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HYDROXYLASE ENZYMES OF THE VITAMIN D PATHWAY: Expression, Function, and Regulation

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■ **Abstract** Vitamin D is a secosteroid that is metabolically activated and degraded through the actions of three cytochrome P450 hydroxylase enzymes. Bioactivation occurs through the sequential actions of cytochromes P450C25 and P450C1, resulting in synthesis of the pleiotropic hormone 1,25-dihydroxyvitamin D (1,25VD), which regulates over 60 genes whose actions include those associated with calcium homeostasis and immune responses as well as cellular growth, differentiation, and apoptosis. Inactivation of 1,25VD occurs by C23/C24 oxidation pathways that are catalyzed by the multifunctional cytochrome P450C24 enzyme. Both P450C1 and P450C24 are highly regulated enzymes whose differential expression is controlled in response to numerous cellular modulatory agents such as parathyroid hormone (PTH), calcitonin, interferon gamma, calcium, phosphorus, and pituitary hormones as well as the secosteroid hormone 1,25VD. Most thoroughly studied at the molecular level are the actions of PTH to upregulate *P450C1* gene expression and 1,25VD to induce the expression of P450C24. The regulatory action of PTH is mediated through the protein kinase A pathway and involves the phosphorylation of transcription factors that function at the proximal promoter of the *P450C1* gene. The upregulation of P450C24 by 1,25VD has both a rapid nongenomic and a slower genomic component that are functionally linked. The rapid response involves protein kinase C and mitogen-activated protein kinase (MAPK) pathways that direct the phosphorylation of nuclear transcription factors. The slower genomic actions are linked to the binding of 1,25VD to the vitamin D receptor (VDR) and the interaction of the VDR-1,25VD complex with its heterodimer partner retinoid-X-receptor and associated coactivators. The regulatory complex is assembled on vitamin D response elements in the proximal promoter of the *P450C24* gene and functions to increase the transcription rate.

CONTENTS

INTRODUCTION

Major industrial cities were shielded from direct sunlight during the industrial revolution of the nineteenth century owing to environmental particulates. During this period the bone-mineralizing disease rickets became nearly epidemic in children living in cities at higher latitudes. Although cod-liver oil and sunlight were used as antirachitic treatments in the 1800s (76, 96), it was not until the 1930s that the antirachitic agent was identified as vitamin D, a secosteroid whose synthesis in the skin is UV-light dependent (33, 143). Because the physiological source of vitamin D is acquired via sunlight rather than food, the term *vitamin D* has been somewhat misleading. The structure of vitamin D is derived from cholesterol (or ergosterol for plant derivatives) and resembles steroid hormones. However, the B-ring is cleaved

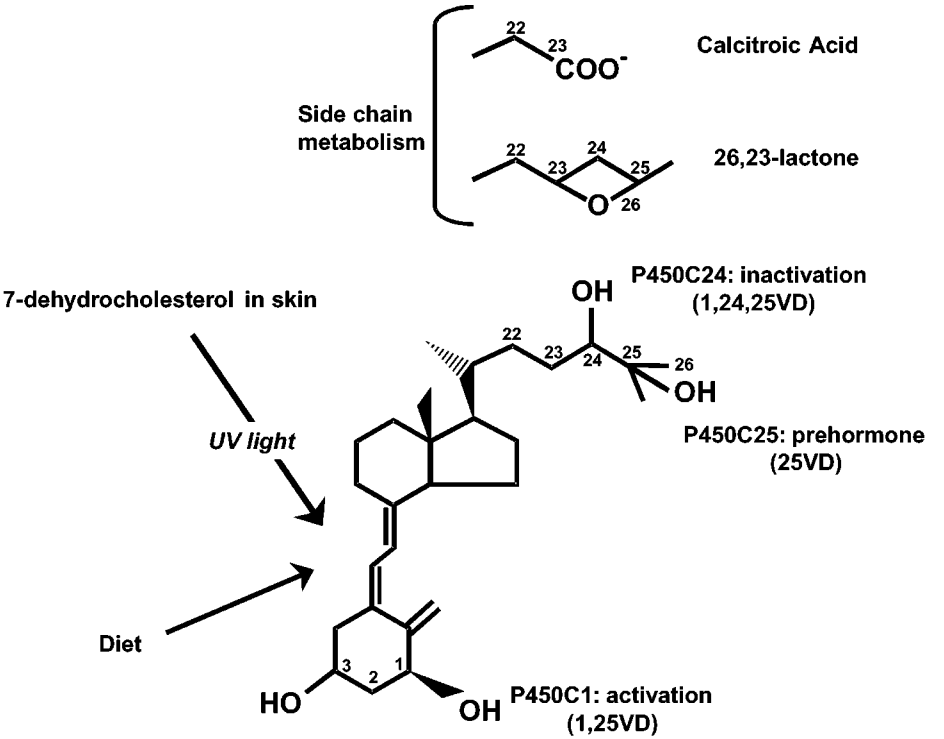

Figure 1 The structure of vitamin D metabolites. The chemical structures of major vitamin D metabolites are shown with the sites of hydroxylation and associated cytochrome P450 hydroxylases that direct the vitamin's bioactivation (sequential hydroxylations at C25 and C1) and side-chain inactivation (C23/C24 oxidation pathways).

(hence the term secosteroid) during the photolytic process of vitamin D synthesis in the skin (Figure 1). Vitamin D was demonstrated to cure rickets through the stimulation of intestinal calcium and phosphate absorption and the subsequent mineralization of bone. The tissue localization of vitamin D was determined using high-specific-activity radiolabeled vitamin D in which liver, intestine, kidney, and bone were discovered as major target sites. From these studies, it was determined that vitamin D was metabolized in tissues to a series of more polar oxidized metabolites in which the biologically inert parental vitamin was bioactivated through the addition of specific hydroxyl groups. Most notable was the modification at carbons 1 and 25 that resulted in the synthesis of 1,25-dihydroxyvitamin D (1,25VD), the hormonally active form of vitamin D (Figure 1).

Vitamins D_2 (plants) and D_3 (animals) are major forms of the vitamin that are biologically functional in man and animals with a US recommended daily allowance (RDA) of 400 IU/day that should be increased or decreased in accordance

with the level of sunlight exposure (53). Natural food products represent a limited source of the vitamin, which has led to the supplementation of selected dairy and cereal products with vitamin D that functioned to eliminate the high prevalence of vitamin D–deficiency rickets. However, rickets and the adult disease osteomalacia are still a subject of medical concern owing to sunlight avoidance, improper diet, and genetic and age-related disorders in selected populations (72). Intestinal absorption of vitamin D is mediated by bile salts and fatty acids in which vitamin D enters the plasma as a chylomicron/liproprotein complex through the intestinal-lymphatic system (16). Vitamin D synthesized in the skin is carried as a sterol-complex with the plasma vitamin D binding protein (141) in which liver is the major site for uptake of circulating vitamin.

Vitamin D[1] is bioactivated by specific hydroxylase enzymes that display preferential tissue localization. The process is initiated in the liver by the addition of a hydroxyl group at C-25 to give the prehormone 25-hydroxyvitamin D (25VD), which is the major circulating form of vitamin D (15–60 ng/ml) (Figure 2). Subsequent hydroxylation of 25VD at C-1 occurs mainly in the kidney and results in synthesis of the hormonally active 1,25VD that is released to the circulation (20–60 picogram/ml) (54) and functions to regulate cellular processes in a host of target tissues. It is now appreciated that 1,25VD is also synthesized at extrarenal sites where local production of 1,25VD can act in a paracrine/autocrine fashion to regulate cell-specific processes. Prominent actions of 1,25VD are evident in calcium homeostasis, the immune response, reproduction, and cellular differentiation and proliferation. The action of vitamin D hydroxylase enzymes to regulate circulating and cellular 1,25VD levels by enzymes of the vitamin D pathway is the topic of this review. The review focuses on properties of the vitamin D hydroxylases, the biological function of their metabolic products, notably 1,25VD and regulation of the enzymes' activity through control of gene expression.

PROPERTIES OF VITAMIN D HYDROXYLASES

The vitamin D pathway consists of three hydroxylase activities that function in a coordinated manner to metabolize vitamin D and its 25-hydroxylated metabolites. Vitamin D is initially hydroxylated at C-25, and the resultant 25-hydroxyvitamin D (25VD) functions as a substrate for the 1-hydroxylase and 24-hydroxylase enzymes (Figures 1, 2). Each enzyme has been isolated and cloned, and based on their sequence-alignment were found to contain heme-binding and other functional domains typical of cytochrome P450 hemoprotein enzymes. Therefore, the vitamin D enzymes have a P450 notation that follows nomenclature rules derived for the cytochrome P450 superfamily (83). We use the terms P450C25, P450C1, and P450C24 to describe the three enzymes. The enzymes are also referred to

[1]In this review the term vitamin D denotes both vitamin D_2 and vitamin D_3, with exceptions noted in the text.

as CYP27A1, CYP27B1, and CYP24, respectively, where CYP represents cytochrome P450. Each enzyme is discussed with respect to its cellular expression, sequence characteristics, and catalytic properties.

25-Hydroxylase (P450C25)

Using radiolabeled vitamin D, it was possible to establish the liver as the major tissue for expression of vitamin D 25-hydroxylase (105) (Figure 2). Subsequent studies revealed a broader tissue distribution of 25-hydroxylase activity including skin, kidney, and intestine. Initial subcellular localization studies in the liver found most enzyme activity associated with the microsomal fraction (15), although activity was also evident in mitochondria. Work on the mitochondrial 25-hydroxylase enzyme converged with previous studies of the sterol 27-hydroxylase from the bile-acid pathway (i.e., P450C27 or CYP27A1) (6). Although P450C27 displays preferential cholesterol-27-hydroxylase activity in the bile-acid pathway, it also displays a host of minor activities (103), one of which involves the 25-hydroxylation of vitamin D. Therefore, the enzyme originally referred to as mitochondrial 25-hydroxylase (P450C25) is in reality P450C27. However, the P450C25 notation is used in this review to retain clarity when discussing 25-hydroxylase activity within the vitamin D pathway.

The human microsomal P450C25 enzyme has yet to be identified; however, the pig microsomal enzyme has been isolated and characterized (55). The primary structure of the porcine microsomal enzyme differs significantly from CYP27A1 and it displays $>70\%$ similarity to the CYP2D family. It is, therefore, named CYP2D25 and contains an endoplasmic-reticulum targeting sequence and a P450-reductase binding site for electron transfer used in the microsomal hydroxylation process. The enzyme 25-hydroxylates both vitamin D_2 and D_3 in contrast to the pig mitochondrial CYP27A1 enzyme, which does not hydroxylate vitamin D_2.

The mitochondrial and microsomal enzymes have strikingly different Km values (i.e., substrate required for activity at one half of the maximal velocity). The Km for P450C27 with vitamin D as substrate is $\sim$3–10 μM, whereas the pig microsomal enzyme has a higher substrate affinity (Km $\sim$0.1 μM) (142), which is nearly identical to the $\sim$0.1 μM concentration of vitamin D in the liver (55). Therefore, the microsomal enzyme seems quite capable of functioning at the physiological substrate concentration in the liver, whereas the mitochondrial enzyme would appear to operate far below its preferred higher-substrate level, thereby functioning as a minor contributor to the circulating 25VD pool. A minimal role for the mitochondrial enzyme is supported further by results in which no changes were observed in serum 25VD levels for patients with a bile-acid genetic enzyme disorder (cerebrotendinous xanthomatosis) (18) or for mice whose *CYP27A1* gene was inactivated (110). Resolution of the conflict regarding the "real" 25-hydroxylase in the vitamin D pathway requires the isolation and characterization of the human microsomal enzyme.

1-Hydroxylase (P450C1)

The 1-hydroxylase enzyme (Figures 1, 2) is expressed in a number of tissues that include kidney, skin, intestine, macrophage, and bone (150). Enzyme expression is highest in kidney, where induced mRNA levels can be 40–50-fold higher than those observed in extra-renal sites (J Omdahl, personal observation). Enzyme expression occurs in both the proximal and distal convoluted tubules. An endocrine function has been ascribed to the proximal site of hormone synthesis, whereas a local or autocrine/paracrine function has been attributed to the distal tubule 1,25VD synthesis (20, 149). The primary sequence of the 1-hydroxylase enzyme was derived from cloning data for the rat and mouse proteins (119, 124, 127). The enzyme ($\sim$52 kDa) is a mixed-function oxidase that displays the hallmark features of mitochondrial P450 enzymes. It is attached to the inner mitochondrial membrane, receives NADPH-reducing equivalents from an iron-sulfur protein, and uses molecular oxygen during the hydroxylation catalytic cycle. P450C1 has greatest sequence similarity to CYP27A1, from which the CYP27B1 notation was derived (119). P450C1 is highly selective for 25-hydroxylated vitamin D metabolites and is not catalytically active with adrenal or gonadal steroids. The broad specificity P450C27 enzyme (i.e., CYP27A1) expresses a low level of 1-hydroxylase activity (115). However, P450C1 is the major enzyme for 1,25VD synthesis, because circulating levels of 1,25VD are not maintained in the mouse *P450C1*-gene knockout model, in which *P450C27* gene expression is unaltered (32, 98).

P450C1 catalyzes the C-1 hydroxylation of 25-hydroxylated vitamin D secosteroid substrates. The bioactivation of 25VD to 1,25VD proceeds at variable rates owing to regulation of the enzyme's expression level. The apparent affinity of the cellular or partially purified enzyme for 25VD differs between species and tissues with Km values ranging from 1 to 16 μM (46, 82, 114), suggestive of a broad level of vitamin D requirements. The substrate concentration for optimal catalysis can differ with altered metabolic states as observed in chronic uremia in which apparent Km values can increase several-fold in monocytes (42). The major circulating dihydroxy metabolite 24,25-dihydroxyvitamin D (24,25VD) is also readily hydroxylated by P450C1. The dihydroxy metabolite is the preferred substrate compared with 25VD; however, owing to the $\sim$10-fold higher concentration of 25VD, the rate of 1,25VD synthesis is greater than that observed for its counter product 1,24,25-trihydroxyvitamin D.

24-Hydroxylase (P450C24)

The 24-hydroxylation of 25-hydroxyvitamin D is a major enzyme activity in cell lines pretreated with 1,25VD (93). Nearly all cells express 24-hydroxylase activity in vivo, and similar to the 1-hydroxylase enzyme, the highest activity is observed in the kidney. In the rat, P450C24 expression is observed predominantly in the proximal tubule of the kidney, with limited expression in more distal segments (57). Using 24-hydroxylase antibody to the purified rat protein, the enzyme was cloned and determined by derived-primary-sequence data to be a member of the

cytochrome P450 superfamily (90). Although similar in size (~53 kDa) to existing mitochondrial P450s, its sequence is different enough to constitute a new member of the P450 family, and therefore the enzyme was given the cytochrome notation of CYP24 (or P450C24). The substrate binding domains for the P450C24 and P450C1 enzymes have low similarity, whereas other regions of the enzymes have high sequence homology. Such differences are to be expected when considering the diametric orientation of substrate molecules within the different secosteroid-binding sites for the two enzymes. It is evident from similarity molecular-modeling studies that the side-chain of the 25-hydroxy substrate is aligned with heme in the substrate-binding pocket of P450C24. In contrast, the substrate's A-ring occupies the heme-active site in P450C1, and the side-chain domain extends away from the active site into the substrate access channel (45; J Omdahl & K Bobrovnikova, personal observation).

P450C24 is a multicatalytic enzyme that catalyzes the side-chain oxidation of 25-hydroxyvitamin D metabolites, notably 25VD and 1,25VD. The vitamin D 24-hydroxylase is distinct from the cholesterol 24-hydroxylase (CYP46) (73), although both enzymes catalyze side-chain hydroxylations at C24. The catalytic P450C24 process is initiated by side-chain hydroxylation of 1,25VD at C-23 or C-24, which shows species preference. The C-23 pathway is preferentially expressed in humans and the guinea pig (14, 100, 113) and involves sequential 23- and 26-hydroxylations followed by formation of a 26,23-lactol and the final 25VD-26,23-lactone product (Figure 1). Oxidation by the C-24 pathway is the main activity of the rat enzyme and is a secondary pathway expressed by the human enzyme (14, 113). Side-chain cleavage occurs in the C-24 pathway and involves five separate steps that begin with 24-hydroxylation, followed by oxidation to C24-oxo, 23-hydroxylation to C23-OH/C24-oxo, followed by side-chain cleavage to the C23-alcohol, and finally oxidation to the C23-carboxylic acid. Four aliphatic hydrocarbons are cleaved in the C-24 pathway during the oxidation of 1,25VD, which results in synthesis of the water-soluble oxidized-end-product calcitroic acid that is filtered and excreted in the kidney. Similar C23/C24 oxidation pathways exist for 24,25VD (90). Interestingly, the side-chain cleavage of plant vitamin D_2 metabolites (i.e., those that contain a double bond at C22 and a methyl group at C24) with rat P450C24 does not proceed past the initial 24-hydroxylation step (R. Horst, personal communication). In this case, it appears that other enzymes are required for the complete side-chain metabolism of D_2 metabolites. The importance of the correct chemistry at C23 and C24 for side-chain metabolism is further evident from studies in which a C23-yne (triple bond) modification inhibits side-chain metabolism (109).

Apparent cellular kinetic measurements for P450C24 have addressed the enzyme's initial C24 hydroxylation step, with no information currently available on rate constants for the later four reactions in the multi-step process. Apparent Km values for the 24-hydroxylation of 25VD range from ~0.5 to 3 μM (25, 91, 129), whereas the enzyme appears to display a preference for 1,25VD with Km values that are approximately 10-fold lower (0.1–0.25 μM) (25). However, recent

results with mitochondrial-extracted rat enzyme reported a higher preference for 25VD than for 1,25VD (129). Yet, substrate-induced spectral shifts with purified rat P450C24 show a higher affinity for 1,25VD binding (J Omdahl & K Bobrovnikova, personal observation) (92), which supports the concept that 1,25VD is the preferred substrate for P450C24 and that the enzyme plays an important role in controlling ambient 1,25VD levels.

VITAMIN D P450s AND METABOLITE IN HEALTH AND DISEASE

Most biological functions for vitamin D are directed by 1,25VD, whose synthesis is regulated by the P450C1 bioactivation enzyme. The 1,25VD hormone acts in the regulation of (*a*) calcium homeostasis; (*b*) cellular differentiation, growth, and apoptosis; and (*c*) the immune system. The pleiotropic actions of 1,25VD are mediated through the VDR as described in the regulatory section later. Several articles address target-cell actions of 1,25VD that are not addressed in this review (24, 59, 87, 102, 126).

Calcium Homeostasis

Ambient calcium is highly regulated in order to support its roles in bone mineralization and cellular processes that include neuromuscular activity, intracellular signal transduction, and blood coagulation. Central regulators of this endocrine system are parathyroid hormone (PTH) and 1,25VD, which function coordinately to stimulate the cellular uptake and retention of calcium. The synthesis and secretion of PTH is stimulated in response to the parathyroid gland's calcium sensing receptor, which detects decreases in extracellular calcium levels and initiates the synthesis and secretion of PTH (94). PTH acts in the kidney to stimulate transcellular calcium reabsorption (41) and inhibit renal-phosphate reabsorption by decreasing Npt2 transporter expression (130). PTH also upregulates P450C1 expression in the kidney (discussed in regulatory section). In bone, 1,25VD stimulates osteoclast biogenesis (133) and PTH stimulates the cell's bone-resorptive function (44). Such actions lead to a net increase in serum calcium when coupled with PTH's inhibition of renal phosphate reabsorption. The secosteroid hormone can also express a minor action to stimulate phosphate reabsorption (130) that is significantly less than PTH's inhibitory action. The active absorption of dietary calcium and phosphorus from the intestine is mediated by 1,25VD through specific calcium and phosphorus transport systems (52, 130). Bone mineralization is indirectly linked, therefore, to the action of 1,25VD to maintain ambient calcium and phosphate levels required for the mineralization of extracellular bone matrix. Increased levels of 1,25VD and calcium function in a coordinated manner to suppress PTH synthesis and secretion, which in turn functions to feedback-inhibit the development of hypercalcemia and attendant tissue damage.

Cellular Differentiation

P450C1 is expressed in mice embryonic stem cells and the fetal tissues of kidney, bone, and intestine. Such an observation is consistent with the linkage of 1,25VD to cellular development and differentiation (97). Such an action for P450C1 appears to be associated with the action of 1,25VD to sustain cellular calcium needs during development. For example, in mice there is no obvious effect on growth and development in the fetal period for pups that lack P450C1 or its VDR effector or both (i.e., null mice) owing to calcium nutriture provided by the mothers. Abnormal growth and development is only observed after birth when the placental nutrient support is removed, which can be corrected by feeding the pups high-calcium diets (58; R. St. Arnaud, personal communication).

The hormone 1,25VD dramatically influences the in vitro differentiation of many normal and cancerous cells (e.g., epithelial cells, keratinocytes, mammary, prostate, and colon cells). Skin keratinocytes are the main model used to document the action of 1,25VD to promote differentiation in nonmalignant cells. Cultured keratinocytes contain all enzymes required for the synthesis of 1,25VD from 7-dehydrocholesterol (67, 116). Acting by an autocrine-type function, the locally synthesized 1,25VD promotes cellular differentiation through a calcium-dependent process that involves phospholipases C and D and protein kinase C (17, 21). The 1,25VD-dependent elevation in cellular calcium is mediated through the calcium sensitive receptor that results in the synthesis of specific proteins associated with cellular differentiation (132). Interestingly, 1,25VD is used as an antiproliferative agent in the treatment of psoriasis even though psoriatic skin expresses P450C1 throughout the dysregulated growth area. Such an observation is consistent with a limited availability of 25VD substrate to the psoriatic cells (49).

Cancer

Expression of vitamin D hydroxylases in malignant tissue displays a variable pattern. In general, P450C1 expression decreases with tumor progression, as observed in colon and prostate cancer (13, 56). Suggested by such results is the possibility that sustained 1,25VD biosynthesis could function as a deterrent to cancer onset and progression. In that regard, an inverse relationship has been established between UV-sunlight exposure, dietary vitamin D, and various cancers (71, 117). These observations are the basis for current studies on the preventive and therapeutic actions of 1,25VD in cancer. The hormone's actions to inhibit the growth and differentiation of cancer were observed 20 years ago in leukemia and melanoma cells (1, 31). Several different signaling pathways (i.e., membrane and nuclear receptors) are involved in the anticancer actions of 1,25VD. The hormone is documented to regulate cell-cycle regulators (e.g., cyclin-dependent kinases), growth factors (e.g., inhibition of IGF-1 signaling pathway), apoptosis (e.g., bcl-2 down-regulation), differentiation (e.g., increases with a decrease in growth), telomerase (e.g., decreased activity), and the metastatic cascade (e.g., decreased invasive action) (48). Recent studies have addressed the synthesis of 1,25VD-analogs that

retain the hormone's anticancer action without its attendant hypercalcemic actions. Analogs with demonstrated clinical efficacy include seocalcitol (EB1089, a dimethyl side-chain analog) and maxacalcitol (OCT, a 22-oxy side-chain analog), as well as analogs containing C-16 and C-19 derivatives (48).

Immune System

P450C1 activity is present in peripheral macrophages, precursor monocytes and granulomatoses (36). P450C24 is present in peripheral monocytes; however, differentiation of monocytes into macrophages results in suppression of P450C24 expression by an interferon-gamma–mediated process. The 1,25VD receptor (i.e., VDR) for mediating the autocrine actions is expressed by most immune cells in which the hormone functions as a strong immunomodulatory agent. The immune regulatory function of 1,25VD is evident by its action in several animal models to prevent autoimmune diseases, extend graft survival (28), and downregulate immune responses in general (95). Immunosuppresive actions of 1,25VD appear to be mediated through dendrite cells in which the hormone inhibits the differentiation and maturation of dendrite cells that result in decreased T-cell responsiveness (101). The lack of 1,25VD in P450C1 null mice lead to the development of ectopic CD4+ lymph nodes and an alteration in the distribution of peripheral T cells, which is consistent with a role for 1,25VD in modulating immune function. Cytokine production in activated T-lymphocytes is also regulated by 1,25VD in which the hormone can induce actions that are both stimulatory (IL-4 and transforming growth factor-beta) and inhibitory (IL-2 and interferon-gamma) (5, 26).

The autoimmune disease sarcoidosis can be linked to hypercalcemia and high serum levels of 1,25VD. P450C1 over-expression and excess 1,25VD synthesis occur in the associated hyperactive macrophages (2). P450C1 is elevated to such an extent that the sarcoid tissue can express a hormone-endocrine function that accounts for elevated circulating levels of 1,25VD in sarcoidosis. Owing to the lack of negative feedback by 1,25VD, the P450C1 in sarcoid tissue functions as a constitutively activated enzyme without a limiting P450C24 degradative pathway (36).

Deletion of P450C1 Activity

The pleiotropic actions of 1,25VD are clearly evident from human *P450C1* genetic disorders and enzyme knockout studies in the mouse (32, 62, 98). The autosomal-recessive genetic disorder pseudo vitamin D–deficiency rickets has been linked to mutations in the *P450C1* gene locus on human chromosome 12 (12q13.2–13.3) that are associated with an inefficient or dysfunctional P450C1 enzyme in humans (65, 124, 139) and the pseudo vitamin D–deficiency rickets Hannover pig model (29).

Individuals with pseudo vitamin D–deficiency rickets are normal at birth but develop symptoms of vitamin D deficiency that include low calcium, phosphorus, and 1,25VD serum values and elevated serum PTH with low bone mineralization,

all of which are associated with normal circulating vitamin D levels (139). Similar characteristics were observed in vivo for P450C1 knockout (null) mice, in which no circulating 1,25VD could be detected and rachitic bone lesions were evident (32, 98).

The ovary has been observed by RNase-protection and PCR analysis to contain a significant level of *P450C1* mRNA (J Omdahl, personal observation) (3). A role for vitamin D in reproductive physiology was also evident in the P450C1-null mice studies in which supplemental dietary calcium was required for reproduction by mice homozygous for the gene deletion (R. St. Arnaud, personal communication; 32, 98). Similar observations were also observed in VDR-null mice in which reproductive difficulties were corrected by calcium supplementation (58). Such findings are consistent with a decreased reproductive capacity observed in vitamin D–deficient rats (47) and suggest that low cellular calcium is a contributory factor to the vitamin D–directed reproductive aberrations in mice knockout models for VDR and P450C1. However, tissue-specific inactivation studies of the *P450C1* gene are required in order to determine whether the enzyme's expression is functionally critical as part of a 1,25VD paracrine/autocrine system in specific tissues and organs.

It is also noteworthy that alopecia is observed in humans with hereditary vitamin D–resistant rickets (mutations in VDR) and also in VDR-null mice, but the hair follicle disorder is not observed in humans or mice with a dysfunctional *P450C1* gene. A high-calcium, high-lactose diet normalizes the phenotype of VDR-null mice with the exception of alopecia (68). Molecular aspects of these findings are discussed later.

Deletion of P450C24 Activity

In contrast to mutations in the *P450C1* gene, there are no diseases that implicate a genetic or acquired disorder in the *P450C24* gene. However, the *P450C24* gene has been identified by comparative-genomic-hybridization analysis as a candidate gene for breast cancer (4), which suggests that the enzyme could have a role in the dysregulation of cell growth through the lowering of cellular 1,25VD. To address such possibilities, mice null for the *P450C24* gene have been developed and studied (123). Homozygote mice exhibited a high level of circulating 1,25VD with associated signs of vitamin D toxicity including soft-tissue calcification in the kidney. It has been speculated that P450C24 synthesis of 24,25-dihydroxyvitamin D (24,25VD) may be associated with a physiological role for the metabolite in bone development. In apparent support of this, it was observed that intramembranous bone formation was impaired in *P450C24*-null mice. However, this phenotype was rescued by mating the mice with VDR-null mice (i.e., VDR is required for the major actions of 1,25VD), indicating that the bone abnormality was due to elevated 1,25VD and not to the lack of 24,25VD. In the context of these studies, a possible role for 24-hydroxy metabolites in bone mineralization seems less likely. Nevertheless, a seminal role is clear for P450C24 to modulate the normal ambient level of circulating 1,25VD and therefore intracellular calcium levels.

ACTIVATION OF TARGET GENES BY 1,25-DIHYDROXYVITAMIN D

Genomic Actions

The regulation of >60 genes by 1,25VD occurs through alterations in the rate of target gene transcription. Transcription regulation is initiated by 1,25VD binding to the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily that acts as a ligand-inducible transcription factor (49, 59, 74, 102). Ligand-bound VDR functions as a heterodimeric complex with the 9-cis retinoic acid nuclear receptor retinoid-X-receptor (RXR). VDR comprises a DNA binding domain with two zinc fingers linked to the ligand binding domain that binds 1,25VD and interacts with RXR (102). The liganded heterodimeric VDR/RXR complex binds to a specific control element [vitamin D response element (VDRE)] in the promoter (i.e., regulatory region) of target genes. A large coactivator complex subsequently assembles on the heterodimeric complex and functions to both remodel locally condensed chromatin and to communicate with RNA polymerase II located at the transcriptional start site (75, 81). Such actions function to alter the rate of gene expression (Figure 3). Many coactivator complexes for VDR-mediated transcription have been identified (106, 107, 151). These coactivator complexes probably act in a two-step process. The first coactivator complex with histone acetyltransferase activity modifies chromatin and is then replaced by another coactivator complex that lacks this enzyme activity but interacts with RNA polymerase II (108).

With most or all 1,25VD-target genes, unliganded VDR/RXR first binds to the VDRE to recruit a corepressor, which represses basal transcription through an associated histone-deacetylase whose activity functions to facilitate a closed chromatin structure (37, 104). Induction by 1,25VD then proceeds from this suppressed level when subsequent 1,25VD binding to VDR leads to replacement of the repressor by the coactivator complex. That is, VDR can adopt a dual role of acting as a repressor in the absence of ligand and then subsequently as a coactivator when ligand binds.

In the context of VDR acting in the absence of ligand, it is relevant to mention here that alopecia is observed in VDR-null mice but not P450C1-null mice, and recent data support the contention that VDR is important for prevention of alopecia but in a 1,25VD independent manner (112). Perhaps the unliganded VDR represses basal activity of certain genes in keratinocytes. Alternatively, unliganded VDR could increase expression of certain genes through binding of specific coactivators. Such a precedence exists in which the thyroid hormone receptor binds coactivators in a thyroid-hormone independent manner (89).

Nongenomic Actions

The genomic action of 1,25VD can be preceded by more rapid nongenomic actions that occur in minutes and involve membrane-associated events such as increased calcium transport and protein kinase C (PKC) and MAPK activation (84–88, 122).

It appears that 1,25VD acts through a plasma-membrane-associated VDR linked to intracellular signaling pathways. We have observed in kidney COS-1 cells that 1,25VD addition leads to a rapid induction of PKC and MAPK activities, and these nongenomic actions are critical for subsequent transcriptional activation of *P450C24* gene transcription (PP Dwivedi, GE Muscat, PJ Bailey, JL Omdahl, & BK May, submitted). The end result of the nongenomic action, therefore, is an alteration of gene transcription. Based upon sequence analysis of the nuclear VDR, there does not appear to be a membrane-spanning domain. However, it is possible that the VDR could associate with a tyrosine kinase receptor or G protein coupled receptor on the interior face of the plasma membrane as documented for estrogen (61). Alternatively, there may be a novel VDR protein (85, 87). Identity of the putative plasma membrane VDR is currently a major issue.

Studies with VDR-null mice have provided molecular insights to the action of 1,25VD. Osteoblasts prepared from the calvaria of VDR-null mice, in which the second zinc finger of the DNA binding domain of VDR was ablated (69), displayed nongenomic activities in response to 1,25VD (138). On the other hand, 1,25VD-directed nongenomic responses were abrogated in osteoblasts prepared from *VDR*-null mice in which the first zinc finger of the DNA was deleted (40). It is possible that the first but not the second zinc finger is required for functional activity of the nuclear VDR at the membrane.

Vitamin D Analogues and Coactivators

Synthetic vitamin D analogues that bind to VDR have been synthesized and are attracting considerable attention as possible therapeutic agents in hyperproliferative disorders (48). Many of these analogues are vastly more potent than the natural ligand as inhibitors of cell growth and activators of cell differentiation, yet they remarkably have less severe calcemic side effects. The mechanism that separates the calcemic activities from the differentiation-inducing activities of vitamin D analogues is currently of interest. In this regard, the most popular hypothesis involves an analogue-dependent conformation change in VDR that favors the binding of a specific coactivator. Availability of a specific coactivator within the cell would determine whether a particular set of genes is activated selectively by the analogue. However, this proposal has been excluded at least for two 20-epi vitamin D analogues. The crystal structures of the ligand-binding domain of VDR occupied with either 1,25VD or with the vitamin D analogues KH1060 or MC1288 have shown that the protein's conformation is identical for all three ligands (134). However, these analogues make new contacts within the ligand-binding domain that provide the basis for increased stability, a longer half-life, increased transcriptional activity, and also resistance to limited in vitro proteolytic digestion of VDR with bound analogue (27). These results do not provide a ready explanation for selective transcriptional activity. Recently, a variant of human VDR (126) was identified, and this could play a role in preferential activation of genes. The different contacts between VDR and vitamin D analogues form the basis for the recent treatment of hereditary vitamin D–resistant rickets with 1,25VD analogues (43).

NUTRIENTS AND HORMONES IN THE MOLECULAR REGULATION OF RENAL VITAMIN D HYDROXYLASES

It is generally considered that 25VD production in the liver is unregulated and dependent on vitamin D substrate availability. However, hepatic and renal CYP27A1 activity is altered in response to gender, development, and sunlight exposure (132). Also, the half-life and expression of mRNA for mitochondrial CYP27A1 is inhibited by 1,25VD in kidney and intestine but not liver (12, 132). There are no reported studies on regulation of the pig microsomal 25-hydroxylase (CYP2D25), the key enzyme thought to be involved in hepatic 25VD production.

Under normal physiological conditions, the level of circulating 1,25VD is stringently controlled by regulating renal P450C1. PTH, calcium, phosphate, 1,25VD, and calcitonin function to regulate the enzyme's expression. The two most important physiological regulators are PTH and 1,25VD. Renal CYP24 expression is also controlled by these regulators but in a reciprocal manner (59, 93). This section on renal enzymes focuses on P450C1 and P450C24, because P450C25 appears to be of minor importance.

Parathyroid Hormone

PTH acts to increase *P450C1* mRNA levels in the kidney (80, 124). The mRNA upregulation involves a cAMP dependent protein kinase A activity pathway (80). Increased activation of the human *P450C1* promoter underlies the PTH response as revealed by transient expression analyses (22, 66, 79). Sequence analysis of the *P450C1* promoter has revealed several binding sites for transcription factors that could be phosphorylated by cAMP (22, 66). A PTH target site has been identified in the −0.5 kb region of the human *P450C1* promoter that involves a novel vitamin D inhibitor receptor (VDIR) present in kidney (78). It is proposed that VDIR functions in the absence of 1,25VD and in the presence of protein kinase A to upregulate *P450C1* gene expression. When present, 1,25VD would allow the VDR/RXR heterodimeric complex to interact with VDIR and inhibit gene expression by promoting recruitment of a corepressor (78).

Whereas PTH induces renal P450C1 expression, it functions in a diametric manner to inhibit renal P450C24 expression (118). PTH represses 1,25VD-induced expression of *P450C24* mRNA in the porcine kidney cell line AOK-B50, an action that can be mimicked by forskolin (153). This repression was not due to changes in VDR level that indirectly could modulate P450C24 induction. The first 1.4 kb of the *P450C24* promoter was insensitive to PTH action as determined by transient reporter assays. This suggested that PTH may act posttranscriptionally, and a preliminary report indicates that PTH action leads to a decrease in the stability of the *P450C24* mRNA (152) and thereby the enzyme's functional level in the kidney. Such an action functions to upregulate the renal-endocrine process involved with hormone release into the ambient circulation.

Calcium

There is evidence that extracellular calcium modulates P450C1 expression by a mechanism independent of PTH. In parathyroidectomized PTH-replete rats, the elevation of blood ionized-calcium inhibits circulating 1,25VD levels, indicating a role for ambient calcium in regulating renal P450C1 expression (140). Based upon studies with kidney cell lines, it has been possible to demonstrate a reciprocal relationship between extracellular calcium and P450C1 expression (19, 20). These cells express calcium sensing receptor protein, which monitors calcium fluctuations and may link P450C1 expression with external calcium levels (20, 51). Several downstream signaling pathways including PKC and MAPK are known to be activated by the calcium sensing protein (64) and could result in the activation of the P450C1 promoter. P450C24 activity was also unexpectedly increased in the presence of low calcium (19, 20), which remains to be clarified.

1,25-Dihydroxyvitamin D

The action of PTH to induce P450C1 expression in the kidney and in kidney cell cultures is subject to negative feedback-inhibition by 1,25VD (79, 80, 127). VDR is essential for the hormone's inhibitory action as observed in VDR-null mice in which no downregulation of P450C1 expression was observed in the presence of high 1,25VD levels (127, 128). Several negative VDREs have been reported (e.g., PTH gene) (111). Although PTH induction of the human *P450C1* promoter in kidney is inhibited by 1,25VD, no sequence related to known negative VDREs has been identified. As mentioned above, a novel mechanism of repression has been proposed in which a VDIR bound to the *P450C1* promoter facilitates recruitment of a corepressor in the presence of 1,25VD (78). It is interesting that the high levels of circulating 1,25VD seen in P450C24-null mice presumably reflect a lack of inhibition of renal P450C1 by 1,25VD (123). Levels of renal VDR are not suppressed by the high serum 1,25VD (123). Perhaps the repression mechanism only operates on PTH induced expression of P450C1, although the mechanism remains to be determined.

The feedback repressive effect of 1,25VD on P450C1 expression is accompanied by a marked induction of P450C24 expression in the kidney, thereby providing additional control of circulating 1,25VD levels (3, 93). P450C24 induction is dependent on ligand-charged VDR and in both P450C1 and VDR-null mice, expression of P450C24 is markedly reduced (39, 127). The rat *P450C24* promoter is unique among currently characterized 1,25VD responsive promoters in that it contains two tandem VDREs (63, 92). In the absence of 1,25VD, the heterodimer VDR/RXR binds to one or both VDREs to recruit a corepressor and lower basal expression (37, 104). Upon ligand binding to the VDR, the corepressor is replaced by a coactivator complex. At elevated levels of 1,25VD, both VDREs are utilized in a synergistic manner to ensure high levels of P450C24 expression and efficient side-chain inactivation of the hormone (63). The ubiquitous protein

Ets-1, a member of the Ets family of transcription factors (146), binds adjacent to the proximal VDRE and contributes to 1,25VD-dependent transactivation (38). For this activity, Ets-1 must be phosphorylated at threonine residue 38 (38).

Our recent studies have demonstrated that 1,25VD-directed nongenomic responses are critical for the transcriptional induction of the *P450C24* gene in COS-1 kidney cells (P Dwivedi, C Hii, A Ferrante, C Der, J Omdahl, H Morris & B May, submitted). We have found that 1,25VD treatment results in the induction within minutes of the ERK1/2 and ERK5 MAPK pathways (99). Specific blockage of these pathways inhibited phosphorylation of Ets-1 and RXR alpha, and 1,25VD induction of the *P450C24* promoter (Figure 3). Specific antagonists of PKC inhibit 1,25VD induction of *P450C24* promoter activity, whereas PKC activators (e.g., the phorbol ester TPA) function to enhance the 1,25VD induction of P450C24 expression in kidney cultures (8, 30, 93). These data can now be explained as a 1,25VD-dependent stimulation of PKC and subsequent activation of the downstream MAPK pathways.

Phosphate

Hypophosphatemia in rats caused by phosphate deprivation results in increased serum 1,25VD with induction of renal *P450C1* mRNA and activity (148). The induction mechanism is independent of PTH, which is usually reduced under low dietary phosphate conditions (121). It has been postulated that phosphate regulation of 1,25VD synthesis is dependent on phosphate transport by renal tubules. However, this does not seem to be the case, because dietary phosphate continues to appropriately regulate renal P450C1 expression in hypophosphatemic mice that are null for the Npt2 phosphate transporter (131). Hypophysectomy completely abolishes the induction of renal *P450C1* mRNA in hypophosphatemia; however, enzyme induction can be partially restored by administration of growth hormone (148). Hence, under low phosphate concentrations, the signal for the kidney to synthesize more 1,25VD is likely to be transduced via hormones of the pituitary gland. Growth hormone replacement in hypophysectomized rats only partially restores *P450C1* gene expression; therefore, other factors must be involved in the complex process.

Dietary phosphate restriction leads to a substantial decrease in renal *P450C24* mRNA and activity (144). Despite the large increase in circulating 1,25VD, owing to increased P450C1 during hypophosphatemia, *P450C24* mRNA levels are not induced but lowered. This resistance to 1,25VD induction of P450C24 can be attributed to a reduced VDR content. In support of this concept, VDR mRNA levels decreased in parallel with *P450C24* during phosphate restriction (144). The precise molecular mechanism by which phosphate alters *P450C24* (or *VDR*) mRNA expression remains to be established.

X-linked hypophosphatemia in humans is the most commonly inherited disorder of phosphate transport and is characterized by rachitic bone disease, hypophosphatemia, and renal defects in the reabsorption of phosphate. X-linked

hypophosphatemia (Hyp and Gy) mice with this disorder respond to dietary phosphate restriction in a paradoxical fashion, with a decrease in both 1,25VD and renal P450C1 activity rather than the expected increase (131). In X-linked hypophosphatemia there are deletions in the Phex gene, which encodes an endopeptidase that may lead to an increase in the recently identified FGF23 protein. This protein is a member of the fibroblast-growth-factor family and a possible candidate for the elusive phosphate-transport hormone *phosphatonin* (125). An increase in FGF23 could, therefore, be responsible for two independent renal effects that involve the inhibition of renal phosphate uptake and the dysfunctional regulation of renal P450C1 expression. How FGF23 would modulate P450C1 expression is not clear.

Calcitonin

Calcitonin stimulates P450C1 in distal parts of the nephron under normocalcemic conditions (120). In support of this observation, 1,25VD synthesis is increased by calcitonin in a cortical-collecting-duct cell line but not in a proximal tubule cell (20). The mechanism by which calcitonin upregulates *P450C1* mRNA production through the hormone's receptor involves the PKC signaling pathway rather than the protein kinase A pathway employed by PTH (80, 147). The *P450C1* promoter control elements that direct activation have not been identified (79). Interestingly, calcitonin does not affect expression of P450C24 in the cortical-collecting-duct cell line in which the hormone induces P450C1 expression (20).

Lipopolysaccharide

Recently, it was revealed that the mitogen lipopolysaccharide (LPS) promotes a potent induction of P450C1 activity in the cortical-collecting-duct cell line (HCD) but has no effect in the proximal tubular cell line HKC-8 (20). Unlike PTH induction, the inductive action of LPS was insensitive to feedback inhibition by 1,25D in HCD cells. The molecular reasons underlying this lack of inhibition by 1,25VD become apparent when the action of LPS on the promoter is defined. The response of HCD cells to LPS resembles that of activated macrophages as described below. It can be speculated, therefore, that in the distal nephron P450C1 fulfills an immunomodulatory action (20).

NONRENAL REGULATION OF VITAMIN D HYDROXYLASES

Intestine

The mRNA for *P450C1* is higher in mouse fetal intestine than in adult, which is consistent with the adult tissue's low contribution to circulating 1,25VD (97). *P450C1* mRNA and enzyme activity are present in normal and malignant colon tissue, and locally produced 1,25VD may be important for cellular proliferation and

cancer protection. PTH plays no role in the regulation of vitamin D hydroxylases in the intestine, which lacks the PTH receptor but expresses VDR. The intestine is a major site of P450C24 induction by 1,25VD. In vivo there are differences in the kinetics and levels of induction of *P450C24* mRNA in the intestine compared with kidney (34, 50). Induction in the intestine is more responsive to acute changes in secosteroid level. Such induction is likely to be important for downregulating transient elevations in circulating 1,25VD. As observed in kidney, phorbol ester and PKC activity enhances the action of 1,25VD to induce P450C24 expression in intestinal cells (7, 9).

Osteoblasts

Osteoblasts express both VDR and PTH receptors. It is evident from in situ hybridization studies that *P450C1* mRNA is expressed in osteoblast cells (97). Locally produced 1,25D is likely to be important for controlling the proliferation and differentiation of osteoblast cells (70). We have also shown that *P450C1* mRNA can be induced by PTH in primary cultures of chick bone cells (J Omdahl & W Ramp, unpublished), perhaps reflecting the roles of 1,25VD in osteoblast differentiation and bone resorption.

P450C24 mRNA is induced in UMR-106 osteoblast cells by 1,25VD, but unlike the hormone's actions in kidney and intestinal cells, PKC activity is not involved (11, 145). In contrast, PTH has a clear action in UMR-106 cells that involves PKC isozymes (35), which may play a role in the synergistic action between 1,25VD and PTH in UMR-106 cells (7, 10). The synergistic action of PTH to enhance the *P450C24* inductive action of 1,25VD is ultimately expressed at the transcriptional level as shown by promoter-transfection studies in our laboratory and is not likely due to increased VDR amounts (B May & J Omdahl, unpublished). The precise promoter site for PTH's synergistic action is currently under investigation. Synergism would produce a decrease in cellular 1,25VD attendant to an increase in the production of 1,25VD oxidation metabolites by P450C24, which collectively could exert specific biological actions in osteoblasts (50, 135, 136).

Macrophages

PTH and calcitonin have no effect on *P450C1* mRNA expression in macrophages as expected owing to the lack of hormone receptors. However, the immune stimulator interferon-gamma (INF-γ) stimulates *P450C1* mRNA and enzyme production (77, 95). INF-γ is known to stimulate gene expression through the JAK-STAT (Janus kinase-signal transducers and activators of transcription) pathway with activation of STAT1 homodimers (23). However, no consensus sequence for STAT1 homodimer binding is present in the human *P450C1* gene promoter (77), and the mechanism by which INF-γ induces *P450C1* expression remains unknown.

Little or no feedback inhibition by 1,25VD on *P450C1* expression is seen in activated macrophages (77, 95). This lack of inhibition by 1,25VD is apparently

not due to the secosteroid's degradation by P450C24, because expression of the 24-hydroxylase enzyme is inhibited when INF-γ-activated STAT1 homodimers interact with liganded VDR to prevent VDR/RXR binding to the *P450C24* promoter (137). The lack of 1,25VD repression may reflect tissue specificity with inhibition of P450C1 only occurring in the kidney.

CONCLUDING REMARKS ON FUTURE DIRECTIONS

This review describes actions of the P450C25, P450C1, and P450C24 enzymes to modulate the cellular and circulating levels of the bioactive 1,25VD secosteroid. It also discusses the responses of the enzymes to nutrient and hormonal regulators and the target-tissue actions of 1,25VD in a number of cellular processes in health and disease. It is evident from this review that a molecular comprehension of the functional and regulatory dimensions of the vitamin D hydroxylases is rapidly coming into focus. As a result of cloning the vitamin D hydroxylases, it is now possible to determine the structure and function of the enzymes and the molecular mechanisms whereby they are differentially expressed. Studies using natural and directed mutagenesis are in progress to map the enzymes' active sites and identify residues involved in the mechanism of action. Through such studies, it will be possible to design enzymes for genetic engineering purposes that direct the metabolism of natural and synthetic vitamin D analogs to preferred activated or end products. Based upon gene-regulatory investigations, the tissue-specific expression of the hydroxylase enzymes is understood at a fundamental level. Yet, details of the complex molecular-regulatory network whereby nutrient and hormonal factors function to control the vitamin D endocrine/paracrine/autocrine systems remain to be determined. Such information can be obtained by using cell-specific null- and transgenic-mice models and following the functional effects when enzyme expression is turned on or off in particular cell types. The ability to alter enzyme levels in a tissue-specific manner will have a profound impact on future nutrient and pharmacological therapeutic regimens used in the treatment of hyperproliferative (i.e., cancer) and age-related (i.e., osteoporosis) disorders.

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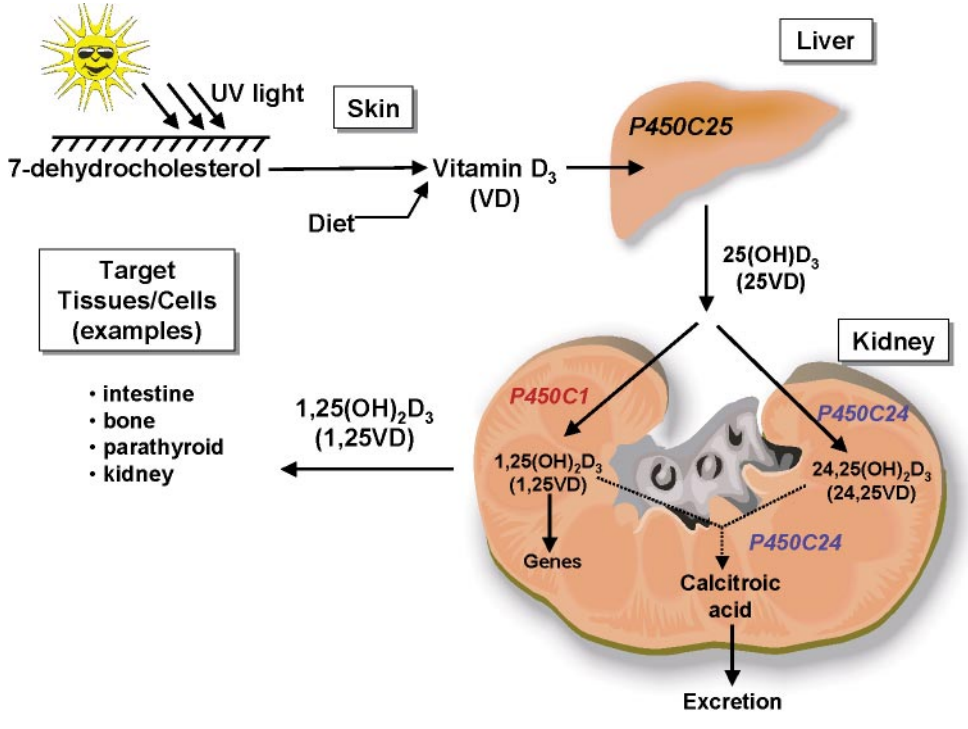

Figure 2 Bioactivation, function and degradation of vitamin D metabolites. Parental vitamin D_3 from sunlight action on the epidermis or dietary sources is hydroxylated in the liver by P450C25 and then activated in kidney by P450C1 to give 1,25-dihydroxyvitamin D (1,25VD), which acts on renal genes or enters the circulation to act on target tissues (intestine, bone, parathyroid). P450C24 plays a dual role to remove 25-hydroxyvitamin D (25VD) from the circulation and excess 1,25VD from kidney. Circulating 1,25VD is also be removed by other tissues, notably the intestine.

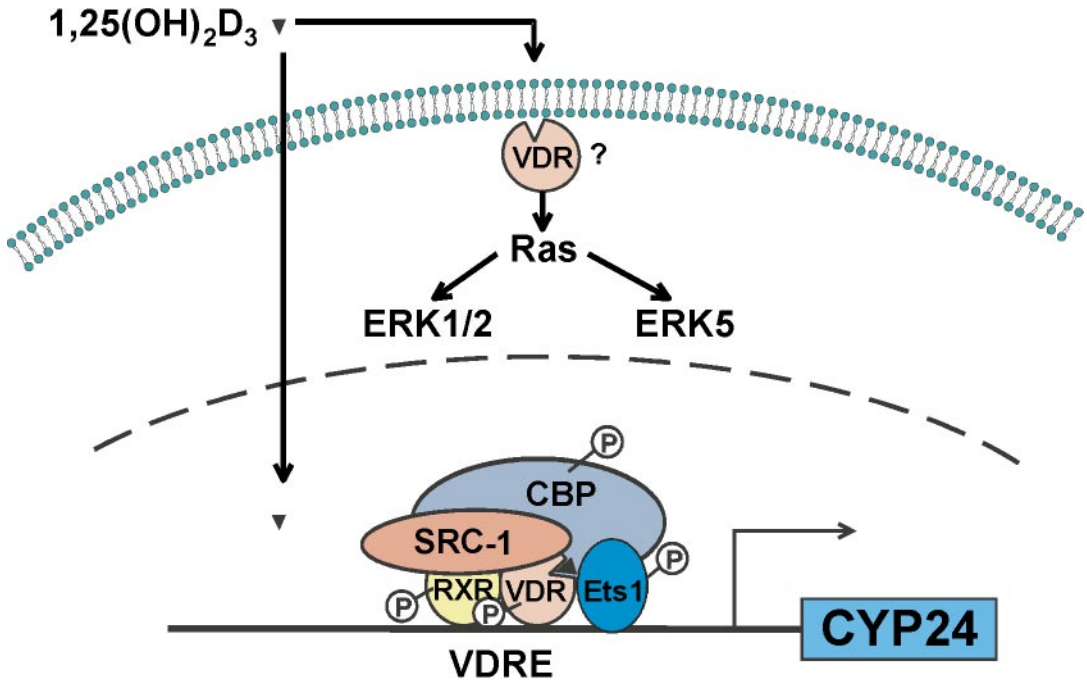

Figure 3 Schematic for 1,25-dihydroxyvitamin D (1,25VD) upregulation of *P450C24* gene expression in the kidney. 1,25VD binds to a putative plasma membrane vitamin D receptor (VDR) that may be the nuclear VDR or a novel protein. Activation of Ras and mitogen-activated protein kinases can lead to the phosphorylation and activation of regulatory proteins that interact and bind at the P450C1 promoter [e.g., VDR, retinoid-X-receptor (RXR), Ets-1, and coactivators SCR-1 and CBP]. 1,25VD binding to VDR facilitates coactivator association with the VDR/RXR heteroduplex (and possibly Ets-1) that is attached to response elements within the promoter region. Only one of the two vitamin D response elements in the P450C24 promoter is shown.