The Vitamin D_3 Pathway in Human Skin and its Role for Regulation of Biological Processes

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Abbreviations: UVB, ultraviolet B; 7-DHC, 7-dehydrocholesterol, pre-D_3, previtamin D_3; D_3, vitamin D_3; 25OHD_3, 25-hydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; C, Calcitriol; DBP, vitamin D binding protein; VDR, vitamin D receptor; mVDR, membrane bound vitamin D receptor; CYP, cytochrome P450; RXR, retinoid X receptor; VDRE, vitamin D response element; PCNA, proliferating cell nuclear antigen; RFLP, restriction fragment length polymorphism; INF-γ, interferon γ; IL, interleukin; Th, T helper cell; IgE, immunoglobulin E; TNF-α, tumor necrosis factor α; IU,international unit; MED, minimal erythema dose;

Abstract

The skin is the only tissue yet known in which the complete UVB-induced pathway fom 7-dehydrocholesterol to hormonally active calcitriol (1α,25-dihydroxyvitamin D_3, 1α,25$(OH)_2D_3$) occurs under physiological conditions. Epidermal synthesis of calcitriol could be of fundamental relevance because calcitriol regulates important cellular functions in keratinocytes and immunocompetent cells. Because of their antiproliferative and prodifferentiating effects, calcitriol and other vitamin D analogs are highly efficient in the treatment of psoriasis vulgaris. The known antipsoriatic effect of UVB light could, at least in part, be mediated via UVB-induced synthesis of calcitriol. In addition, mounting evidence indicates now that cutaneous vitamin D_3 synthesis is of high importance for the prevention of a broad variety of diseases, including various malignancies. New but controversially discussed sun-protection guidelines were established for the prevention of internal cancers. A better understanding of the metabolism of vitamin D in the skin opens new perspectives for therapeutic applications of vitamin D analogs.

The vitamin D_3 pathway in human skin

A photochemical reaction with maximum spectral effectiveness at about 297 nm results in generation of previtamin D_3 from 7-dehydrocholesterol (provitamin D_3, 7-DHC) in basal and suprabasal layers of the skin (1). Dependent on temperature and time, previtamin D_3 is then isomerized to vitamin D_3. After binding to carrier proteins, in particular vitamin D binding protein (DBP), vitamin D_3 is transported to the liver where it is enzymatically hydroxylated by CYP27A1 at the C25 position , generating 25-hydroxyvitamin D_3 (calcidiol, $25OHD_3$). More recently, it has been found that in all six cytochrome P450 isoforms (CYP27A1, CYP2R1, CYP2C11, CYP3A4, CYP2D25 and CYP2J3) exhibit vitamin D_3 25-hydroxylation activities (2, 3). 25-Hydroxyvitamin D_3, bound to DBP, is then transported to the kidney, and is finally hydroxylated by CYP27B1 at C1α position to hormonally active calcitriol (1α,25-dihydroxyvitamin D_3, $1\alpha,25(OH)_2D_3$). Calcitriol acts in the kidney but is also transported by DBP to vitamin D receptor (VDR) positive target tissues (mainly bone, intestine and parathyroid gland) to act in a genomic or nongenomic manner. There is substantial evidence for additional extrarenal sites of calcitriol synthesis. *In vitro*, many nonrenal cells, including bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes and several cancer cells (e. g. from lung, prostate and skin) can enzymatically convert $25OHD_3$ to $1\alpha,25(OH)_2D_3$. A five-step inactivation pathway from calcitriol to calcitroic acid is attributed to a single multifunctional CYP, CYP24A1, which is transcriptionally induced by the action of calcitriol in a very sensitive manner. The physiological importance of a second catabolic pathway which encloses the conversion of $1\alpha,25(OH)_2D_3$ to $1\alpha,25(OH)_2D$-3epi-D_3 is less clear.

Skin cells (keratinocytes, fibroblasts and other cells) express VDR, an absolute prerequisite for regulation of genomic effects of calcitriol and other synthetic vitamin D analogs. Experimental and clinical findings have shown that the serum concentration of calcitriol (10^{-11} to 10^{-10} M) is too low to induce VDR mediated hormonal effects in the skin (4, 5). It should be noted that more than 99% of the total circulating $1\alpha,25(OH)_2D_3$ is bound to carriers such as DBP and albumin. In the normal human only 0.4% of the circulating $1\alpha,25(OH)_2D_3$ is free (6).

According to the “free hormone hypothesis” (7), it is commonly accepted that only the free, and not total 1α,25$(OH)_2D_3$ regulates genomic processes within keratinocytes. This suggests that free plasma calcitriol approximates around 6 x 10^{-13} M. It has been shown in several studies that calcitriol, at concentrations higher than 10^{-8} M (equivalent to a highly unphysiological concentration of approximately 2.5 x 10^{-6} M total calcitriol in the circulating blood), is a potent growth inhibitor of normal human keratinocytes *in vitro*. In addition, it has been suggested that cutaneous metabolism of circulating 25OHD_3 to 1α,25$(OH)_2D_3$ does not play a significant role *in vivo* because the amount of free 25OHD_3 which penetrates the cell membrane of epidermal keratinocytes is too small to induce formation of sufficient amounts of 1α,25$(OH)_2D_3$ (5). 25OHD_3 is very tighly bound to DBP (K_d = 10^{-10} to 10^{-12} M) in circulating blood. Due to this tight binding and the high plasma concentration of DBP (0.3 to 0.5 mg/ml), virtually all 25OHD_3 molecules in the circulation are present in a complex with DBP. Only approximately 0.03% of the metabolite is found in free form (8). Furthermore, the deeper layers of the epidermis are not vascularized which additionally impairs the passage of the 25OHD_3 x DBP complex from blood to epidermal keratinocytes. Accordingly, no therapeutical effects were observed in covered involved psoriatic skin after whole body UVB irradiation, in spite of increased 25OHD_3 level in circulating blood (5). On the other hand, has been known for a long time that cultured keratinocytes can metabolize exogenously added (free) 25OHD_3 to substantial amounts of 1α,25$(OH)_2D_3$ which is subsequently catabolized in these cells (9). It was found a few years ago *in vitro* (10-12) and *in vivo* (13) that human keratinocytes have an autonomous vitamin D_3 pathway. This pathway encloses not only the well known UVB-induced synthesis of vitamin D_3 but also its further enzymatically regulated metabolism, which results in generation of hormonally active calcitriol (Fig. 1). Thus, keratinocytes are the only cells in the body with the whole pathway from 7-DHC to 1α,25$(OH)_2D_3$. Cutaneous production of calcitriol may exert intracrine and/or autocrine effects on keratinocytes and paracrine effects on neighboring cells. This hormone may regulate growth, differentiation, apoptosis and other biological processes. There are a number of genes in keratinocytes

(Tab. 1) which are regulated by calcitriol (12). Regulation of genes associated with growth and differentiation of keratinocytes argues in particular for a link of therapeutical effect of UVB radiation in the treatment of psoriasis with the cutaneous vitamin D_3 pathway. Interestingly, Su et al. (14) have previously demonstrated that free concentrations of calcitriol as low as 10^{-12} M (equivalent to approximately 2.5 x 10^{-10} M total calcitriol in circulating blood) increased involucrin and transglutaminase mRNA levels in keratinocytes *in vitro*. These sensitive effects of calcitriol might primarily contribute to differentiation of keratinocytes *in vitro* and *in vivo*. Thus, it becomes clear that the epidermal keratinocyte is both: the site of calcitriol synthesis and target of this hormone.

Recently, *in vitro* investigations have shown that dermal fibroblasts express one of the potential 25-hydroxylases (CYP27A1), but not the 1α-hydroxylase (CYP27B1). Therefore, fibroblasts might play an important role in supply of calcitriol precursors (vitamin D_3 and $25OHD_3$) for keratinocytes and possibly for circulating blood (15).

It is commonly assumed that most of calcitriol formed by extrarenal cells serves an intracrine, autocrine or paracrine regulation within the cells in which it is produced. However, it remains to be shown whether and to what extent extrarenal synthesis of calcitriol, in particular in the skin, modulates cellular proliferation, differentiation, apoptosis, and immunological processes.

Biologic effects of calcitriol in the skin

Inhibition of proliferation and induction of differentiation in keratinocytes

Numerous *in vitro* and *in vivo* studies demonstrate dose-dependent effects of vitamin D analogs on cell proliferation and differentiation. At low concentrations, calcitriol promotes proliferation of keratinocytes *in vitro*, at higher pharmacological doses ($\geq 10^{-8}$ M) keratinocyte proliferation is inhibited (16). Several vitamin D analogs (e.g. calcitriol, calcipotriol, tacalcitol and maxacalcitol) have been synthesized for topical psoriasis therapy. These agents (Fig. 2) show antiproliferative and prodifferentiating effects on human keratinocytes *in vtro* and *in vivo*. Both the hydroxyl group in C1α position and in the side chain are essential for the

biological effects of vitamin D analogs. It has been demonstrated that the immunohistochemical staining pattern for various markers of epidermal proliferation (e.g. proliferating cell nuclear antigen [PCNA], Ki-67-antigen) and diifferentiation (e.g. involucrin, transglutaminase K, filaggrin, cytokeratin 10) changes in lesional psoriatic skin along with topical treatment with vitamin D analogs almost completely to the staining pattern characteristic for nonlesional psoriatic or normal skin (17,18). Although the mechanisms underlying the antiproliferative and prodifferentiatiative effects of vitamin D analogs on keratinocytes are not completely understood, it is well known that these effects are at least in part genomic and mediated via VDR. Consequently, it has been shown that keratinocytes from VDR-deficient mice do not respond to the antiproliferative effects of vitamin D analogs. In lesional psoriatic skin, the clinical improvement correlates with an increase of VDR mRNA in vitamin D treated skin areas (19). However, not all patients with psoriasis respond to treatment with vitamin D analogs. It has been demonstrated that a responder can be discriminated from a non-responder by the increase in VDR mRNA in treated skin areas (19). Results from immunohistochemical and molecular biology studies indicate that the antiproliferative effects of topical calcitriol on epidermal keratinocytes are more pronounced compared to the effects on dermal inflammation. One reason for this observation may be that the bioavailability of this potent hormone in the dermal compartment may be markedly reduced compared to the epidermal compartment. The target genes of topical calcitriol responsible for its therapeutical efficacy in psoriasis are still unknown. Principal candidates for calcitriol target genes responsible for growth inhibition and differentiation of keratinocytes are listed in Table 1. Data analyzing VDR genotype in psoriasis is conflicting, some studies report a correlation between individual VDR genotypes (BsmI, FokI, or ApaI RFLP) and skin eruptions of psoriasis or efficacy of treatment with vitamin D analogs (19).

Immunmodulatory effects in the skin

Over the past decade, new and important immunomodulatory effects of vitamin D analogs have been characterized (20). Under experimental conditions systemic administration of

calcitriol is strongly immunsuppressive and improves various Th1-triggered diseases including autoimmuneencephalomyelitis and autoimmune diabetes in mice and psoriasis in humans; calcitriol may even prevent rejection of allografts. Calcitriol also seems to directly promote Th2 differentiation leading to a Th2 phenotype with augmented production of IL-4, IL-5, and IL-10 and reduced synthesis of INF-γ in antigen-stimulated and CD3/CD28 stimulated CD4+ lymphocytes. Lymphocytes treated with calcitriol express enhanced levels of the transcription factors *c-maf* and GATA-3, which explains the strong Th2-driving effect (21). Calcitriol also abolishes the pathogenicity of autoreactive Th1 cells as injection of activated lymphocytes treated with calcitriol have a significantly impaired potential to induce psoriasis in a skin xenograft model of psoriasis (22). These observations show that calcitriol and its analoga may be effective in the therapy of Th1-mediated diseases like psoriasis by promoting IL-4 production and Th2 development (23). Psoriasis is considered by many experts to be an autoimmune disease. However, the antigen or specific endogenous factors responsible for activation of T cells have not yet been identified. Also, agents such as vitamin D analogs and retinoids which affect the growth and differentiation of keratinocytes are very effective in the treatment of psoriasis, while they are ineffective in other T-cell- mediated skin diseases, such as atopic dermatitis or contact dermatitis (24). On the other hand, an association between vitamin D_3 and pathogenesis of atopic dermatitis is currently being discussed. Epidemiologic studies have demonstrated that patients with atopic dermatitis have a lower intake of vitamin D_3 as compared to controls (25). Additionally, it has been demonstrated that vitamin D analogs suppress IgE-production *in vitro* and IgE-mediated cutaneous reactions *in vivo* (26, 27). Various cell types involved in immunologic reactions (e.g. monocytes, activated T- and B-lymphocytes, Langerhans cells) do not only express VDR, but moreover possess enzymatic activity of CYP27B1 for the local synthesis of $1\alpha,25(OH)_2D_3$ from $25OHD_3$ (20). The local synthesis of calcitriol in immune cells may contribute to regulation and control of immune responses. It is evident that calcitriol inhibits activation of T-cells and induces the generation of CD25+/CD4+ regulatory T-cells. Calcitriol inhibits maturation of dendritic cells and induces a phenotype that promotes tolerance and

suppresses immunity after stimulation with antigen (20). Furthermore, in dendritic cells, calcitriol abolishes expression of MHC II molecules and costimulatory molecules including CD40, CD80 and CD86, increases the production of IL-10 and inhibits formation of IL-12, leading to suppression of T-cell activation.

These immunomodulatory effects identify calcitriol and vitamin D analogs, in particular new vitamin D analogs with selective immunomodulatory activity, as promising new drugs for the prevention and therapy of inflammatory skin diseases including psoriasis and perhaps atopic dermatitis as well as allergic contact dermatitis (28).

Regulation of apoptosis in keratinocytes

Calcitriol has been shown to induce the neutral Mg^{2+}-dependent sphingomyelinase which converts sphingomyelin to ceramide (29). Interestingly, ceramide simulates the prodifferentiating effect of calcitriol on keratinocytes (30). In addition, this agent plays a crucial role in the induction of apoptosis in a number of cells including keratinocytes (31). It has been demonstrated that physiological concentrations of calcitriol do not initiate apoptosis in cultured keratinocytes but in opposite cause resistance against proapoptotic ceramides, ultraviolet radiation and tumor necrosis factor-α (TNF-α) (32). The cytoprotective / antiapoptotic effect of calcitriol is obviously linked with generation of sphingosine-1-phosphate. Accordingly, the antiapoptotic effect of calcitriol is completely suppressed after exogeneous addition of N,N-dimethylsphingosine, which is an inhibitor of the sphingosine kinase (32). In contrast, pharmacological concentrations of calcitriol ($\geq 10^{-6}$ M) exert a proapoptotic effect on keratinocytes. Similar effects were observed in the regulation of growth of keratinocytes, where low calcitriol concentrations of about 10^{-11} M stimulate and higher concentrations dose-dependently decrease the proliferation of these cells (16).

Antioxidative effects of calcitriol

Interestingly, calcitriol seems to exert a photoprotective effect on keratinocytes *in* vitro. In keratinocytes calcitriol induces the synthesis of the protein metallothionein, which is a well

known antioxydant (33, 34). Possibly, this is a mechanism of protection directed against UVB-induced synthesis of harmful oxygen radicals.

Vitamin D_3 synthesis in the skin and sun protection - how much sunlight do we need?

Quality and quantity of UV-radiation are of critical importance for the biologic effects of sunlight in the skin. It is well known that UV radiation provokes bio-positive (e. g. vitamin D_3 synthesis and healing effects) but also bio-negative effects (e. g. development of skin cancer and skin aging). At most Central European latitudes a very short (about 7 min for skin-type-2 adult) and unprotected exposure to solar radiation is enough to achieve sufficient vitamin-D_3 levels (35). Exposure of the body in a bathing suit to one minimal erythemal dose (MED) of sunlight is equivalent to ingesting about 10,000 IU of vitamin-D_3 (250 µg). It has also been reported that exposure of less than 18% of the body surface (hands, arms, and face) two to three times a week to a third to a half of an MED in the spring, summer, and autumn is more than sufficient for adequate synthesis of vitamin D_3. On the other hand, caution is recommended even at relative short UV-exposure because UVB radiation represents the main trigger factor for development of non-melanoma skin cancer. Recently, it has been postulated that cancer mortality could be reduced by moderate unprotected UV-exposition and / or oral substitution with vitamin D_3 (35, 36). UVB radiation causes an increase of serum $25OHD_3$ which is converted to calcitriol in several internal organs. It is assumed that local production of calcitriol regulates cell growth, which may ultimately decrease risk of developing cancers in these tissues (36, 37). Several independent epidemiological studies have surprisingly shown that sunlight may reduce the risk of non-Hodgkin lymphoma (38, 39) and may be associated with increased survival rates in patients with early-stage melanoma (40, 41). It is speculated that the apparently beneficial relationship between sun exposure and reduced risk of non-Hodgkin lymphoma as well as survival from melanoma could be mediated by vitamin D_3. Epidermal keratinocytes can convert vitamin D_3 to its hormonal form, calcitriol, which in turn particularly stimulates the differentiation of keratinocytes (14), raising the hope that calcitriol may prevent the development of cancer in these cells (42). As matters

stand, a compromise has to be found between protected and unprotected sun exposure to obtain sufficient amounts of vitamin D_3. In each case, no more than approximately 0.5MED of sunlight should arrive on unprotected skin. Application of higher radiation doses can result in more or less harmful effects in the skin, and the use of sun protection (shade, sunscreens and clothing) is urgently recommended.

What does vitamin D_3 insufficiency mean? The serum level of $25OHD_3$ describes the nutritional vitamin D status. Population-based reference-values for $25OHD_3$ vary by season (43), latitude (43), age (44), and skin pigmentation (45). Health-based reference values for serum $25OHD_3$ have been proposed to replace population-based reference-values with a cutoff at 20 ng/mL serum defining vitamin D insufficiency (46, 47). This cutoff protects from secondary hyperparatherodism but is still considered too low by many specialists (37, 48-50). A consensus has to be reached in this question. More recently, vitamin D_3 deficiency has been defined based on data from various biomarkers (PTH, calcium absorption, bone-mineral density, insulin resistence and beta-cell function) as circulating levels of $25OHD_3$ that are less than 32 ng/mL or 80 nmol/L (50).

It has to be emphasized that in populations with high risk of developing vitamin-D_3 deficiency (e.g. nursing-home residents; patients with skin type I or patients under immunosuppressive therapy that must be protected from sun exposure), vitamin D status should be determined by analytical methods (37). It seems clear that the current food supply, supplementation practices, and dietary patterns of most countries cannot adequately compensate for the existing cautionary guidelines to limit solar exposure to prevent skin cancer (51). Vitamin D_3 deficiency should be redressed by giving vitamin D_3 orally as recommended - a dose of 2,000 IU vitamin D_3 per day (52), or a single dose of 50,000 IU vitamin-D_3 once a week for 8 weeks (37) are efficient and safe to treat vitamin-D_3 deficiency. Another means of guaranteeing vitamin D_3 sufficiency, especially in nursing-home residents, is to give 50,000 IU of vitamin D_3 once a month (37). Of note, orally administered vitamin D_3 increases the serum vitamin D status ($25OHD_{3/2}$) more efficiently (factor = 1.7) than equimolar amounts of vitamin D_2 (53). The assumption that vitamins D_2 and D_3 have equal nutritional value is

probably wrong and should be reconsidered. Care should be taken to specify the type of vitamin D used for nutritional studies (53). Vitamin D_3 intoxication is usually seen only when daily doses in excess of 10,000 IU are ingested (37, 54). It should be mentioned, however, that effects and consequences of long-term vitamin D_3 supplementation on humans are poorly understood until now. It has been reported that long-term vitamin D_3 supplementation may have adverse effects on serum lipids (55). Altogether, it is dificult to create a “golden rule” for vitamin D_3 intake.

Outlook

The physiological function of UVB-induced cutaneous calcitriol synthesis needs to be clarified in the near future. Exactly how calcitriol and other vitamin D analogs act to produce effects on cellular differentiation, proliferation, apoptosis and immune system in the skin is not known. The elucidation of the mechanisms involved will be an active area of further research. Calcitriol may mediate these effects by genomic and nongenomic mechanisms (Fig. 1). New target genes and details of signaling pathways will be identified which should expand our understanding of the sequences involved in vitamin D-mediated mechanisms. New insights may also be obtained concerning different transcription factors which are involved in mediating these diverse biological responses.

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Figure legends

Fig. 1 Functional vitamin D_3 pathway in keratinocytes (7-DHC, 7-dehydrocholesterol; pre-D_3, previtamin D_3; D_3, vitamin D_3, DBP, vitamin D-binding protein; CYP27A1, (27)25-hydroxylase; CYP27B1, 1α-hydroxylase; CYP24A1, 24-hydroxylase; 25OHD_3, 25-hydroxyvitamin D_3; VDR, vitamin D-receptor; mVDR, membrane bound vitamin D-receptor; C, calcitriol; RXR, retinoid X receptor; VDRE, vitamin D response element)

Fig. 2 Structure formulas of vitamin D analogs (calcitriol, calcipotriol, tacalcitol and maxacalcitol)

Table 1: Calcitriol-regulated genes in keratinocytes

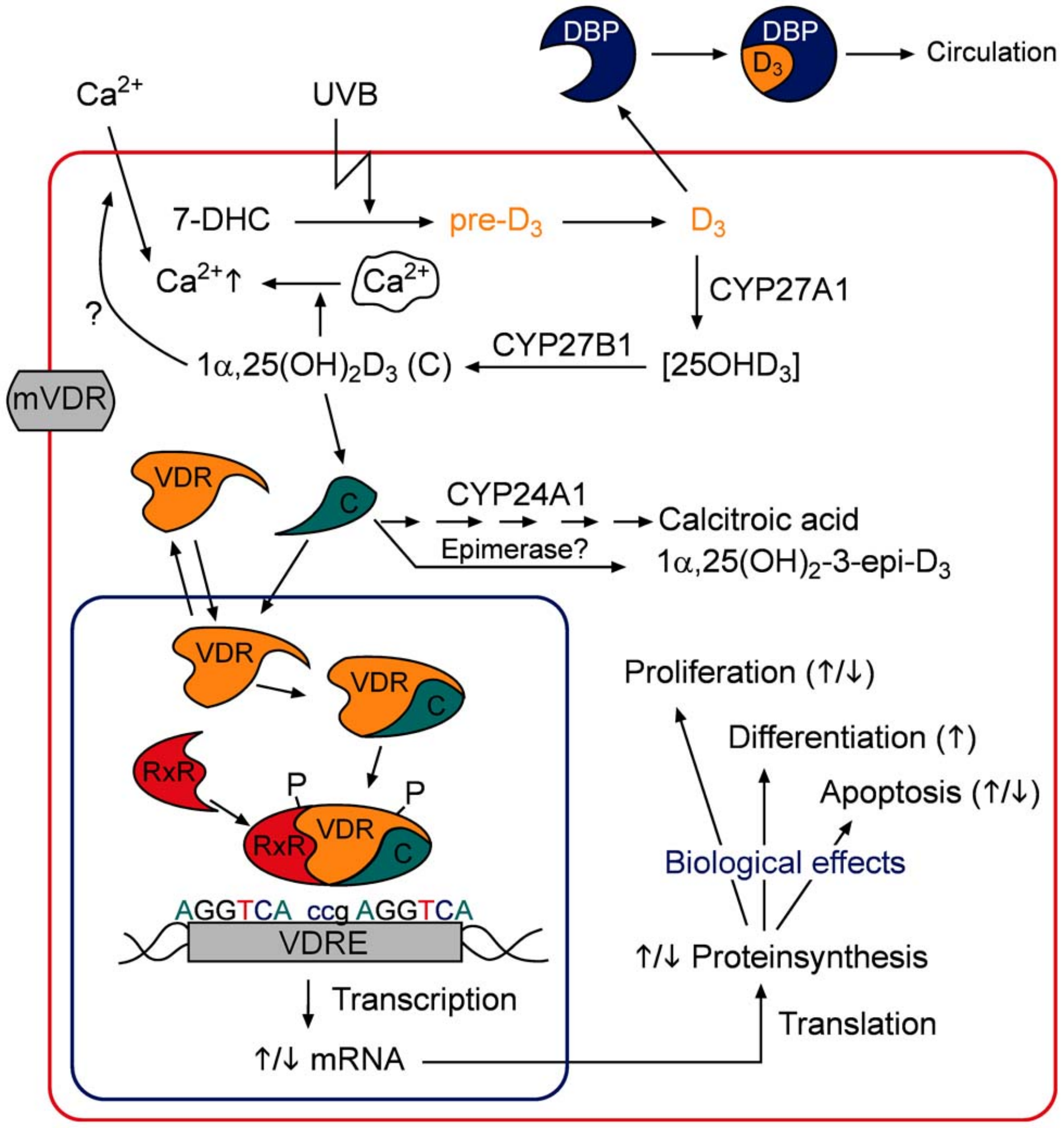
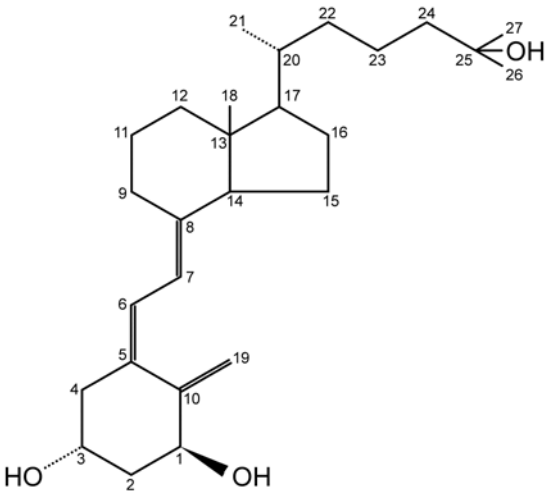
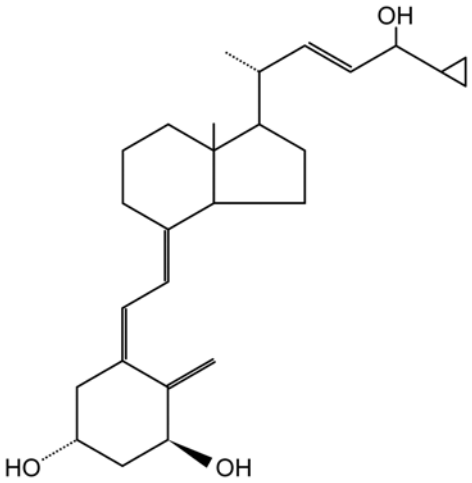

DBP
DBP
D3
Circulation
Ca2+
UVB
7-DHC
pre-D3
D3
Ca2+↑
Ca2+
?
CYP27A1
CYP27B1
1α,25(OH)2D3 (C)
[25OHD3]
mVDR
VDR
C
CYP24A1
Calcitroic acid
Epimerase?
1α,25(OH)2-3-epi-D3
VDR
VDR
C
RxR
P
P
RxR
VDR
C
AGGTCA ccg AGGTCA
VDRE
Transcription
↑/↓ mRNA
Proliferation (↑/↓)
Differentiation (↑)
Apoptosis (↑/↓)
Biological effects
↑/↓ Proteinsynthesis
Translation

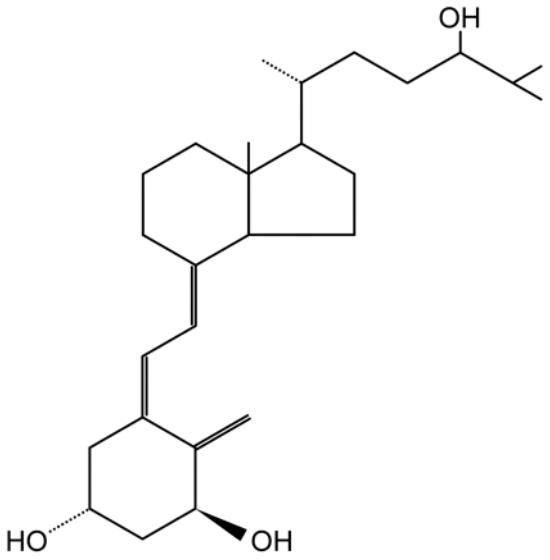
Fig. 2



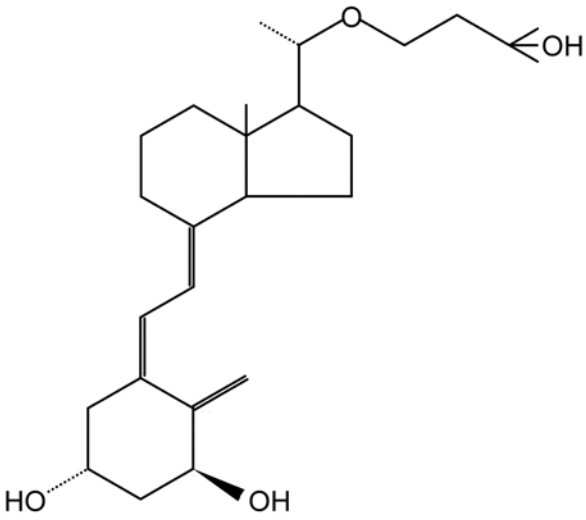
Calcitriol



Calcipotriol



Tacalcitol



Maxacalcitol

Table 1: Vitamin D regulated genes in keratinocytes

Effect of $1\alpha,25(OH)_2D_3$ on	mRNA	Protein	VDRE
<u>Proliferation associated genes</u>			
c-myc	↓		
c-fos	↑		+
Cyclin D1	↓		
TGF-$\beta_{1/2}$	↑	↑	+(β_2)
cdk4	↓		
$p21^{WAF1/CIP1}$	↑	↑	+
$p27^{KIP1}$		↑	
PTHrP	↓	↓	
β_3-Integrin			+
<u>Differentiation related genes</u>			
Involucrin	↑	↑	+
Transglutaminase I	↑	↑	
u- and t-plasminogen activator	↓	↓	+
PLC (β, γ, δ)	↑	↑	+(γ1)
<u>Vitamin D/calcium metabolism related genes</u>			
Vitamin D receptor	↑↓	↑	
24-hydroxylase	↑	↑	++
Calcium receptor	↑		
<u>Inflammation related genes</u>			
TNFα	↑	↑	+
IL-1α		↓	
IL-6	↓	↓	
IL-8		↓	
IL-10 (IL-10 receptor)	↑(↑)		
RANTES		↓	
i-NOS			+
5-Lox			+
<u>Miscellaneous</u>			
Osteopontin	↑		+
Fibronectin	↑		+
Metallothionein	↑	↑	
17β-OH-Steroiddehydrogenase	↑	↑	