Rapid Nontranscriptional Effects of Calcifediol and Calcitriol

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Classically, a secosteroid hormone, vitamin D, has been implicated in calcium and phosphate homeostasis and has been associated with the pathogenesis of rickets and osteomalacia in patients with severe nutritional vitamin D deficiency. The spectrum of known vitamin D-mediated effects has been expanded in recent years. However, the mechanisms of how exactly this hormone elicits its biological function are still not fully understood. The interaction of this metabolite with the vitamin D receptor (VDR) and, subsequently, with the vitamin D-responsive element in the region of specific target genes leading to the transcription of genes whose protein products are involved in the traditional function of calcitriol (known as genomic actions). Moreover, in addition to these transcription-dependent mechanisms, it has been recognized that the biologically active form of vitamin D3, as well as its immediate precursor metabolite, calcifediol, initiate rapid, non-genomic actions through the membrane receptors that are bound as described for other steroid hormones. So far, among the best candidates responsible for mediating rapid membrane response to vitamin D metabolites are membrane-associated VDR (VDRm) and protein disulfide isomerase family A member 3 (Pdia3).

vitamin D   calcitriol   calcifediol   non-genomic actions   vitamin D receptor

1. Introduction

Calcitriol (1α,25-dihydroxyvitamin D_3 or 1α,25$(OH)_2D_3$), the biologically active form of vitamin D_3, is a sterol hormone that is involved in the regulation of phosphate and plasma-ionized calcium levels by modulating their renal excretion, intestinal absorption, and calcium bone mobilization [1].

When low serum calcium concentrations are detected through the calcium-sensing receptor (CaSR) on the parathyroid glands, a G-protein coupled receptor, parathyroid hormone (PTH) secretion is triggered, thus activating calcitriol synthesis [2].

In addition to repressing parathyroid gene expression and cell proliferation by interacting with the vitamin D receptor (VDR), 1α,25$(OH)_2D_3$ maintains ideal serum calcium levels through its direct actions on the gut, the kidney, and the bone tissues [3].

Vitamin D_3 is typically produced in the skin, where provitamin D_3 (7-dehydrocholesterol (7-DHC)) is transformed into previtamin D_3 by the exposure to ultraviolet B (UVB) radiation, which then undergoes a thermal isomerization

to produce vitamin D_3 [4] (**Figure 1**). Subsequently, vitamin D_3 enters the bloodstream and reaches the liver where it is promptly hydroxylated by the 25-hydroxylase, a member of cytochrome P450 enzyme subfamily, thus forming 25-hydroxyvitamin D_3 or calcifediol $(25(OH)D_3)$ [5]. This compound shows an average plasma life of about three weeks, and therefore its serum levels are now currently used as indicators of the body vitamin D storage and status of patients. However, calcifediol is metabolically inactive and, once produced, it is secreted into blood bound up with vitamin D binding protein (DBP). In this case, the calcifediol needs a renal 1α-hydroxylation to obtain the biologically active form of vitamin D_3, $1α,25(OH)_2D_3$ [1] (**Figure 1**).
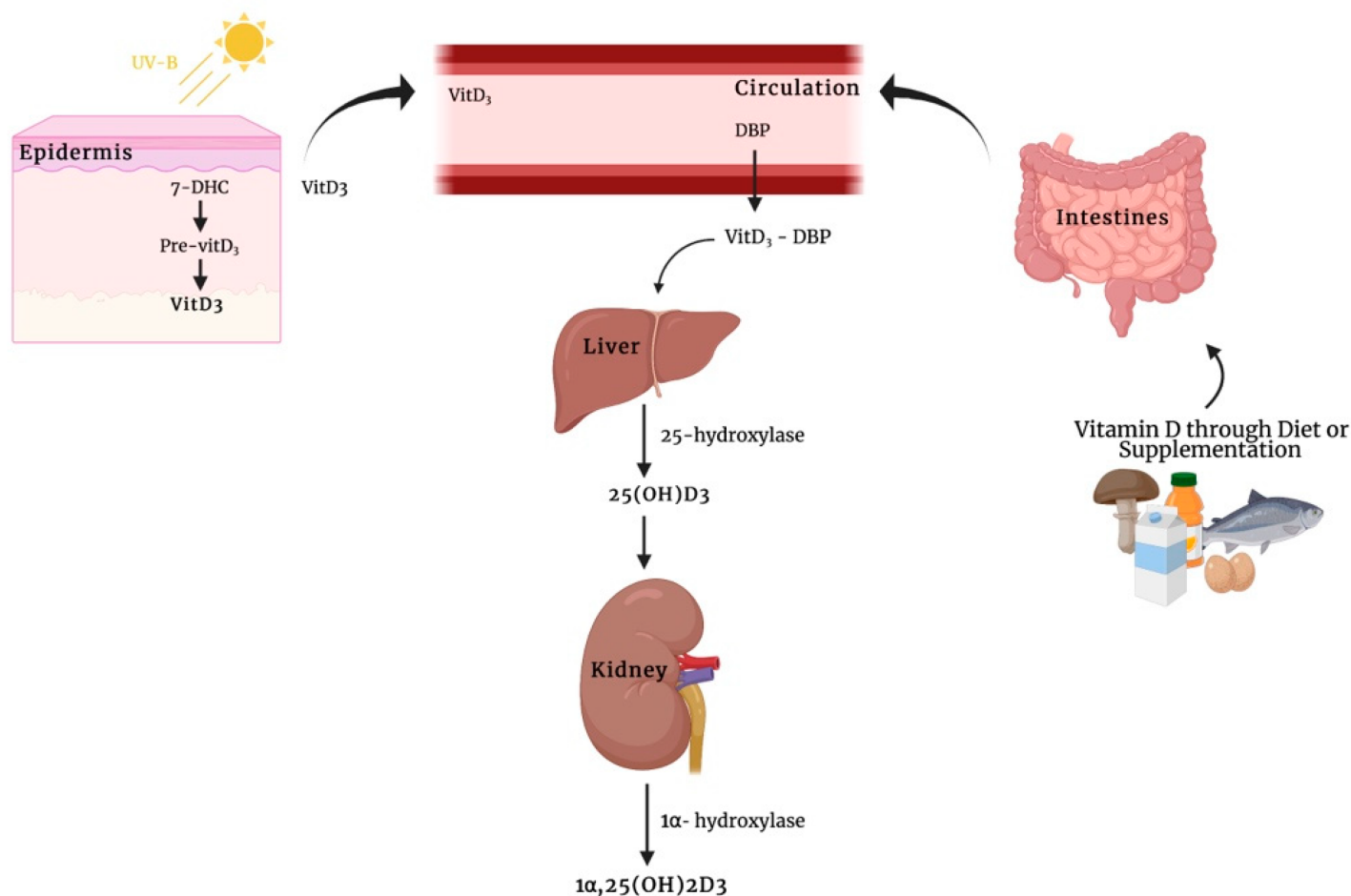
Figure 1. Schematic representation for the classical synthesis pathway of the biological active form of vitamin D_3, $1α,25(OH)_2D_3$. 7-DHC: 7-dehydrocholesterol; DBP: vitamin D binding protein. Image created by BioRender (https://app.biorender.com (accessed on 14 February 2022)).

Calcitriol acts through the binding with a VDR, which has been described in different species including humans, rats, and chickens [6]. It has a ligand-binding domain, named E-domain, a DNA-binding domain named C-domain, and an F-domain, which is one of the active domains. It acts by binding vitamin D-responsive elements (VDREs), repeated sequences located in the proximity of the start site of the target gene [7]. Upon the interaction between the ligand and the VDR, there is a conformational rearrangement of the receptor, preventing the bonding of the repressor. This results in the formation of a heterodimer between the VDR and the retinoid X receptor (RXR) at the VDREs, thus either initiating or suppressing the gene transcription whose protein products are involved in the

controlling of calcium homeostasis (i.e., cytochrome P450 family 24 (CYP24), alkaline phosphatase (ALP), type I collagen (COL1A1), osteocalcin (bone gamma-carboxyglutamate protein, BGLAP), and transient receptor potential vanilloid type family member 6 (TRPV6)) [1][8][9]. Since VDR has been found in nearly all cell types, this could clarify the different vitamin D_3 actions on several types of tissues [8].

In these circumstances, the increase or reduction of protein expression levels through gene transcription regulation from steroid hormones is the consequence of genomic steroid action [10]. These actions are not acute but delayed, because they require time for newly synthesized proteins and their processing [10]. In addition, molecules inhibiting of transcription and protein synthesis, such as cycloheximide or actinomycin D, may completely block these delayed effects if they do not take place at the time when steroid molecules are coupled to large proteins, thus preventing their cell entry [10].

2. Rapid, Non-Genomic Steroid Actions

The first non-genomic action of steroid molecules was described in 1942 when Hans Selye observed the anesthetic effect of progesterone immediately after its injection into peritoneum of rats and mice differently from what was observed with respect to its main effect that took place only within hours after its administration [11]. Subsequently, Spach and Streeten demonstrated that Na^+ ion variation occurred within few minutes after aldosterone administration in dog erythrocytes, offering new compelling evidence on non-genomic effects of this hormone because these cells lack nuclei, and therefore the observed in vitro effects might be attributable exclusively to its non-genomic mechanism [12]. However, these rapid hormone effects were not clarified until their recent recognition for several steroid hormones, including $1\alpha,25(OH)_2D_3$ [10].

Recently, $25(OH)D_3$, for a considerable time deemed only a metabolic precursor of $1\alpha,25\text{-}(OH)_2D_3$, has been proved to be an agonist ligand of VDR and capable of initiating rapid, non-genomic actions [13][14].

As opposed to their genomic counterpart, non-genomic rapid responses appear rapidly (within a range of seconds or minutes), are not susceptible to cycloheximide or actinomycin D, and also occur in response to steroids coupled to macromolecules which block their cell entering [15]. Therefore, a major difference for discerning between genomic and non-genomic actions is the time course and the sensitivity of transcription and protein synthesis inhibitors.

3. Mechanisms of Membrane-Associated Proteins for $1\alpha,25(OH)_2D_3$-Mediated Rapid, Non-Genomic Actions

Concerning the existence of non-genomic calcitriol actions, the earliest observations for these actions were described by Nemere et al. in 1984 [16], who observed that this hormone was able to induce a rapid increase of intracellular calcium (Ca^{2+}) concentrations both by promoting its release from intracellular stores and by stimulating its intestinal uptake in the vascularly perfused duodenum of normal, vitamin D-replete chicks. They observed that calcitriol significantly increased Ca^{2+} transport within 14 min compared with controls in a mechanism independent

of genome activation and de novo protein synthesis. This rapid effect on transepithelial Ca^{2+} movement across the intestine has been termed transcaltachia.

It is established that the biologically active vitamin D_3 metabolite stimulates different signaling molecules, including phospholipase A2 (PLA2), phospholipase C (PLC), and phosphatidylinositol-3 kinase (PI3K), and promotes the generation of second messengers, such as Ca^{2+} ions, phosphatidylinositol (3,4,5)-trisphosphate (PIP3), and cyclic AMP (cAMP), culminating in the activation of several downstream protein kinases (protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II gamma (CaMKIIG), Src, and mitogen-activated protein (MAP) kinases) [17][18][19][20][21]. In addition to the above-mentioned non-genomic actions of $1\alpha,25(OH)_2D_3$, this secosteroid hormone also mediates the opening of Ca^{2+}, Cl^-, and Pi channels.

Early studies performed by Norman and colleagues [22][23] suggested that a distinct membrane VDR could mediate non-genomic actions in response to $1\alpha,25(OH)_2D_3$. The biologically active form of vitamin D_3 promotes the generation of non-genomic responses and, especially in the case of transcaltachia, in its 6-s-cis configuration. By contrast, binding of secosteroid in the 6-s-trans form could be responsible for genomic responses.

This rapid effect was also observed in primary muscle cell cultures isolated from a chicken embryonic heart [24]. In particular, $1\alpha,25(OH)_2D_3$ induces a fast increase of both tissue Ca^{2+} uptake and cAMP levels within 10 min in primary myocytes. Moreover, scholars found that this effect was inhibited by a specific protein kinase A (PKA) suppressor, suggesting that the regulation of Ca^{2+} ion channel activity by the biologically active form of vitamin D_3 was mediated by the second messenger cAMP.

Baran et al. [25] demonstrated that the secosteroid $1\alpha,25(OH)_2D_3$ not only evokes a rapid opening of Ca^{2+} channels but also a rapid activation of phospholipase C (PLC) in ROS 24/1 osteoblastic cells lacking the VDR, implying that these effects occur independently of the VDR signaling mechanisms.

This could suggest that, in addition to membrane-associated VDR, the existence of other membrane receptors in conjunction with vitamin D might be essential for rapid, non-genomic effects of $1\alpha,25(OH)_2D_3$.

Given the highly liposoluble nature of vitamin D_3, $1\alpha,25(OH)_2D_3$, can penetrate biological membranes, and when inside the cells, may interact with heat shock proteins (HSPs) so as to be transferred to the nucleus and the mitochondria [26]. Alternatively, vitamin D_3 metabolites bound to a specific vitamin D_3 binding protein (DBP) can undergo endocytosis through an LDL receptor-related protein 2 (LRP2)- and cubilin (CUBN)-mediated mechanism. Subsequently, $25(OH)D_3$ bound to DBP is transported into the proximal tubule epithelium of the kidneys via megalin-mediated endocytosis, where it undergoes a hydroxylation step to transform into $1\alpha,25(OH)_2D_3$ [27][28]. In this light, additional studies should be performed to clarify the physiological significance and the potential mechanistic aspect for vitamin D_3 transporters.

One of best membrane-associated proteins able to bind vitamin D_3 compounds is the protein disulfide isomerase family A member 3 (Pdia3), also known as $1\alpha,25(OH)_2D_3$-membrane-associated rapid response to steroid

(MAARS), which has been described as a crucial protein in $1\alpha,25(OH)_2D_3$-initiated rapid membrane non-genomic signaling pathways [29].

This protein was first purified in the study of Nemere et al. [30], describing the existence of a putative plasmalemmal receptor for the biologically active form of vitamin D_3 involved in the transcaltachia on the basal-lateral membranes (BLM) of chick intestinal epithelium. This conclusion was supported by the observation that an altered, but still present, specific binding for [^{3}H] $1\alpha,25(OH)_2D_3$ was discovered in membrane fractions purified from vitamin D-deficient chicks with respect to the corresponding fractions obtained from normal animals. In addition, the BLM-VDR exhibited down-regulation of specific [^{3}H] $1\alpha,25(OH)_2D_3$ binding upon exposure to nonradioactive $1\alpha,25(OH)_2D_3$.

Later studies showed that this candidate plasmalemmal receptor essential for the rapid responses by $1\alpha,25(OH)_2D_3$ is Pdia3 [31][32]. Its primary function is to catalyze the formation, reduction, and isomerization of disulfide bonds, interacting with lectin-like molecular chaperones, calnexin (CANX) and calreticulin (CALR), to ensure the correct folding of newly synthesized glycoproteins [33].

4. 25(OH)D$_3$-Mediated Rapid, Non-Genomic Actions

In the study conducted by Lou et al. [14], it was assumed that calcifediol is an agonist ligand of VDR with anti-proliferative effects and gene regulatory functions, despite the fact it binds to VDR with a lower affinity compared to the biologically active metabolite of vitamin D_3.

Another study by Asano et al. showed an interesting non-genomic mechanism of $25(OH)D_3$, where this compound could regulate lipogenesis, thereby reducing the risk of metabolic disease-associated complications by altering sterol regulatory element-binding proteins (SREBPs) activation via the ubiquitin-mediated proteasomal degradation of SREBP cleavage-activating protein (SCAP) [34].

In recent years, $1\alpha,25(OH)_2D_3$, the biologically active metabolite of vitamin D_3, has attracted attention due to its involvement in several biological processes, including the regulation of the serum levels of calcium and phosphate, as well as its influence on bone and mineral metabolism.

There is now compelling evidence that $1\alpha,25(OH)_2D_3$ affects the target cells through genomic pathways and membrane receptor-mediated rapid, non-genomic responses. This latter mechanism has also been described for virtually all the steroid molecules, such as aldosterone, testosterone, estrogens, and cortisol [13][29][35].

Recently, even the direct metabolic precursor of $1\alpha,25(OH)_2D_3$, named calcifediol, it has been revealed able to activate rapid, nontranscriptional actions, such as an acute and sustained rise in intracellular Ca^{2+} levels, similarly to that observed with the biologically active form of vitamin D_3 [13][36].

Although growing evidence has led to a significant knowledge concerning calcifediol and calcitriol rapid, non-genomic activities, their impact on physiological processes needs to be clarified. In fact, it is difficult to identify vitamin D-deficiency-associated diseases which occur exclusively due to aberrations of its rapid actions.

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