Vitamin D metabolites; protective versus toxic properties: molecular and cellular perspectives

Mohamed S. Ahmed, Ahmed Shoker
Department of Medicine, Royal University Hospital, University of Saskatchewan, Saskatoon, SK, Canada

Abstract

Vitamin D plays an essential role in bone metabolism. The discovery that the vitamin D receptor (VDR), a member of the nuclear receptor superfamily, is expressed in most tissues led researchers to investigate other biological actions of vitamin D. These effects were found to include anti-inflammatory effects and anti-atherogenesis, decreased renin activity and biosynthesis, induction of cell differentiation, inhibition of cell growth, and immunomodulation. In spite of the plethora of evidence on the protective effects of vitamin D, the reports on its intoxication still are considerably few. Therefore, in this review, we aim to summarize the molecular and cellular bases of the protective and toxic vitamin D actions that are mediated mostly by VDR. This review will also shed light on vitamin D metabolites other than the active metabolite calcitriol and particularly 25-hydroxy vitamin D (25(OH)D), putting emphasis on its magnifying role in vitamin D intoxication. One of the important themes we discuss is defining serum levels of beneficial or toxic effects of other exogenous vitamin D administration and its impact on 25(OH)D serum levels in animals and human subjects.

Introduction

Vitamin D was discovered a long time ago as an essential nutrient for calcium and phosphate homeostasis, and thus is essential for bone health. Over the last decade, a plethora of literature confirmed the existence of the vitamin D receptor (VDR) on multiple tissues, and therefore it is accepted now that vitamin D functions extend above and beyond bone homeostasis. It has been demonstrated that among the desirable and protective functions of vitamin D, it has anti-inflammatory and anti-atherogenesis effects, and decreased renin activity and biosynthesis. Vitamin D also induces cell differentiation, and inhibits cell growth. On the other hand, high levels of vitamin D metabolites may lead to undesirable effects such as oxidative insult. Calcitriol (1,25-dihydroxyvitamin D) and calcidol (25-hydroxy vitamin D) are among the most important metabolites of vitamin D. Both metabolites are thought to exert physiological functions and pathological effects, respectively. In clinical settings, it was reported that low levels of 25(OH)D and 1,25(OH)D were associated with prevalent myocardial dysfunction, heart failure, and sudden cardiac death in two cross-sectional studies. In addition, in a prospective cohort study involving 3258 patients, it was indicated that there was an independent association of low serum 1,25(OH)D and 25(OH)D levels with all-cause and cardiovascular mortality. In this review, we shall focus our discussions on defining serum levels of vitamin D metabolites that exert beneficial versus toxic effects particularly on the available exogenous compounds approved for human use.

Vitamin D

Vitamin D metabolism

Vitamin D is a 9,10-secosteroid. Vitamin D has two distinct forms: vitamin D2 and vitamin D3. Vitamin D2 is a 28-carbon molecule derived from the plant sterol ergosterol. Vitamin D3 is a 27-carbon derivative of cholesterol. Vitamin D2 (D2 and D3) from the skin and diet enters the blood circulation and slowly transfers to vitamin D binding protein (DBP), which carries vitamin D to the liver and kidney for bioactivation. Vitamin D2 has a relatively low affinity for DBP, in contrast to vitamin D3 that is made during skin synthesis. The concentration of DBP in human and rat plasma is 5-6 µmol/L and 3.7 µmol/L, respectively. Vitamin D is metabolized in the liver to 25(OH)D and 25(OH)D under the action of a microsomal cytochrome P450, 25-hydroxylase (CYP2R1), or the action of mitochondrial cytochrome P450, CYP27A1. The levels of 25(OH)D increase in proportion to vitamin D intake, and thus plasma 25(OH)D levels are commonly used as an indicator of vitamin D status. Plasma 25(OH)D and D are metabolized in the kidneys by the enzyme 25(OH)D-1α-hydroxylase (CYP27B1) to the active form, 1,25(OH)2D (calcitriol). Vitamin D compounds are catabolized primarily by the oxidation of the side chain under the effect of CYP24A1. The 24-hydroxyl- lation of 1,25(OH)2D is the first catabolic step in the elimination of the active hormone leading to the formation of 24,25(OH)2D and 25(OH)D26,23-lactone. Further oxidative reactions of these catabolic metabolites lead to progressive loss of biological activity and finally to the production of water-soluble calcitriolic acid, which is excreted in urine.

Vitamin D–vitamin D receptor binding

VDR, like other members of the nuclear receptor family, is composed of distinct structural domains within the protein. These include a ligand binding domain (LBD) and DNA binding domain (DBD). LBD is responsible for hormone binding, strong receptor dimerization and interaction with co-repressors and co-activators, which all together are critical for the regulation of transcriptional activities. The core sequence of DBD is highly conserved among nuclear hormone receptors (NHRs). The binding of calcitriol to VDR activates heterodimerization with
Vitamin D as a protective agent

Vitamin D and renin-angiotensin system activity

The renin-angiotensin system (RAS) is a regulatory cascade that plays an essential role in the regulation of blood pressure, electrolyte, and volume homeostasis. Decreased VDR activity increases circulating renin levels and blood pressure, and it causes left ventricular and myocyte hypertrophy in genetically manipulated mouse models. Zhou et al. indicated that administration of 1,25(OH)2D3 to 10(OH)-ase mice not only normalized serum calcium and phosphorus levels but also normalized the RAS, blood pressure, cardiac hypertrophy, and cardiac functions. In addition, they reported that normalization of serum calcium and phosphorus in 10(OH)-ase mice on a diet to rescue serum levels of calcium and phosphorus failed to normalize the RAS and cardiac abnormalities. They concluded that 1,25(OH)2D3 regulates the RAS and cardiac function by a calcium and phosphorus-independent mechanism.

These data were consistent with previous reports that showed that mice with targeted ablation of the VDR gene also develop hypertension owing to up-regulation of RAS. Plasma 1,25(OH)2D3 is inversely correlated with plasma renin activity in patients with essential hypertension and normotensive subjects. Administration of 1,25(OH)2D3 has also been reported to reduce blood pressure, plasma renin activity, Ang II, and myocardial hypertrophy as well as endothelium-induced atrial natriuretic peptide levels. Recent clinical studies show that calcitriol-induced reductions in PTH, atrial natriuretic peptide, and renin-angiotensin II are associated with amelioration of left ventricular hypertrophy in patients receiving dialysis.

The finding that 1,25(OH)D3 represses gene transcription provides a good basis to use vitamin D analogs for the suppression of the compensatory renin increase because the analogs directly inhibit renin biosynthesis.

It has long been known that cyclic AMP (cAMP) is a major intracellular signal that stimulates renin production in JG cells. Intracellular cAMP is converted from ATP by adenylyl cyclase activated by membrane receptors. Cyclic AMP binds to the regulatory subunit of protein kinase A (PKA) to free the catalytic subunit. This in turn enters the nucleus and phosphorylates cAMP response element binding protein (CREB) at serine 133 or cAMP response element modulator (CREM) at serine 117. This leads to the recruitment of ubiquitous co-activators CBP/p300 to promote gene transcription (Figure 1). In fact, a number of cAMP response elements (CREs) have been identified in renin gene promoters that play crucial roles in renin gene transcription.

From a molecular point of view, Yuan et al. developed a model of 1,25(OH)2D3-induced transcription of renin gene expression. They indicated that the cAMP-PKA pathway plays a key role in stimulating renin gene expression. This pathway activates CREB by phosphorylation, leading to recruitment of CBP/p300. In the presence of 1,25(OH)2D3, liganded VDR interacts with CREB and blocks its binding to CRE, leading to a reduction of renin gene transcription (Figure 1). Overall, Yuan et al. demonstrated that 1,25(OH)2D3 down-regulates renin gene transcription by suppressing, at least in part, CRE-mediated transcriptional activity in the renin gene promoter, as CRE is activated by cAMP-PKA signaling. Yuan et al. identified a major regulatory pathway as the target in vitamin D inhibition of renin synthesis.

Vitamin D and insulin resistance

As it has been established already that hypovitaminosis D enhances renin produc-

Figure 1. Model of 1,25(OH)2D3-induced transrepression of renin gene expression. The cAMP-PKA pathway plays a key role in stimulation of renin gene expression. This pathway activates CREB by phosphorylation, leading to recruitment of CBP/p300. This complex enhances renin gene transcription particularly because of CRE-CREB binding. In the presence of 1,25(OH)2D3, liganded VDR interacts with CREB and dissociates it from binding to CRE, leading to the reduction of renin gene transcription. P: phosphorylation; D, 1,25(OH)2D3; Pol II, RNA polymerase II. Modified with permission from Yuan et al., 2007.
tion, Ang II levels are expected to be elevated. The elevated Ang II is reported to contribute to the development of insulin resistance. Although molecular mechanisms implicating Ang II in the pathogenesis of insulin resistance are not fully elucidated, the existing evidence shows that its action is mediated by various pathways in the insulin signaling cascade including the insulin receptor, the insulin receptor substrate (IRS), and phosphatidylinositol 3-kinase (PI3-K). Insulin resistance is characterized by impairment of insulin ability to enhance glucose uptake in insulin sensitive tissues, especially in skeletal muscle tissue, leading to the major pathogenetic pathway for the development of type II diabetes mellitus.

**Vitamin D as an anti-inflammatory and immunomodulatory agent**

It is well established that calcitriol has an immunomodulatory capacity in vitro and in vivo. Vitamin D deficient animals and humans have a higher risk of infection, probably related to deficient macrophage function. The monocytic function of antigen presentation is also decreased, and it is well known that monocytes are functionally modulated by interferons (IL-1) and TNF-α. Key targets for anti-inflammatory and immunomodulatory properties for vitamin D are dendritic cells (DCs) and T cells. DCs are professional presenting cells (APCs) with potent stimulatory capacity in the primary mixed leukocyte reaction.

**Anti-inflammatory effects of vitamin D receptor agonists in dendritic cells**

Earlier indications for the capacity of VDR agonists to target APCs were corroborated by their ability to inhibit the production of IL-12, an APC-derived cytokine critical for Th1 cell development. They also cause a markedly decreased IL-12 and enhanced IL-10 production, resulting in inhibition of T cell activation. In addition, it was emphasized that they are responsible, at least in part, for the induction of DCs with tolerogenic properties. Plasma 1,25(OH)2D3 utilizes different mechanisms to regulate cytokine production by DCs. IL-12 secretion is inhibited by targeting the nuclear factor (NF)–κB pathway, via NF-κB proteins such as RelB and cRel.

**Vitamin D3 and dendritic cell differentiation and maturation**

A modulatory effect of 1,25(OH)2D3 on DC differentiation and maturation has also been identified in both human and murine culture systems. Maturation of DCs is inhibited by physiological levels of 1,25(OH)2D3 and a related analog, 25(OH)16-ene-23-yne-26, 27-hexafluoro-19-nor-vitamin D3. The differentiation and maturation arrest of DCs may also be indicated by decreased expression of maturation markers such as CD40, CD80, and CD86, as well as increased antigen uptake.

**Anti-inflammatory effects of vitamin D receptor agonists in T cells**

VDR agonists can target T cells directly and indirectly, selectively inhibiting T cell subsets able to mediate inflammation and tissue damage. VDR agonists modulate DC function, thus shaping T cell activation and development. They can also have direct effects on T cells. Plasma 1,25(OH)2D3 was shown to inhibit antigen-induced T cell proliferation and cytokine production. Several key cytokines are direct targets for VDR agonists in T lymphocytes, in particular Th1-type cytokines, such as IL-2 and IFN-γ. Plasma 1,25(OH)2D3 proved to enhance the development of Th2 cells via a direct effect on naive CD4+ cells. This could contribute to the beneficial effect of VDR agonists in the treatment of inflammatory conditions.

Both Th1 and Th2 cells can be targets of VDR agonists but this depends on the activation and differentiation status of the cells. Treatment with VDR agonists also inhibits T cell production of IL-17. This is a pro-inflammatory cytokine recently shown to be produced by pathogenic T cells in various models of chronic inflammation and immune-mediated tissue injury, including organ-specific autoimmunity in the brain, heart, synovium, and intestines, allergic disorders of the lung and skin, and microbial infections of the intestines and the nervous system. In conclusion, 1,25(OH)2D3 in vivo appears primarily to inhibit pro-inflammatory, pathogenic T cells, such as Th1 and Th17.

**Vitamin D as an anti-thrombogenic agent**

**Vitamin D toxicity**

Vitamin D toxicity in humans is characterized by markedly elevated serum levels of 25(OH)D. However, levels of the active metabolite 1,25(OH)2D have been reported to be either normal to decreased or elevated.

**Hypotheses of vitamin D toxicity**

Three major hypotheses have been proposed to explain vitamin D toxicity. All involve increased concentrations of a vitamin D metabolite reaching the VDR in the nucleus of target cells and causing exaggerated gene expression. The three hypotheses are:

1. **Raised plasma 1,25(OH)2D3 concentrations lead to increased intracellular 1,25(OH)2D3 concentrations.**
2. **Vitamin D intake raises plasma 25(OH)D3 to concentrations that exceed the DBP-binding capacity, and “free 25(OH)D3” enters the cell, where it has direct effects on gene expression.**
3. **Vitamin D increases the concentrations of many vitamin D metabolites, especially vitamin D itself and 25(OH)D3.** These concentrations exceed the DBP-binding capacity and release of “free” 1,25(OH)2D3, which enters target cells.

Hypothesis 1) is not widely supported as many studies revealed that vitamin D toxicity is associated with normal or marginally elevated 1,25(OH)2D3. It was only Mawer et al. who reported elevated 1,25(OH)2D3 with vitamin D toxicity. Hypothesis 2) suggested that high dietary vitamin D intake increases plasma 25(OH)D3 rather than 1,25(OH)2D3. The low affinity of 1,25(OH)2D3 for the transport protein DBP and its high affinity for VDR dominate normal physiology. This makes it the only ligand with access to the transcriptional signal transduction machinery. However, in vitamin D intoxication, loading by various vitamin D metabolites significantly compromises the capacity of the DBP by allowing other metabolites to enter the cell nucleus. Of all the inactive metabolites, 25(OH)D3 has the strongest affinity for the VDR, and thus at suf-
ficiently high concentrations, could stimulate transcription.\textsuperscript{96} Hypothesis iii) proposed that the “free” form of the hormone that crosses the cell membrane is responsible for vitamin D toxicity (Figure 2).\textsuperscript{97,98} Accordingly, in hypervitaminosis D, vitamin D metabolites, such as vitamin D$_3$; 25(OH)D$_3$; 24,25(OH)$_2$D$_3$; 25,26(OH)$_2$D$_3$; and 25(OH)D$_2$-26,23-lactone, saturate the DBP in the bloodstream and the free concentration of some of these metabolites, such as 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$, could increase significantly owing to their displacement from DBP.\textsuperscript{99,100}

**Safe vitamin D dosing in normal subjects**

Vieth \textit{et al.}\textsuperscript{101} administered 100 µg vitamin D$_3$/day for ≤5 months to 18–56-year-old healthy subjects living at high altitudes. They found that the mean serum 25(OH)D$_3$ concentration plateaued at 96 nmol/L (with an extreme of almost 140 nmol/L) without concomitant changes in serum calcium (≤2.75 mmol/L)\textsuperscript{102} or the urinary calcium-to-creatinine ratio (≤1.0),\textsuperscript{103} which were used as indices of hypervitaminosis D. They concluded that “consumption of vitamin D$_3$ at intakes ≥100 µg/d causes no harm.”\textsuperscript{104} To ensure a serum 25(OH)D$_3$ concentration of ≥75-100 nmol/L (desirable effective concentration), a total vitamin D supply of 100 µg (4000 IU/d) is required. This suggests that this is a physiological limit as this is the concentration at which PTH approaches a minimum in the relationship with 25(OH)D.\textsuperscript{95,96} On the other hand, vitamin D deficiency is not well characterized, and varying 25(OH)D$_3$ thresholds has been used to achieve the target dosing. Malabanan \textit{et al.}\textsuperscript{105} evaluated the effect of vitamin D deficiency on blood concentrations of PTH by increasing 25(OH)D$_3$ levels above 25 nmol/L with vitamin D therapy.\textsuperscript{106} They concluded that the value of 20 ng/mL (to convert values for 25(OH)D from ng/ml to nmol/L, multiply by 2.5) of 25(OH)D can be chosen as the definition of hypovitaminosis D.\textsuperscript{107,108} at which the risk of developing secondary parathyroidism is expected to be high.\textsuperscript{109} Serum 25(OH)D$_3$ concentrations of <40-50 nmol/L are considered to be insufficient\textsuperscript{110,111} as this leads to an elevated level of PTH. However, Malabanan \textit{et al.}\textsuperscript{112} and Lips\textsuperscript{113} indicated that the safe lower reference limit for serum 25(OH)D$_3$ applicable under most circumstances is 50 nmol/L and this level may be influenced by dietary calcium intake.

**Toxic vitamin D dosing in normal subjects and renal patients**

Vitamin D intoxication such as hypercalcemia does not occur until oral vitamin D intake and serum 25(OH)D$_3$ exceeds 250 µg/day and 500 nmol/L, respectively.\textsuperscript{114} Vieth\textsuperscript{115} has reported that long-term daily vitamin D consumption of more than 40,000 IU (1000 µg) is needed to cause hypercalcemia in healthy individuals.

In the clinical setting, hypercalcemia and hyperphosphatemia are still a concern particularly in patients with chronic kidney disease (CKD) since the discovery of 1,25(OH)$_2$D$_3$ (calcitriol).\textsuperscript{116,117} Therefore, vitamin D analogs were thought to be the solution to overcome this burden. Paricalcitol (Zemplar, Abbott Laboratories) was found to lower the incidence of sustained hypercalcemia and/or elevated Ca/P product while it decreases PTH to the target range more rapidly than in patients receiving calcitriol.\textsuperscript{118,119} In addition, patients who receive paricalcitol while undergoing long-term HD appear to have a significant survival advantage over those who receive calcitriol.\textsuperscript{120,121} Some other vitamin D analogs such as doxercalciferol, maxacalcitol, and falecalcitol to replace calcitriol in ESRD patients for a better control of PTH and calcium serum levels were also described (reviewed by Wu-Wong \textit{et al.}\textsuperscript{122}).

**Toxic vitamin D effects in animal studies**

Numerous studies have been conducted on different animal species, including rats, cows, pigs, rabbits, dogs, and horses.\textsuperscript{123-128} In these studies, vitamin intoxication resulted in elevation of the plasma concentrations of vitamin D$_3$; 25(OH)D$_3$; 24,25(OH)$_2$D$_3$; 25,26(OH)$_2$D$_3$ and 25(OH)D$_2$-26,23-lactone, but rarely raised plasma 1,25(OH)$_2$D$_3$.\textsuperscript{129,130} According to Shephard and DeLuca\textsuperscript{131} vitamin D$_3$ and 25(OH)D$_3$ concentrations rose to micromolar levels in plasma of rats given the highest intakes of vitamin D$_3$, resulting in marked hypercalcemia. All dihydroxylated metabolites, including 24, 25(OH)D$_3$; 25, 26(OH)D$_3$; and 25(OH)D$_2$-26,23-lactone, also rose to concentrations higher than 100 nmol/L, but the level of plasma 1,25(OH)$_2$D$_3$ remained within the normal range.

**Stages of vitamin D status**

Conclusively, according to Zittermann,\textsuperscript{132} dif-
ferent stages of vitamin D status can be classified based on serum levels of 25(OH)D as deficiency (0-25 nmol/L), insufficiency (>25-50.0 nmol/L), hypovitaminosis D (>50-70 to 100 nmol/L), adequacy (70-100 to 250 nmol/L), and toxicity (>250 nmol/L). Lips124 also concluded that a substrate reduction in serum calcitriol levels may occur if the circulating serum 25(OH)D falls below 30-40 nmol/L.

What is unique about 25(OH)D?

Investigators, including basic and clinical researchers, focussed mainly on the active metabolite 1,25(OH)2D3, which has abundant roles in modulating many biological and physiological functions. Rajasree et al.119 listed several reasons for choosing the measurement of 25(OH)D above calcitriol in a study on ischemic heart disease. First, 25(OH)D3 shows the highest concentration of all vitamin D metabolites. Second, its levels are stable for almost two weeks (Table 1). Third, vitamin D toxicity is thought to be a function of 25(OH)D3 levels rather than of calcitriol. Fourth, if serum levels of 25(OH)D3 are increased beyond the normal range, vascular calcification is still expected even with normal levels of 1,25(OH)2D3. In addition, Wang et al.125 concluded that serum 25(OH)D3 is the best indicator of vitamin D status in individuals without kidney disease, because it is the substrate for the renal and non-renal production of 1,25(OH)2D3. Serum 25(OH)D3 has a longer half life than 1,25(OH)2D3,125 Shephard and DeLuca92 indicated that 25(OH)D3 in large amounts can replace 1,25(OH)2D3 to stimulate bone calcium mobilization. Although nephrectomy abolishes a response to physiological doses of 25(OH)D3, large doses (1000-fold greater) of 25(OH)D3 can stimulate intestinal calcium absorption and bone calcium mobilization in nephrectomised rats.111,125 Shephard and DeLuca also added that pharmacological doses of vitamin D or its other metabolites are capable of substituting for 1,25(OH)2D3, which acts as an active form of vitamin D under physiological circumstances (Table 1) on the receptors of intestine and bone, causing 1,25(OH)2D3-like responses.111 Counts et al.112 have reported a case of hypercalcemia and vitamin D intoxication in an anephric human receiving vitamin D in doses of up to 100,000 IU/day for several months. The plasma concentration of 25(OH)D3 was found to be as high as 635 ng/mL (20-fold over normal circulating levels); 1,25(OH)2D3 was assumed absent, as anephric humans have been reported to have undetectable plasma 1,25(OH)2D3.113 Hughes et al.114 studied vitamin D intoxication in two human patients with normal kidney function. Tests showed that both patients demonstrated elevated plasma 25(OH)D3 levels (500-600 ng/mL); about 16- to 18-fold over normal concentrations, while the 1,25(OH)2D3 plasma concentrations were only slightly increased above normal from 40 to 56 pg/mL.

Low versus high plasma 25(OH)D levels and mortality rate

Low 25(OH)D levels have been associated with multiple cardiovascular disease (CVD) risk factors in the Third National Health and Nutrition Examination Survey (NHANES III) population,127 as well as with hypertension,128 congestive heart failure,129 and diabetes mellitus;130 all of which are risk factors for CVD and all-cause mortality.131 According to Melamed et al.,132 the lowest 25(OH)D quartile (<12.5 nmol/L) is associated with a higher risk of all-cause mortality in the general U.S. population. The inverse link between higher all-cause mortality and lower 25(OH)D levels in HD patients strongly suggests that vitamin D deficiency is a mortality risk factor that may be reversed by its correction.133 On the other hand, Rajasree et al.119 indicated that 25(OH)D of >222.5 nmol/L was observed in 59.4% of cases of ischemic heart disease compared to 22.1% in controls. Vieth and Rao134 indicated that Rajasree et al.119 presented what was certainly a world record high for a median 25(OH)D concentration achieved without vitamin D supplementation. This level was much higher than the mean concentration previously reported for lifeguards, farmers in the Caribbean, and others living at the equator.135 Vieth135 thought that methods used by Rajasree et al.119 may be not accurate, providing that the 25(OH)D concentrations averaged four times as high as those presented in other reports from India. We and others136 believe that the most likely cause of elevated plasma 25(OH)D is vitamin D toxicity.

Conclusions

Binding of the active metabolite, calcitriol (1,25(OH)2D3) to VDR exerts physiological functions. Other vitamin D metabolites, in particular 25(OH)D3, play an integral part in vitamin D metabolism and calcium homeostasis. Calcitriol regulates RAS and cardiac function by a calcium- and phosphorus-independent mechanism. Vitamin D intoxication is controlled by 25(OH)D3 levels in plasma. Taken together, vitamin D physiology is mostly governed by 1,25(OH)2D3 while vitamin pathology (deficiency, insufficiency, and excess) is usually governed by 25(OH)D3.

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