Vitamin D metabolism and molecular modes of action: new insights into vitamin D activities

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ABSTRACT
Reports of significant health benefits from an adequate vitamin D status continue to generate considerable interest. Knowledge of the molecular actions of the vitamin D system, including the nuclear vitamin D receptor and other receptors for 1,25-dihydroxyvitamin D and vitamin D status, provides evidence for vitamin D system to exert biological activities with physiological sensitivities and specificities across a wide range of tissues in a similar manner to other nuclear receptor hormones. This knowledge provides physiological plausibility for the wide range of activities claimed for vitamin D from largely observational studies and supports proposals for well-conducted clinical trials.

Key words: vitamin D metabolism, vitamin D receptor, VDR protein-protein interactions, VDR genomic actions, vitamin D non-genomic actions

Introduction
An adequate vitamin D status has been claimed to provide health benefits over a wide range of clinical conditions. This has led to a high level of interest in vitamin D testing for clinical laboratories. Over recent years, a large body of basic physiological data on various aspects of vitamin D biology including metabolism within a variety of tissues and activities of the vitamin D receptor (VDR) has been generated [1]. This knowledge has provided new insights into plausible mechanisms by which vitamin D could exert activities across a wide range of tissues. Many organs and tissues express the VDR and can respond to the active form of vitamin D, providing plausible physiological and molecular mechanisms for a diverse array of activities and tissue responses which could improve health. The regulation of vitamin D metabolism and its various modes of action to modulate biological pathways are here reviewed.

Vitamin D metabolism
Vitamin D₃ (also known as cholecalciferol) largely arises from sunlight exposure of the skin where UVB radiation converts 7-dehydrocholesterol to pre-vitamin D₃ which at body temperature isomerises to vitamin D₃ [2]. Small amounts of vitamin D can be obtained from the diet from both animal (vitamin D₃) and plant sources (vitamin D₂ also known as ergocalciferol). In the circulation, vitamin D and each of its metabolites are largely bound to the vitamin D binding protein (DBP).

Vitamin D is activated by two consecutive hydroxylation reactions catalysed by specific P450 enzymes [3]. The first hydroxylation at the carbon 25 position is catalysed by the enzyme vitamin D-25 hydroxylase (CYP2R1) to form the pro-hormone 25-hydroxyvitamin D (25D) [4]. This enzyme is expressed in the liver which appears to be the major site of synthesis for plasma 25D [5]. However, many tissues express CYP2R1, and therefore synthesis of 25D can occur locally in many tissues [5]. A number of enzymes can catalyse this 25-hydroxylation reaction, although with lower affinity than the CYP2R1 enzyme, but their biological role is uncertain [6]. The level of 25D in serum best reflects vitamin D status because of its properties with regard to solubility and binding to vitamin D binding protein [7].

The second hydroxylation in this pathway occurs at the carbon 1 position and is responsible for synthesis of the biologically active metabolite, 1,25-dihydroxyvitamin D (1,25D). This metabolite has the highest affinity of any of the vitamin D metabolites for the nuclear VDR.
[3]. The renal activity of the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) is responsible for synthesising plasma 1,25D and in the healthy, non-pregnant state renal synthesis appears to be the sole source of plasma 1,25D [8]. During pregnancy, some consider that the placenta makes a contribution to the plasma levels [9]. Furthermore, plasma 1,25D levels can be increased to pathological levels by various diseases through synthesis at extra-renal sites. This synthesis occurs with increased numbers of plasma cells including macrophages and white blood cells which express the CYP27B1 gene to levels sufficient to cause hypercalcaemia [10]. Many tissues express the CYP27B1 gene [11].

A third step in vitamin D metabolism is hydroxylation at the carbon-24 position by the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24). This enzyme is largely responsible for catabolism of vitamin D metabolites through its multi-catalytic activity [3]. This is the first step in a series of reactions involving the sequential cleavage of four aliphatic hydrocarbons to synthesise calcitroic acid which is eliminated via the kidney. The renal CYP24 activity is responsible for synthesis of plasma 24,25-dihydroxyvitamin D (24,25D) as well as contributing to regulation of plasma 1,25D levels through the clearance of vitamin D metabolites including 1,25D [8]. Biological activity for 24,25D has been proposed by way of a specific membrane-associated receptor involved in bone fracture repair [12].

**Regulation of plasma 1,25-dihydroxyvitamin D levels**

Regulation of renal CYP27B1 and CYP24 gene expression play key roles in plasma calcium and phosphate homeostasis through regulating plasma 1,25D levels. Renal CYP27B1 expression is regulated by each of the calcium- and phosphate-regulating hormones. Many of these regulatory factors including localisation of gene expression to the renal proximal tubular cells of the kidney, are contained within the proximal 1503 base pairs (bp) upstream of the human CYP27B1 gene [11]. PTH markedly stimulates renal CYP27B1 expression in these cells. Two mechanisms have been described for PTH stimulation, one acting through a vitamin D inhibitory receptor (VDIR), which binds to a site approximately –500 bp upstream of the CYP27B1 promoter region [13]. A second mechanism acts through a cyclic-AMP dependent protein kinase A activity by way of a CCAAT box site within the proximal –305 bp upstream of the CYP27B1 promoter region [14]. These actions occur under conditions of hypocalcaemia, a condition when plasma PTH levels are highest.

Inhibition of CYP27B1 expression also plays a critical role in regulating plasma 1,25D levels. 1,25D is a negative regulator of renal CYP27B1 expression through a novel mechanism involving the VDIR located around –500 bp within the proximal promoter [13]. This mechanism has only been demonstrated in kidney cells. Another important inhibitor of renal CYP27B1 expression is fibroblast growth factor 23 (FGF23), a plasma hormone produced largely by mature osteocytes. [15].

As mentioned above, the regulation of catabolism of 1,25D through expression of CYP24 in the renal proximal convoluted tubules is also critical for regulating plasma 1,25D levels as demonstrated by the phenotype of the CYP24-null mouse [16]. The inverse relationship between CYP24 and CYP27B1 expression in the kidney is unusual and appears to be limited to renal tissue [8]. CYP24 is considered to be expressed in all cells expressing VDR [3] and in extra-renal cells a positive relationship between CYP27B1 and CYP24 expression has been demonstrated [8]. This inverse relationship in renal cells lends support to the concept that the renal proximal tubules metabolise 25D for synthesis of plasma 1,25D and subsequent endocrine activities. In extra-renal tissues, the positive relationship between expression of these genes suggests that such local synthesis of 1,25D promotes autocrine or paracrine activities.

In the kidney, the reciprocal relationship between CYP27B1 and CYP24 mRNA levels is largely the product of reciprocal actions of the calcitropic hormones on the expression of these genes. For example, the major stimulator for CYP24 expression is 1,25D which as discussed above directly inhibits renal CYP27B1 expression. CYP24 induction by 1,25D involves activation of VDR through binding to tandem vitamin D responsive elements (VDREs) in the proximal CYP24 promoter [17], although variation of regulation occurs between humans, rats and other species [18]. 1,25D also stimulates intracellular signalling (i.e. non-genomic) pathways to activate transcription factors that are dependent on the VDR signalling to further enhance CYP24 transcriptional activity [19]. Activation of these pathways ultimately results in phosphorylation of a transcription factor such as one of the E-twenty six (Ets) family members which binds to the gene promoter region in the region of the VDREs to further stimulate transcription. Each of these mechanisms is dependent on a liganded VDR and interacts to markedly increase CYP24 enzyme levels in the presence of 1,25D. Such increases of CYP24 enzyme activity presumably minimise toxic activities by rapidly reducing plasma 1,25D levels.

Increased PTH levels decrease CYP24 activity in kidney cells, although the mechanism of this effect remains controversial. PTH has been reported to destabilise CYP24 mRNA and reduce CYP24 enzyme activity [20]. Calcitonin induces CYP24 expression in a human embryonic kidney cell line through a combination of protein kinase A and protein kinase C activities. This is possibly physiologically relevant under conditions of hypercalcaemia when calcitonin levels are elevated.
and it would be advantageous to reduce plasma 1,25D levels [21]. FGF23 actions to lower plasma 1,25D levels are also dependent on stimulation of CYP24 expression in addition to inhibition of CYP27B1 expression. The stimulation of CYP24 expression is predominantly dependent on VDR actions through a VDRE unlike the inhibition of CYP27B1 [15].

Molecular actions of vitamin D and vitamin D receptor

Regulation of gene transcription

One mechanism by which vitamin D exerts its biological activity is through binding of the 1,25D metabolite to a protein receptor in a similar manner to other steroid hormones. The best described receptor for vitamin D is the vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor superfamily. The VDR acts to stimulate gene transcription after binding 1,25D and forming a heterodimer with the retinoid-X receptor (RXR) protein which then binds to a vitamin D receptor-specific gene sequence [22]. Vitamin D responsive genes are defined by the genetic coding of a specific control element known as the vitamin D response element (VDRE) in the regulatory region of the genome. Often, but not always, VDREs are situated in physical proximity to the transcriptional start site for a vitamin D-responsive gene. The binding of the 1,25D-VDR-RXR complex to the VDRE initiates the recruitment and assembly of a very large complex of proteins known as coactivators. This complex has at least two functions. It remodels the locally condensed chromatin through the activities of various enzymes to either add moieties such as acetyl or methyl groups to histones or remove these moieties from the histones. It also communicates with the RNA polymerase II enzyme recruiting this enzyme to the transcriptional start site necessary for transcription of vitamin D-responsive genes through mRNA synthesis.

The totality of such transcriptional complexes defines the specificity and sensitivity of steroid hormones such as vitamin D to regulate a multiplicity of biological responses through a wide range of tissues. Currently we understand the contribution of at least four elements of the transcriptional complex [23]. In the case of vitamin D, the nuclear receptor ligand, 1,25D, identifies the physiological specificity of the response. The VDRE identifies the genetic specificity of the response. The various co-activators and other proteins complexing to the liganded VDR-RXR heterodimer bound to the VDRE identify the cell or tissue specificity of the response. Finally, the transcription and translation of the gene and the activity of the specific gene product identifies the biological response.

Rapid actions of vitamin D (non-genomic activities)

It has been recognised for some 30 years that 1,25D exerts biological activities over significantly shorter time periods than have been considered necessary for regulation of gene transcription as described above or at least can be detected with current techniques. These activities take place over minutes, whereas the genomic synthesis of mRNA and translation into proteins following 1,25D activation requires some hours before detectable protein levels can be measured. These rapid activities are sometimes termed ‘non-genomic actions’ but this is a misnomer as very often such actions modulate the level of transcription of vitamin D responsive genes. These activities include modulation of intracellular calcium levels as well as activation of intracellular signalling through phosphate kinases and phosphatases. These rapid activities appear to activate different intracellular signalling pathways in different cell types [19, 24]. Ultimately, the end result of much of these activities is an alteration of gene transcription [19].

There is evidence of 1,25D acting through a distinct membrane-associated, rapid response steroid binding receptor (MARRS) [25] to initiate such rapid activities. This protein belongs to a superfamily of multifunctional glucose-regulated and redox-sensitive proteins that have previously been implicated in binding thyroid hormones and oestrogens in glycoprotein biosynthesis and in immune responses [26]. In addition, the well-characterised nuclear VDR has been found to elicit similar rapid responses presumably in association with plasma cell membrane constituents [19, 27].

Actions of the vitamin D receptor through binding to intracellular proteins

A further mechanism of action for the vitamin D system is direct binding of VDR to intracellular proteins. Many of the proteins which bind with VDR have been identified as either transcriptional co-activators or co-repressors that make up the transcriptional complex necessary for genomic actions as described above or as transcription factors themselves. One of the most intriguing activities described for VDR in this context is its role in hair follicle cycling. It has been well described that humans and mice in which the VDR gene is inactivated develop alopecia along with other disorders, most particularly hypocalcaemia and hypophosphataemia [28]. In contrast, humans and mice in which the CYP27B1 gene is inactivated do not develop alopecia despite sharing the disordered plasma calcium and phosphate homeostasis [29]. The action of the protein β-catenin, which is normally located within the cell cytoplasm, is critical for maintaining the ability to stimulate hair follicle growth in the hair follicle stem
cell (reviewed in [30]). Activation of β-catenin occurs through the well-characterised Wnt signalling pathway which stimulates the translocation of β-catenin from the cytoplasm to the nucleus where it binds to transcription factors of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families. In the hair follicle stem cell, these transcription factors stimulate expression of genes coding for the specific keratins which make up the structural proteins of hair and therefore are critical for hair growth (as reviewed in [30]). In these cells, the binding of VDR to β-catenin is a critical stage for the activation of β-catenin to stimulate transcription of the keratin genes. There is no requirement for 1,25D binding to VDR for this activity, but the question as to whether there is a requirement for the VDR to bind another ligand is as yet unresolved.

VDR binds directly to β-catenin in a number of cell systems modulating β-catenin-responsive genes in some cells and while modulating vitamin D-responsive genes in others [31]. Importantly for skeletal physiology, the Wnt signalling pathway and β-catenin regulate bone formation as indicated by the actions of the Wnt signalling pathway antagonist sclerostin. Sclerostin binds to the LRP5/6 proteins which, in a complex with the frizzled receptor, the first component of the Wnt signalling pathway, markedly inhibits bone formation. Inhibitors of sclerostin are currently being trialled as a treatment for postmenopausal osteoporosis [32]. Do VDR and β-catenin interact in osteoblasts to modulate bone formation and is this activity dependent on 1,25D as a VDR ligand? Preliminary evidence is available from in vitro experimentation with a human osteoblast-like osteosarcoma cell line to indicate that VDR does stimulate β-catenin activity in this cell line. This activity appears to be independent of 1,25D [33]. This osteoblast-like cell activity is in contrast to similar experimentation with a colon cancer cell line where the authors report that the β-catenin-VDR interaction inhibits β-catenin activity.

Most interesting are data identifying VDR antagonists that inactivate the VDR through a VDRE by blocking recruitment of the classical transcriptional complex. Mutations within the VDR gene have been identified which allow the antagonist-VDR complex to interact with β-catenin stimulating biological activities by way of this pathway [34]. Thus specific amino acid residues in the VDR can discriminate between classical genomic activities of VDR through a VDRE and its ability to interact with β-catenin. A mutation in one such amino acid of the VDR inactivates the classical genomic pathway acting through a VDRE but maintains the β-catenin-VDR pathway has been identified in a patient with hereditary vitamin D-dependent rickets. This patient exhibits manifestations of rickets but is not alopecic [35].

Conclusions

The wide variety of diseases associated with a low vitamin D status has tested the credulity of many medical practitioners and biological scientists alike. Much of this interest originates from reports of simple associations between a particular disease state or condition and low vitamin D status. Thus the evidence can be very weak and there are numerous knowledge gaps, although evidence continues to strengthen as the findings of further studies are reported.

An improved vitamin D status can be associated with many attributes of good health relating to mobility and activities providing sunlight exposure without the involvement of a direct biological action of vitamin D. However, during the 21st century there has been a flourishing of knowledge of vitamin D metabolism and its modes of action. This knowledge provides plausibility to these claims.

The VDR has been reported to be widely expressed, with one study identifying its expression in 31 of a total of 39 tissues examined in two genetic strains of mice [36]. Thus the vitamin D system, including VDR activity whether liganded by 1,25D or not, has the capacity to exert biological effects in many tissues. Most steroid hormones exert a multitude of biological activities across a wide range of tissues. The most notable are the glucocorticoid hormones which are in wide clinical use because of their range of activities. Knowledge is currently emerging that the properties determining the tissue specificity and the physiological sensitivity of steroid hormones reside within the totality of the transcriptional nuclear receptor complexes when bound to chromatin [23]. Currently, we understand the contribution of at least four elements of the transcriptional complex to regulate biological activities across a wide range of tissues and responding to a variety of physiological conditions encountered throughout life. In the case of vitamin D, the nuclear receptor ligand, which in the main is 1,25D although other factors may possibly bind to the VDR, identifies the physiological specificitiy of the response. The steroid hormone chromatin response element identifies the genetic specificity of the response. In the case of the vitamin D receptor, this is the vitamin D response element (VDRE). The various co-activators and other proteins complexing to the liganded VDR-RXR heterodimer bound to the VDRE identify the cell or tissue specificity of the response. As well as in the case of the hair follicle stem cell as described in detail above, the VDR binding to another transcription factor also determines the cell or tissue specificity of the response. Whether such binding requires a ligand other than 1,25D or no ligand at all is currently unknown. Finally the transcription and translation of the gene and
the activity of the specific gene product identifies the biological response. Thus we are making significant progress in expanding our fundamental knowledge of the biology of the vitamin D system. This knowledge provides biological plausibility to the claims for various health benefits arising from an adequate vitamin D status.

References