, reports also suggest local production of this compound in many other tissues [187]. Both activating (25- and 1α-hydroxylases) and catabolizing (24-hydroxylase) enzymes as well as VDR and 1α,25D3-MARRS have been found in cells of certain extra-renal tissues. Indeed, evidence for extrarenal generation of 1α,25-dihydroxyvitamin D was reported already about 40 years ago in studies on anephric individuals with sarcoidosis. These patients produced 1α,25-dihydroxyvitamin $D_3$ and also 24,25-dihydroxyvitamin $D_3$ [188–190]. Monocytes/macrophages were found to possess 1α-hydroxylase activity and to be the source of 1α,25-dihydroxyvitamin $D_3$ production in sarcoidosis [191]. Subsequently, a large amount of reports has shown extrarenal vitamin D metabolism in several cells and tissues, including cells in the skin, breast, colon, prostate, lung, brain, bone, and various cells of the immune system. Enzymes for production of 1α,25-dihydroxyvitamin $D_3$ and other vitamin D metabolites have been reported in extrahepatic cells (for a review, see Ref. [187]). However, the characterization of enzyme activity, metabolite formation and regulation of the vitamin D-metabolizing genes for CYP450 enzymes, in cells from other tissues than liver and kidney, is incomplete. This is apparently due to difficulties in assaying the enzyme activities. The enzymes are present in low concentrations and the metabolites formed, particularly 25-hydroxyvitamin $D_3$ and 1α,25-dihydroxyvitamin $D_3$, are difficult to analyze adequately.

The 1α,25-dihydroxyvitamin $D_3$ produced in extrarenal cells is considered to be mainly used in an autocrine (or paracrine) manner as a regulator in the expression of many genes. This autocrine 1α,25-dihydroxyvitamin $D_3$ is believed to bind to VDR and modify gene transcription. For example, genes involved in cell proliferation, differentiation and apoptosis may be regulated by the internal 1α,25-dihydroxyvitamin $D_3$ of the cell. The activation, effects on gene expression and inactivation of 1α,25-dihydroxyvitamin $D_3$ is contained within the host cell. 1α,25-Dihydroxyvitamin $D_3$ seems to be the major player in the internal autocrine actions, but also 25-hydroxyvitamin $D_3$ can be formed within the cell and regulate gene expression [15,59,169, 192–195]. After acting, the metabolites are inactivated by formation of 24-hydroxymetabolites and may be secreted as such into the blood. It is likely that several of the recently reported functions of active vitamin D metabolites are mediated by autocrine or paracrine action. However, more basic research is needed to increase our knowledge of molecular mechanisms behind autocrine vitamin D for local action in extrarenal and extrahepatic cells.

Examples of other reviews on extrarenal formation and metabolism are those by Dusso et al. [196]; Dusso et al. [197]; Christakos and DeLuca [9]; Jones G et al. [187]; DeLuca GC et al. [198]; Eyles et al. [18] and Norlin [199].

## 9. Concluding remarks and future directions

This review describes the considerable progress achieved in identification of the enzymes in formation of circulating vitamin $D_3$ metabolites involved in the endocrine functions of vitamin D. Matters of incomplete understanding regarding the physiological roles of some vitamin D hydroxylases are discussed. Vitamin $D_3$ is reported to be important not only for endocrine functions, such as calcium homeostasis and bone health, but also for autocrine and/or paracrine functions in extrarenal cells. The following areas are, according to the authors, intriguing and relevant for future research.

### *9.1. Extrarenal formation of 1α,25-dihydroxyvitamin $D_3$*

The potential physiological role of extrarenal bioactivation of vitamin $D_3$ in different tissues and cells would be an area of future interest. Particularly, there is little information on e. g. the nature and roles of 1α,25-dihydroxyvitamin $D_3$ and other endogenously formed vitamin $D_3$ metabolites in various extrarenal cells. Further studies on the molecular mechanisms involved in the control of the expression of the vitamin D hydroxylases in different tissues and cells are required. The functions of vitamin $D_3$ are dependent on the bioactivating and metabolizing enzymes producing active forms of vitamin $D_3$ that can activate receptor(s) in vitamin D signaling pathways.

### *9.2. Detection of enzymatically formed vitamin D metabolites that are formed in small amounts in different cell types*

New and better methods of measuring the extrarenal enzyme activities in production of endogenous vitamin $D_3$ metabolites, formed in very small quantities, would lead to extended understanding of the role of locally formed metabolites, including those other than 1α,25-dihydroxyvitamin $D_3$. The roles of locally formed 25-hydroxyvitamin $D_3$ in extrarenal and extrahepatic cells deserve further research. Also, future research on the roles of 20-hydroxylated active metabolites is important. Studies in these areas should lead to increased basic knowledge of molecular mechanisms behind autocrine vitamin D action.

### *9.3. Receptors for 1α,25-dihydroxyvitamin $D_3$ and other active vitamin $D_3$ metabolites*

The nuclear vitamin D receptor (VDR) plays a central role in 1α,25-dihydroxyvitamin $D_3$ signaling. However, signaling by interaction of 1α,25-dihydroxyvitamin $D_3$ and other active vitamin $D_3$ metabolites with membrane receptors is less known. The role of 1α,25D3-MARRS/PDIA3, membrane-associated VDR, and potential other receptors deserve further attention. Potential cell-specific interactions of 1α,25-dihydroxyvitamin $D_3$ or other vitamin D metabolites with different vitamin D receptors may explain how vitamin D molecules can have such different actions in so many physiological processes.

### *Conflict of interest*

The authors declare no conflict of interest.

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